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REMOTELY ACTIVATED BIOMATERIALS FOR OPTIMIZING THE TEMPORAL PROFILES OF THERAPEUTIC MOLECULES

Tania T. Emi
University of Rhode Island, etaniatabassum@gmail.com

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REMOTELY ACTIVATED BIOMATERIALS FOR
OPTIMIZING THE TEMPORAL PROFILES OF
THERAPEUTIC MOLECULES

BY
TANIA T. EMI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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DOCTOR OF PHILOSOPHY DISSERTATION
OF
TANIA T. EMI

APPROVED:

Dissertation Committee:

Major Professor Samantha A. Meenach

Geoffrey D. Bothun

Sara Rosenbaum

Nasser H. Zawia

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

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ABSTRACT

Remotely activated hydrogel-based biomaterials can maximize the therapeutic effects by providing localized deliveries and minimizing off-target side effects. Smart hydrogels respond to an externally applied stimulus by delivering therapeutic molecules. While showing promise in releasing molecules in a precise spatial manner, they do not inherently provide temporally dynamic delivery profiles (e.g., controlled timing, rates, and sequences) required to direct sequences of biological events.

Biological processes are characterized by a sequence of events regulated by a complex interplay of signaling factors. For example, in the instance of ischemia (where a tissue no longer receives oxygen and nutrients due to a lack of blood perfusion), the establishment of new vascular networks in/out of the ischemic tissue is needed. To achieve this, angiogenic sprouts are instructed to emerge from existing nearby blood vessels through the presentation of proangiogenic signaling factors from the ischemic tissue. After entering into the ischemic tissue, angiogenic sprouts are instructed to become mature blood-perfusing vessels through localized presentations of pro-maturation signaling factors. In another coordinated biological process (i.e., the wound healing process), specific progenitor cells are recruited to the injury site where they must then be instructed to proliferate and differentiate into a robust population of tissue-specific cell types. Spatially, temporally, and sequentially coordinated presentation of cell-specific recruitment, proliferation, and differentiation factors are critical to the proper therapeutic outcome. Thus, in treatments that require proper wound healing and tissue re-vascularization, it is important to locally deliver therapeutics in spatiotemporally nuanced manners while hydrogels are well-suited for

providing localized deliveries, traditional biomaterials not to provide the spatiotemporally nuanced delivery profiles needed to direct spatiotemporally complex biological processes, such as those that underly injuries and disease.

In addition to the regeneration of tissues and vascular networks in wound healing applications, cancer treatments can potentially be improved through spatiotemporally coordinated therapeutic deliveries. Localized deliveries of chemotherapeutics at tumor sites, for instance, can reduce off-target side-effects of toxic chemotherapeutics, and hydrogel-based materials are well-suited for providing these localized deliveries. However, the localized and sustained deliveries provided by polymeric hydrogels are not optimal for cancer treatments, especially regarding tumor cells' abilities to develop resistance to chemotherapeutic agents when subjected to constant chemotherapeutic concentrations over protracted periods. Emerging cancer treatment strategies often employ temporally dynamic deliveries of single or multiple anticancer agents to prevent adaptive resistance and improve tumor regression.

In this dissertation, three types of polymeric hydrogel-based systems were developed - i) calcium crosslinked alginate hydrogels, ii) biphasic alginate ferrogels, iii) two-compartment hydrogel systems consisted of a gelatin outer compartment and ferrogel inner compartment. These hydrogel-based biomaterial systems were investigated to determine their potential for explicitly directing complex biological processes by providing temporally complex therapeutic delivery profiles. For instance, calcium-crosslinked hydrogels were subjected to short ultrasound pulsations (repeated on/off) to minimize hydrogel erosion and temperature increase while providing consistent and statistically significant chemotherapeutic release rates vs. time.

Additionally, the sequential release of anticancer agents was demonstrated. Pulsatile mitoxantrone delivery profiles were also demonstrated from biphasic ferrogels when exposed to graded magnetic fields. Chemotherapeutic release rates could be explicitly controlled by stimulating at different magnetic field frequencies. Sequential release of protein signaling factors was also demonstrated by incorporating these magnetically responsive ferrogels into a compartmentalized hydrogel system. The outer gelatin compartment resulted in burst release of one cytokine (GM-CSF, VEGF, SDF-1 α), whereas the inner ferrogel compartment provided on-demand, delayed the release of separate cytokines (HSP27, PDGF, BMP2) when subjected to magnetic stimulations. In both these ultrasonically and magnetically responsive gel systems, while hydrogels were stimulated by external energy fields, a safe region of operation was maintained. Ultrasound parameters (amplitude, frequency, duration, number of repeated pulses) were chosen such that gel heating does not exceed 45⁰C temperature (8⁰C above body temperature) critical for structural integrity of proteins and maintenance of biological homeostasis. The ferrogel-based systems required that only simple, benign, hand-held magnetics be used for stimulation and could be operated without thermally damaging tissues.

In summary, stimuli-responsive hydrogels systems were developed here, characterized, and demonstrated to provide a number of different therapeutic delivery profiles that are likely to have beneficial impacts on treatment strategies. These stimuli-responsive hydrogel-based biomaterials not only could generate these temporally complex deliveries, but they also could be triggered to produce these types of deliveries in an on-demand manner. This may be of particular clinical utility, in that

clinicians will gain the flexibility to alter the course of therapies in real time, according to updates in patient prognosis. Finally, the hydrogel systems developed here will enable rapid optimization of complex delivery strategies in cancer treatments, wound healing, regenerative therapies, and in any treatment where control over complex biological processes is needed for improved therapeutic outcome.

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Dedication

My grandparents, Dr. M. Abdul Baquee Bhuiyan
and Late Achia Khatun,

Thank you for giving me a beautiful childhood

PREFACE

This dissertation is written in the manuscript format. The first chapter is a general introduction to the motivation and research objectives to address the problems stated throughout this dissertation. The second chapter is a review of the literature on current ultrasound-based drug delivery systems written as part of an invited review in preparation to be submitted to the journal *Acta Biomaterialia*. The third chapter entitled as “Ultrasonic generation of pulsatile and sequential delivery profiles from calcium-crosslinked alginate hydrogels” is a published article in the “Stimuli-Responsive Polymeric Materials” special issue of journal *Molecules* (*Molecules* 2019, 24, 1048; doi:10.3390/molecules24061048). The fourth chapter entitled as “Pulsatile Chemotherapeutic Delivery Profiles Using Magnetically Responsive Hydrogels” is a published article on *ACS Biomaterials Science and Engineering* (*ACS Biomater. Sci. Eng.* 2018, 4, 2412–2423; DOI: 10.1021/acsbiomaterials.8b00348). The fifth chapter named as “Magnetically responsive biomaterial system enables on-demand, sequential delivery of biomolecules for variety of biomedical applications” is a manuscript in preparation for the journal *Biomaterials Science*. The final chapter includes primary conclusions, importance, and future research directions related to this dissertation.

TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENTS.....	vi
DEDICATION.....	viii
PREFACE.....	ix
TABLE OF CONTENTS.....	x
LIST OF FIGURES.....	xvi
CHAPTER 1: INTRODUCTION.....	1
1.1. Background and Significance.....	1
1.1.1 Cancer Chemotherapy.....	1
1.1.2 Re-vascularization in regenerative therapies.....	4
1.2. Research objectives.....	7
1.3. References.....	9
CHAPTER 2: REVIEW OF LITERATURE.....	12
2.1. Introduction.....	12
2.2. Mechanism.....	14
2.2.1 Hyperthermia.....	14
2.2.2 Cavitation.....	16
2.2.2.1 Enhanced convection and diffusion.....	17
2.2.2.2 Disruption of the drug carrier.....	18
2.3. Tunable parameters.....	20
2.3.1 Sonication time, frequency and intensity.....	20

2.3.2 Effect of transducer type and position.....	23
2.3.3 Pulse number and pulse ratio.....	24
2.3.4 Biomaterial construction.....	25
2.3.5 Effect of microbubbles.....	28
2.4. Complex profiles.....	29
2.4.1 Existing strategies.....	29
2.4.2 Prospected strategies.....	35
2.5. References.....	38
CHAPTER 3: Manuscript 1: Ultrasonic generation of pulsatile and sequential delivery profiles from calcium-crosslinked alginate hydrogels.....	47
3.1. Abstract.....	48
3.2. Keywords.....	48
3.3. Introduction.....	48
3.4. Results.....	51
3.4.1 Impact of ultrasonic intensity and duration on molecular release from Ca ⁺² crosslinked hydrogels.....	51
3.4.2 Erosion and heating of scaffold due to ultrasound stimulation.....	57
3.4.3 Triggered release delivery by pulsing the ultrasound stimulation.....	59
3.4.4 Ultrasonically generating pulsatile temporal delivery profiles.....	61
3.4.5 Sequential 5-fluorouracil and irinotecan delivery on melanoma cells in vitro.....	65
3.4.6. Generation of the sequential 5-fluorouracil and irinotecan deliveries....	67
3.5. Discussion.....	70

3.6. Materials and Methods.....	74
3.6.1 Materials.....	74
3.6.2 Calcium Crosslinked Hydrogel Fabrication.....	75
3.6.3 Release Studies.....	76
3.6.4 Sequential Release Studies.....	77
3.6.5 Cell Culture and Maintenance.....	77
3.6.6 In Vitro Melanoma Cell Survival Studies.....	77
3.6.7 Data Representation and Statistical Analysis.....	78
3.7. Conclusions.....	79
3.8. Author Contributions.....	80
3.9. Funding.....	80
3.10. Acknowledgments.....	81
3.11. References.....	82
CHAPTER 4: Manuscript 2: Pulsatile Chemotherapeutic Delivery Profiles Using Magnetically Responsive Hydrogels.....	89
4.1. Abstract.....	90
4.2. Keywords.....	91
4.3. Introduction.....	91
4.4. Materials and Methods.....	94
4.4.1 Materials.....	94
4.4.2 Cell culture and maintenance.....	94
4.4.3 In vitro melanoma cell survival studies.....	94
4.4.4 Biphasic ferrogel fabrication.....	96

4.4.5 Electron microscopy imaging of ferrogels.....	97
4.4.6 Biphasic ferrogel release studies.....	98
4.4.7 Custom magnetic stimulation system.....	99
4.4.8 Data representation and statistical analyses.....	100
4.5. Results.....	101
4.5.1 Continuous vs. pulsatile chemotherapeutic deliveries on tumor cells in vitro.....	101
4.5.2 Biphasic ferrogels for magnetically controlled drug delivery profiles..	105
4.5.3 Generation of pulsatile mitoxantrone profiles from biphasic ferrogels.	107
4.5.4 Regulating chemotherapeutic release rate using the frequency of magnetic stimulation.....	109
4.5.5 Pulsatile delivery schedules with consistent “on” period release rates vs. time.....	113
4.5.6 Extending the duration of Pulsatile deliveries from Ferrogels beyond Three Days.....	115
4.6. Discussion.....	117
4.7. Conclusions.....	120
4.8. Associated Content.....	120
4.9. Funding Sources.....	121
4.10. Acknowledgments.....	121
4.11. References.....	122
CHAPTER 5: Manuscript 3: Magnetically responsive biomaterial system enables on-demand, sequential delivery of biomolecules for variety of biomedical	

applications.....	127
5.1. Abstract.....	128
5.2. Introduction.....	129
5.3. Experimental.....	133
5.3.1 Materials.....	133
5.3.2 Fabrication and characterization of biomaterial system.....	134
5.3.3 Magnetic stimulation of ferrogels.....	135
5.3.4 Protein release studies.....	135
5.3.5 Data representation and statistical analysis.....	137
5.4. Results and Discussion.....	137
5.4.1 Characterization of the two-compartment biomaterial system.....	137
5.4.2 Release characteristics of the outer gelatin scaffold.....	138
5.4.3 Release characteristics of the inner ferrogel.....	140
5.4.4 Strategies for magnetically controlling the rate of delayed release.....	142
5.5. Conclusions.....	151
5.6. Acknowledgements.....	151
5.7. References.....	152
CHAPTER 6: CONCLUSION.....	155
6.1. Importance and Summary.....	155
6.2. Future Directions.....	157
APPENDICES.....	159
Appendix A: Supporting Information of Manuscript 2.....	159
S.1. Loading mitoxantrone in ferrogels and rinsing.....	160

S.2. Movies of magnetic stimulation system.....	163
Appendix B: Manuscript 4: Understanding the influence of magnetic compression frequencies on ferrogels for therapeutic deliveries.....	164
S.1. Abstract.....	165
S.2. Introduction.....	165
S.3. Results and Discussion.....	167
S.4. Conclusions.....	177
S.5. Experimental.....	177
S.5.1 Ferrogel fabrication, imaging, and porosity.....	177
S.5.2 Ferrogel mechanical characterizations and cyclic compressions.....	178
S.5.3 Therapeutic molecule release studies.....	178
S.5.4 Data representation and statistical analysis.....	179
S.6. Acknowledgments.....	179
S.7. References.....	181

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Figure 1. The timing, rate, and sequence of anticancer deliveries are essential parameters in cancer treatment.....	2
Figure 2: Sequential VEGF and PDGF deliveries are required to produce new, mature vasculature.....	5

CHAPTER 2: REVIEW OF LITERATURE

Figure 1: High intensity focused the US can release drugs by increasing temperature on the point-of-interest precisely.....	15
Figure 2: Cavitation in the bubbles can release drug molecules when irradiated with the US. Several cavitation phenomena, i.e., microstreaming, shock waves, and microjets are responsibly increasing the transport of molecules from the depot.....	18
Figure 3: Skin conductivity enhancement of sodium lauryl sulfate due to the presence of the US is a function of time, frequency, and energy density.....	20
Figure 4: Mimosa release concentration in the aqueous solution of the US is a function of (A) time and intensity at constant 43 kHz frequency and (B) time and frequency at constant 10 W intensity.....	21
Figure 5: Release of BSA from NIPAM/MBAm (3.0/0.02 g/g) hydrogels varied with different intensities of the US.....	22

Figure 6: Photographs of the release of iron oxide particles in the PBS solution from alginate capsules when sonicated at various US signals for 5 sec.....	23
Figure 7: The beam spread varies with the diameter and frequency of US.....	24
Figure 8: Release of diclofenac from alginate microcapsules is a function of pulse number and ultrasound power.....	25
Figure 9: Plot of mimosa release concentrations with time when subject to varying cellulose concentrations.....	26
Figure 10: Cumulative release of PEG-AuNPs from (A) alginate hydrogels and (B) alginate microbeads using 9.6 mW cm^{-2} US for 2.5 min h^{-1} up to 5 hrs following overnight storage at 37°C	27
Figure 11: Alginate capsules of varying strength released AuNP particles at different US power.....	28
Figure 12: (A) Release of insulin from methylene chains-coated pHEMA/PEGDMA matrices when exposed to 1.1 MHz in the US for 5 min. Adapted from Kwok <i>et al.</i> [58]. (B) Trypan blue release from liposome-microbubble conjugate in hydrogels <i>in vivo</i> when subjected to US of 6 W/cm^2 intensity for 1 min.....	30
Figure 13: Effect of mitoxantrone dose profiles on the viability of cancer cells.....	31
Figure 14: Cumulative release of (A) BSA (left) and dextran (right) from NIPAM/MBAm hydrogels [74] and (B) diclofenac from alginate microcapsules [70] when subjected to pulsatile US irradiations.....	32

Figure 15: Cumulative release of PEG-AuNPs from alginate microbeads using 9.6 mW cm⁻² US for 2.5 min h⁻¹ up to 5 hrs following five days of storage at 37°C.....33

Figure 16: (A) Percent AuNP release vs. time for when weak and strong capsules were exposed to ultrasound for 200 s on day seven at 20% amplitude followed by at 80% amplitude for 100 s five minutes later. (B) Images of the release of iron oxide loaded capsules integrated into Ca²⁺ crosslinked alginate hydrogels when subjected to US stimulation under the chicken carcass model at 37°C.....34

Figure 17: Prospected strategies for improving the release profiles by US stimulation.....36

CHAPTER 3: MANUSCRIPT 1- Ultrasonic generation of pulsatile and sequential delivery profiles from calcium-crosslinked alginate hydrogels

Figure 1: Ultrasonic amplitude, ultrasonic duration, molecular size, and molecular charge influence the amount of molecular release from Ca⁺² crosslinked hydrogels.....52

Figure 2: The amplitude and duration of ultrasonic stimulation impact the amount of chemotherapeutic released.....55

Figure 3: The amplitude and duration of ultrasonic stimulation impact the amount of signaling factor released.....56

Figure 4: Ultrasound erodes and heats the gels in manners that are dependent on the intensity and duration of the applied ultrasound.....58

Figure 5: Pulsed ultrasound can enhance drug delivery while helping to control gel erosion and heating.....	60
Figure 6: Ultrasound can be used to generate pulsatile chemotherapeutic delivery profiles over a 3-day period, though gel structure is not maintained, and delivery rates change vs. time.....	62
Figure 7: Pulsatile delivery profiles with consistent release rates vs. time can be achieved by altering the US pulse number over time.....	64
Figure 8: 5-fluorouracil and irinotecan delivery sequence had an impact on melanoma cell survival.....	66
Figure 9: Ultrasonically responsive gels can be used to generate a triggered release of irinotecan after an initial burst release of 5-fluorouracil.....	68

CHAPTER 4: MANUSCRIPT 2- Pulsatile Chemotherapeutic Delivery Profiles Using Magnetically Responsive Hydrogels

Figure 1. (A) A timeline describing the <i>in vitro</i> cytotoxicity experiment where B16-F10 cells were seeded and allowed to grow for 3 days before treatment, exposed to mitoxantrone treatment profiles s0 – s4 for 3 days, and allowed to recover for a day. (B) Delivery schedules (s0 – s4) were used on B16-F10 cells where the integrated dose was maintained at 666 ng mL ⁻¹ day ⁻¹	96
---	----

Figure 2. A schematic of the biphasic ferrogel fabrication process. (A) Gels were cast between two glass plates with a magnet on top, (B) allowed to swell in DI water after

gel formation, (C) frozen at -20°C to form ice crystals, and (D) lyophilized to evaporate ice crystals, leaving pores. (E) Photograph of a completed macroporous biphasic ferrogel.....	97
Figure 3. Schematic (top) and photograph (bottom) of the custom magnetic stimulation setup used in these studies.....	100
Figure 4. Pulsatile temporal delivery profiles enhance the toxicity of mitoxantrone exposure when compared to constant delivery profiles.....	102
Figure 5. For pulsatile mitoxantrone deliveries, the number of pulses can have an impact on how many melanoma cells survive treatment.....	104
Figure 6. Magnetically responsive biphasic ferrogels were porous in structure and capable of magnetically triggered drug delivery.....	106
Figure 7. Biphasic ferrogels generate pulsatile delivery profiles when periodically stimulated with hand-held magnets.....	107
Figure 8. The rate of release can be regulated by stimulating at different frequencies.....	110
Figure 9. Stimulation at different frequencies yielded different release rates during “on” periods initially, but significant differences were not achieved at later time points.....	112
Figure 10. “On” period release rates could be more consistent vs. time when progressively higher stimulation frequencies were used.....	114

Figure 11. Pulsatile mitoxantrone delivery schedules from ferrogels can be extended beyond 3 days.....	116
--	-----

CHAPTER 5: MANUSCRIPT 3- Magnetically responsive biomaterial system enables on-demand, sequential delivery of biomolecules for variety of biomedical applications

Figure 1. Sequential delivery of multiple factors is essential for improved outcome in multiple therapies.....	131
--	-----

Figure 2. New Biomaterial system is designed to improve sequential delivery of several factors in an on-demand manner.....	132
--	-----

Figure 3. Dual Compartment biomaterial system with porous outer compartment and magnetically responsive inner compartment.....	138
--	-----

Figure 4. The outer compartment can rapidly release initial factors pertinent to cancer immunotherapy, tissue vascularization, and bone regeneration.....	139
---	-----

Figure 5. Timing of the delivery from ferrogels can be controlled by stimulating at different time points.....	141
--	-----

Figure 6. Rate of the delivery of proteins from ferrogels can be controlled by applying various frequencies.....	143
--	-----

Figure 7. Developed biomaterial system can initially release factor I in fast diffusive manner and delay the release of a second factor until stimulated with magnetic field.....	146
---	-----

APPENDICES: APPENDIX A

Figure S1. The 125 μg of mitoxantrone used in our pulsed delivery studies does not represent a loading that saturated ferrogels with mitoxantrone.....161

Figure S2. When loaded with 125 μg of mitoxantrone, over 80% of the drug integrated into the gels and very little in addition to that was rinsed off during 1-hour rinses.....162

APPENDICES: APPENDIX B

Figure S1: -20°C and -80°C ferrogels shown different morphological and mechanical properties.....168

Figure S2: Rheology of ferrogels changed when subjected to cyclic compressions..170

Figure S3: Ferrogels released model macromolecules when exposed to magnetic stimulation.....172

Figure S4: Magnetic stimulation also triggered the release of chemotherapeutics and proteins from ferrogels.....175

CHAPTER 1

INTRODUCTION

1.1. Background and Significance

A common treatment strategy for a vast array of injuries and diseases involves the deployment of therapeutic agents (i.e., drug delivery), and is an every-growing and ever-evolving field. For example, the fast-track pharmaceutical innovation resulted in 59 new FDA approved molecular entities in 2018—the highest rate of drug approvals in 22 years [1]. Despite this wealth of therapeutics and more anticipated on the horizon, the bench-to-bedside translation of these therapeutics is inherently limited. Administration of drugs is fundamentally designed to enable control over the biological processes underlying injury and disease. However, the biology that underly injury and disease often involve spatially complex sequences of individual biological events. Therapeutic control over these spatiotemporally complex processes thus requires spatiotemporally nuanced therapeutic deliveries. In the following sections, this fundamental roadblock will be discussed in two clinically relevant theaters: cancer chemotherapies and re-vascularization in regenerative therapies.

1.1.1 Cancer Chemotherapy

Cancer brings immense sufferings to 2 million people every year in the USA (i.e., one patient dies, and three new patients will be diagnosed with cancer in every minute in 2019) [2]. In most cases, cancer is treated by oral or systemic administration of chemotherapeutics. Traditional systemic administration is limited in its difficulty in

maintaining therapeutic drug concentration at tumor sites (i.e., maintenance of the therapeutic window) and is plagued by off-target side effects due to the requirement of repeated and large doses and toxic agents (chemotherapeutics are harmful to healthy cells [3]). Implantable biomaterials can help reduce these side effects by releasing drugs locally at the point of interest (i.e., the tumor site) [3-4]. Despite promising work of localized drug deliveries from polymeric hydrogels, the deliveries provided by these hydrogels are often not optimal. Traditional hydrogels release drug molecules in a sustained manner and prevent altering the dose after implantation. This inherently limits clinical flexibility (i.e., the ability to change therapies in real time). Moreover, the relatively constant chemotherapeutic concentrations locally provided by these hydrogels are highly amenable to the development of adaptive resistance [5].

Figure 1: The timing, rate, and sequence of anticancer deliveries are essential parameters in cancer treatment. (A) MDA-MB-231 human breast cancer cells were

exposed to mitoxantrone delivery schedules S1 to S5 with same integrated dose *in vitro* (i), percent viable cells (cell population normalized to untreated controls) over 3 days when exposed to schedule S1 to S5 (ii). Adapted from Huebsch *et al.* [6]. (B) A murine mammary carcinoma mouse model was exposed to a different combination of anticancer drugs- vehicle only, DTX only, DTX and Das simultaneously, and DTX and Das sequentially (i, schedules 1 and 2), results of tumor growth rate (ii) and percent survival (iii). Adapted from Goldman *et al.* [7]. Asterisks (**) indicate statistically significant differences with $p < 0.01$.

There is growing evidence that adaptive resistance can be hindered by pulsatile chemotherapeutic delivery profiles [5-7]. These pulsatile deliveries are inspired by chronotherapy where highly concentrated drugs are applied during short timeframes when tumor cells are most susceptible, but the rest of the body is less susceptible [8-9]. This strategy has been found to be effective because cancer cells have faster metabolic cycles whereas the rest of the body has a slower metabolic cycle [8-9]. A short burst of highly concentrated mitoxantrone deliveries has reported being more effective in killing MDA-MB-231 human breast cancer cells *in vitro* than constant flatline delivery of same integrated dose (**Figure 1A**, comparing the percent cell viability of schedule S1 to schedules S2-S5). In addition to pulsatile deliveries, some emerging cancer treatment strategies involve multiple therapeutic deliveries, often delivered in sequence [7, 10-12]. It has been shown that 5-fluorouracil pre-treatment increases the bioeffect of irinotecan on colon cancer cells [10-12]. Also, the timing of second agent delivery plays an important role. For example, better tumor growth reduction resulted in a mammary murine carcinoma model when dasatinib (Das) was

applied soon after docetaxel (DTX) administered rather than delayed application. (**Figure 1B**, the percent of animal survival was highest for schedule S1 than schedule S2 and simultaneous deliveries). These results motivate the need to develop a drug delivery system that will enable sequential deliveries of multiple therapeutics so that multi-drug delivery strategies can be rapidly optimized.

1.1.2 Re-vascularization in regenerative therapies

Tissue regeneration involves delivery of signaling factors for restoration and functionalization of new tissues and organs. Natural tissue regeneration requires the establishment of growth factor gradients where the timing and direction of these gradients are key to regenerative outcome [13-15]. For instance, wound healing after injury requires the establishment of vascular networks [16]. Growth and development of vascular tissues requires the presentation of several growth factors at different times [17]. Proangiogenic factors like vascular endothelial growth factor (VEGF) promotes endothelial sprouting to the site that requires new vasculature (**Figure 2A**). VEGF, for instance, enhances microvessel density by promoting pericyte cell detachment from the vascular endothelium (**Figure 2A**, 2nd from left). This pericyte detachment destabilizes the vessel, allowing the sprouting of a new, but small, vessel (**Figure 2A**, 3rd from left). This small sprout will continue to sprout, but not mature into a larger, blood-perfusing vessel until pericytes are recruited back to the sprout through platelet-derived growth factor PDGF signaling (**Figure 2A**, 4th from left). This re-recruited pericyte stabilizes the sprout, allowing it to grow, thus facilitating the flow of blood through the tissue (**Figure 2A**, furthest to the right) [18-19]. Therefore, the development of mature vasculature requires sequenced delivery of pro-angiogenic

factors followed by pro-maturation factors (e. g., VEGF followed by PDGF). This motivates the need to develop effective strategies to deliver growth factors in sequence. Besides wound healing applications, vascularization can potentially help to treat cardiovascular diseases which are the leading cause of death in the USA in 2018 (in 38 sec one person dies by cardiovascular diseases) [20]. Tissue engineering can potentially provide powerful strategies for treating a wide variety of cardiovascular diseases by directing the growth of new or repairing defective vascular tissues.

Figure 2: Sequential VEGF and PDGF deliveries are required to produce new, mature vasculature. (A) Schematic of natural angiogenesis and vessel maturation

process. Adapted from Brudno et al. [13]. (B) Comparisons of blood vessel area distributions after administering the indicated combinations of growth factors. Adapted from Richardson et al. [18].

Because of this perceived need to deliver both VEGF and PDGF for proper re-vascularization, previous work has examined its potential. Richardson et al. [19] designed a hydrogel system to deliver both VEGF and PDGF and investigated how different combinations of VEGF and PDGF impacted vascular development and maturation (**Figure 2B**). It was found that in an *in vivo* ischemic tissue model, the percentage of matured (larger) blood vessels was significantly higher when delivering PDGF only (**Figure 2B**, comparing ‘blank’ to ‘PDGF’). However, vessel maturation was not significantly higher than for dual delivery of VEGF and PDGF comparing to delivering PDGF alone (**Figure 2B**, comparing ‘Dual’ to ‘PDGF’). To understand these results, it is important to note that these deliveries were not truly sequential. While the gels used here were designed to deliver VEGF and PDGF at different rates (i.e., with VEGF delivering at a higher rate than PDGF), starting at early time points, both VEGF and PDGF were being delivered to the ischemic tissue. Thus, the presentation of PDGF at the same time as VEGF likely negated the signal to form new vessels (VEGF instructs pericytes to detach while PDGF instructs them to attach). Thus, this conflicting instruction did not give significantly better blood vessel maturation (as indicated by the lumen area distribution) than the system where PDGF was added alone. These studies underscore the strong need for biomaterials that are capable of delivering VEGF and PDGF in truly sequential manners. Furthermore, the optimum time for delivering PDGF after VEGF is not yet understood. Depending on

the disease, injury, or patient, this timing will likely be different. Moreover, the timing and dose for delivering growth factors in clinical situations may need to be altered in response to up-to-date diagnostic information. Being able to remotely and flexibly control these sequential deliveries without changing the biomaterial construct will provide clinical and investigative advantages.

1.2. Research objectives

The principal objective of this study was to develop hydrogel-based systems capable of delivering therapeutic molecules with real-time control over the release profiles, timings of release, and release sequences by external stimuli. The four following objectives were designed to address this overarching principle objective:

Objective 1, Review and understand the use of ultrasonic stimulation for regulating drug delivery profiles from hydrogels.

Objective 2, Develop, characterize, and demonstrate the use of ultrasonically responsive hydrogels for generating pulsatile and sequential delivery profiles.

Objective 3, Investigate the impact of pulsatile anticancer treatment profiles on tumor cells *in vitro* and reproduce the optimized temporally controlled deliveries (pulsatile profiles) from magnetically responsive hydrogels.

Objective 4, Develop and demonstrate sequential release profiles of various protein signaling factors from hydrogel systems containing magnetically responsive hydrogels.

This dissertation was intended to uncover critical information needed to push stimuli-responsive biomaterials towards use in clinical scenarios. This work will provide fundamental characterizations on how gels release their payloads in response

to remotely applied stimuli. For example, drug release from these hydrogels will be characterized in terms hydrogel formulation (polymer and crosslinker concentration, type of crosslinker, mechanical strength, porosity, swelling ratio), molecule properties of the payload (molecular weight, charge density, polarity, hydrogel polymer-molecule affinity, amount of drug remaining in the gel vs. time), stimulation parameters (intensity, duration, number of repetitions, frequency, the type of stimuli (magnetic vs. ultrasonic, for example)), and physical alterations to the gel system over time (temperature vs. time, gel deformations, gel erosion). These extensive characterization can reveal new strategies for generating temporally complex delivery profiles (i.e., pulsatile and sequential). Subsequent demonstrations of pulsatile and sequential deliveries combined with in vitro tests proving that pulsatile and sequential deliveries can improve therapeutic outcome will provide critical ‘proof of concept’ data. Taken altogether, this lays the groundwork for potential improvements in treatment strategies in far-reaching applications, including, but not limited to chemotherapies, regenerative therapies, wound healing, cancer immunotherapies, and beyond.

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CHAPTER 2

REVIEW OF LITERATURE

2.1. Introduction

Ultrasound has a long successful journey in diagnostic imaging applications for monitoring internal tissues in the clinics. Because of its relatively safe, portable, affordable and patient compliant facilities, it's been adopted for therapeutic purposes. In 1954, Fellingner and Schmidt showed that the higher penetration of hydrocortisone with ultrasound for bursitis and tendinitis treatments [21]. In the 1980s, lithotripsy rapidly replaced surgical kidney stone removal that used high-pressure amplitude shock waves [22]. Ultrasound has a high spatiotemporal resolution that's why it has been used to deliver high and low molecular weight proteins, DNA, and gene by invading deep tissues. It is possible to deliver drugs from a payload using ultrasonic stimulation to tissues like the heart, lungs, brain, blood vessels, skeletal muscle, bones, tumors, fetus, etc. Some of ultrasound-based FDA approved treatments include tumor ablation (prostate, uterine fibroid, kidney), adipose tissue removal (ultrasound assisted liposuction), cataract removal (phacoemulsification), transdermal drug delivery, wound and bone healing, hemostasis, analgesia. Ultrasound can be divided into two types: low-intensity ultrasound (20 kHz-100 kHz) and high intensity focused ultrasound (2 MHz-16 MHz). Low-intensity ultrasound undergoes vigorous cavitation, causes a lower temperature increase, has better tissue penetration, and longer wavelengths make it difficult to focus. [23-26] On the other hand, high frequency focused ultrasound (HIFU) has a very precise focal site where the temperature can

increase (up to 60-80°C) rapidly (within 1-20 sec) results in drug carrier perturbation and thermal lesion on tissues [22, 27-28].

There are several commercially available US FDA approved ultrasound devices and products. Sonotherm 1000 (Labthermics Technologies, Inc.) is an ultrasonic hyperthermia device for cancer treatment received approval in 1989. Since then, tools like Exogen 2000+ (Smith & Nephew) for bone healing, SonoPrep (Sontra Medical Corp.) for topical anesthesia, Contour Genesis (Mentor Corp.) for the Lipoplasty device, Shinmed SW-988 (Shinning World Health Care) an ultrasonic nebulizer, etc received market approval in last three decades. ExAblate 2100 (InSightec) and Sonoalleve (Philips) are two instruments that use HIFU to cancer treatment and pain relief. ThermoDox (Celsion Corp.) is a liposome containing doxorubicin that releases by HIFU received FDA approval on 2014 for treating liver cancer.

Although therapeutic ultrasound treatment has well-defined benefits, some undesirable burning and tissue injury may happen because of accumulating a substantial amount of energy to localized areas. Some adverse effects include bleeding, scarring, and infection [22]. Standardization of ultrasound dosimetry and experienced operator can potentially manage the side effects. American Institute of Ultrasound in Medicine provides guidelines for a safe window of parameters. Careful consideration of bioeffects caused by these parameters ensures safe therapeutic applications that lead to government-approved devices and products for clinical practice.

2.2. Mechanism

The biological effects caused ultrasonic energy on cells and tissues can be discussed into two mechanisms:

2.2.1 Hyperthermia

Acoustic waves by ultrasound propagate at a predetermined intensity where a portion of the energy converts into heat during irradiation. The rise of temperature can be very rapid varying from material to material due to different coefficients of ultrasound absorption. This elevated temperature can break the biomaterials carrying drug (nanoparticles, liposomes, micelles, hydrogels) [3, 29-34] (**Figure 1**, nanocarriers (yellow) in the depot releasing drug molecules after getting hit by US beam). The US also increases the temperature of the surrounding tissue that can be calculated by the bioheat transfer equation [28]. Hence, US-induced heating with the unfocused beam is used in physical therapy to fast temperature elevation to tissues like bone [35-36]. Hyperthermia-based heat elevation is widely used for tumor ablation. Tumor tissues are inherently acidic, stressed by low oxygen levels, nutrient deprived, less tolerable to additional stress caused by elevated temperature. Heat dissipation by ultrasound is cytotoxic for tumor cells compared to normal cells due to their dense vascular growth [37]. It is reported that an ultrasound wave at frequency 1 to 3.4 MHz for 30-60 min can increase tumor temperature uniformly to 42-45°C and reduce its growth [38-39].

Figure 1: High intensity focused the US can release drugs by increasing temperature on the point-of-interest precisely. Here, a nanocarrier (yellow) is disrupted by HIFU and exploded to release drug molecules (red). The explosion also causes degradation of the depot.

The applications of hyperthermia-based ultrasound have been modified into high frequency focused ultrasound (HIFU) because of its precise temporal and spatial control. Targeted drug delivery from nano-carriers by ultrasonic hyperthermia is a promising field of research [40]. Temperature sensitive liposomes (TSL) was at first introduced in 1978 by Yatvin *et al.* for drug delivery by phase transition of lipids [33]. The increased cellular uptake of TSL into cancer cells happen by targeting drug-loaded liposomes into the tumor sites by passive accumulation and preferential extravasation of tumor blood vessels (EPR effect) [41]. Ultrasound-assisted hyperthermia triggers drug release from TSL in the tumor vasculature and interstitium. Some liposomes can revert to its previous phase when not irradiated and seals away any unreleased drug [42]. Dromi *et al.* used single focus pulsed HIFU (ISATA=1300 W/cm², 120 pulses, 1 Hz, duty cycle 10%) to release doxorubicin from ThermoDox® in a murine mammary adenocarcinoma BALB/c model for 4-5°C temperature

elevation in 15-20 min [43]. Patel *et al.* used a split focus transducer (TAT 80 W, 120 s pulsed, 1 MHz duty cycle 10%) to release doxorubicin from ThermoDox® in a murine squamous cell carcinoma SCC7 model for 4-5°C temperature elevation in 2 min [29].

HIFU has been recently coupled with MRI for better tissue differentiation and real-time monitoring of temperature changes by focused ultrasound [44]. Smet *et al.* released doxorubicin from a TTSL formulation of ThermoDox® and MRI contrast agent Gd-HP-DO3A (H₂O) with MR-HIFU clinical system (ISATA=117 W/cm², 1.4 MHz, continuous pulses for 15 min twice) at 41.5±0.5 °C in a rat tumor model [32]. Negussie and Ranjan *et al.* found better doxorubicin distribution in the tumor core and periphery using an LTSL with doxorubicin and Gd-HP-DO3A stimulated by an MR-HIFU clinical system (1.2 MHz, 10 min heating, 5 min cooling twice) in a Rabbit VX2 tumor model [30-31]. Staruch *et al.* released doxorubicin from ThermoDox® in a Rabbit bone marrow muscle by MR-guided mechanical scanning (2.8 MHz, 2.5 MPa) at 43°C for 20 min [34].

2.2.2 Cavitation

Cavitation is the dominant reason for the enhanced convection and diffusion by ultrasound [45]. The oscillating media can increase the transport of molecules. This effect can be enhanced by several orders of magnitude if gaseous cavities exist. Formation, growth, and activities of gas-filled cavities are known as cavitation. Stable cavitation involves periodic growth and oscillations of gas bubbles whereas inertial cavitation involves rapid growth and collapse of gas bubbles [46]. Stable cavitation creates acoustic streaming and shear forces on tissues, and inertial cavitation generates

shock waves that can produce very high shear rates to surrounding tissues drug carriers [47]. Pressure gradient in the surrounding fluid of the bubble leads asymmetric collapse that ejects a liquid microjet at a sonic speed capable of penetrating tissues [46]. The mechanical effects exerted by cavitation ultrasound can be used for therapeutic purposes which are discussed below,

2.2.2.1 Enhanced convection and diffusion

The oscillatory motion of the collapsing bubbles capable of inducing transport in the media, extracellular matrix and blood. The continuous growth and collapse of bubbles can make high viscous shear rates around the surface of the bubbles that increase the diffusion coefficient of any drug [48]. This enhanced transport affects the nanocarriers by attracting them to the area of microstreaming due to density difference (nanocarriers usually have a higher density). When the nanocarriers reach that area, they are sheared open by microjets (**Figure 2**, drug-carrying vesicles are breaking by cavitation effects of US and releasing drug molecules from the depot). This effect depends on the degree of oscillations in the area and can be very useful for targeted drug delivery [48-49]. A large number of studies showed enhanced delivery of low and high molecular weight drugs, radionucleotides, siRNA, plasmid DNA into deep muscle tissue (ovary [50], kidney [51]), ocular tissues (retina, cornea) [52], cardiac tissue [53], and brain tissue [54] in vivo. Fujii *et. al.* treated myocardial infarction in Sprague-Dawley rats by targeted delivery of stem cell factor (SCF) and stromal cell-derived factor (SDF-1 α) from pcDNA₃ plasmid DNA with DEFINITY[®] microbubbles using US (15L8 transducer, mechanical index 1.6, 1800-2000 ms bursts for 20 min/day up to 10 days, 2 days interval) [55]. Shimoda *et. al.* reported revascularization

of islet cells and restoration of euglycemia in transplanted islet cells in diabetic mice to treat type 1 diabetes by non-viral plasmid vectors delivering VEGF₁₆₅ gene by a commercial ultrasound transducer (S3, Sonos 55000; 1.3 MHz, mechanical index 1.4, 4 bursts with 85 ms delay) [56]. Chandan *et al.* delivered paclitaxel to murine B16F10 melanoma tumor model in C57BL/6 mice from phosphatidylserine-liposome-nanobubble conjugates (PSPLBC) using US (SP100 sonoporator, 2 W/cm², 50% duty cycle, 60 s) immediately after administration of the formulation [57].

Figure 2: Cavitation in the bubbles can release drug molecules when irradiated with the US. Several cavitation phenomena, i.e., microstreaming, shock waves, and microjets are responsibly increasing the transport of molecules from the depot.

2.2.2.2 Disruption of the drug carrier

Drug carriers like liposomes, micelles, microcapsules, and hydrogel micro composites, etc. are susceptible to ultrasound-induced cavitation. They carry a payload to tissues and release drugs when irradiated. Stable oscillation attracts them to be diffused to the region of interest. Shock waves and microjets can pierce through the vesicles [47-49, 51, 53, 55-57] (**Figure. 2**). Kwok *et al.* introduced an ultrasound responsive coated pHEMA hydrogels for insulin delivery that can reseal itself and can

be used up to 5 days [58]. Liposomal vesicles usually get partially restored which can maintain a sustained release [59-60]. When a drug is not entrapped but attached to the polymer backbone of the carrier, the ultrasonic effect can be seen to pull apart the drug molecules by breaking polymeric chains [60]. Epstein-Barash *et al.* co-encapsulated liposomes with perfluorocarbon (PFC) microbubbles in an *in situ* crosslinking hydrogels in male CD-1 mice and triggered release of trypan blue dye using a 20 kHz US (100% duty cycle, 0-10 W/cm², 0-15 s intervals) [61].

Ultrasound can be very useful for cancer treatment because of its ability to locally deliver chemotherapeutics and induce apoptosis [62-63]. Nelson *et al.* found reduced tumor volume by delivering doxorubicin from Pluronic NanoDelivTM micelles to colon carcinogenic DHD/K12/TRb inoculated rat model by continuous and pulsed US (1-2 W/cm², 20-70 kHz, one h/week up to a month) [64]. Howard *et al.* found 90% cellular proliferation of tumor by releasing paclitaxel from SYP-PM micelles to the MDB7/AD_{mt} breast cancer cell lines *in vitro* and *in vivo* in nu/nu mice with US (1 MHz, 1.7 W/cm², 33% duty cycle, 30-90s durations) [65]. Ultrasound is not only destructing the nano/microvesicles to release payloads; it is also able to liberate payloads from scaffold-based drug depots. Huebsch *et al.* introduced a seal-healing injectable Ca²⁺ crosslinked alginate hydrogel system to trigger the release of mitoxantrone in MDB-MB-231 and MCF7 cell lines and tumors on Nu/J mice models and the US (VCX130 Sonicator, 120 mW/cm², 2.5 min/d) [6]. They also showed these hydrogels could be used to release cytokines VEGF₁₆₅ and SDF-1 σ without altering their bioactivity [6].

2.3. Tunable parameters

2.3.1 Sonication time, frequency and intensity

Inertial cavitation is directly related to the sonication time (**Figure 3A**), frequency (**Figure 3B**), and acoustic pressure amplitude/intensity [26, 66-73]. Initiation of cavitation requires a minimum threshold ultrasound intensity at a given frequency up to a certain decoupling frequency [26, 73]. Beyond this decoupling intensity, cavitation does not increase with increasing intensity due to acoustic decoupling. The threshold frequency increases with increasing frequency because the growth of bubbles becomes difficult at a certain intensity (**Figure 3C**). Tezel *et al.* formulated that energy density can be calculated by multiplying the intensity and net exposure time [26]. At a constant intensity, low frequencies can penetrate more deep tissues than higher frequencies [66, 73]. Increasing the US power intensity directly increases the release of payloads [57, 66, 68, 70, 72-74].

Figure 3: Skin conductivity enhancement of sodium lauryl sulfate due to the presence of the US is a function of time, frequency, and energy density. (A) Plot of conductivity enhancement vs. time at 36.9 kHz frequency at intensities 0.54 ($\square$), 0.60 (Δ), 0.84 ($\blacksquare$), 0.96 ($\diamond$), 1.08 ($\bullet$), 1.26 ($\circ$) W/cm^2 . (B) The plot of conductivity enhancement vs. frequency. (C) The plot of conductivity enhancement vs. energy density at frequencies

19.6 (●), 36.9 (○), 58.9 (■), 76.6 (□) and 93.4 (▲) kHz. Adapted from Tezel *et al.* [26].

Jiang *et al.* found the release concentration of mimosa drug from 1 wt. % cellulose in dimethylacetamide (DMAc/LiCl) hydrogel film increased with increasing US intensity and exposure time at constant 43 kHz frequency (**Figure 4A**, release were higher at 30 W intensity than at 5 W intensity) [72]. They also reported 1.4 times higher rate of mimosa release at a lower frequency when tested at a constant 10 W intensity (**Figure 4B**, 23 kHz frequency resulted in a higher rate than 96 kHz frequency) [72].

Figure 4: Mimosa release concentration in the aqueous solution of the US is a function of (A) time and intensity at constant 43 kHz frequency and (B) time and frequency at constant 10 W intensity. The 1 wt.% cellulose hydrogel film was made from the solution with 0.08 wt.% mimosa. Adapted from Jiang *et al.* [72].

Wu *et al.* reported the release of bovine serum albumin (BSA) from N-isopropylacrylamide (NIPAM) and N,N'-methylenebisacrylamide (MBAm) hydrogel increased significantly when US intensity risen from 0.55 to 3 W/cm² (1 MHz

frequency, 30% duty cycle, for 3 min) [74]. They used temperature control water bath to maintain the temperature of control same as US group to ensure the release were only due to the US, not due to elevated temperature inflicted diffusion by US [74].

Figure 5: Release of BSA from NIPAM/MBAm (3.0/0.02 g/g) hydrogels varied with different intensities of the US. The elevation of temperature by the US was kept similar to the controls by water bath heating to eliminate the effect of temperature-induced diffusion. Adapted from Wu *et al.* [56].

Kennedy *et al.* showed ultrasonic pressure amplitude has a direct relationship with the release of iron oxide microparticles from alginate capsules [68]. At 80% amplitude for 2-sec irradiation made an effect equivalent to 4-sec irradiation of 40% amplitude. The 20%, 40%, and 80% amplitude were reported to be equivalent to 1.8, 6, and 7.4 W/cm² power intensity respectively depending upon the sonicator [68].

Figure 6: Photographs of the release of iron oxide particles in the PBS solution from alginate capsules when sonicated at various US signals for 5 sec. The 13 cm US probe was placed 2 mm above the capsules in glass scintillation vials. Images adapted from Kennedy *et al.* [68].

2.3.2 Effect of transducer type and position

The effects of US vary with the diameter, shape, and distance from the transducer crystal [66, 75-77]. The area of the US field beam is not uniform and changes its nature with distance. The energy of the US beam spreads as it propagates through the material. Therefore, the maximum effects found in the area directly in front of the transducer. Beam spread is higher for low frequencies (**Figure 7A**) and can be reduced by increasing diameter (**Figure 7B**) of the transducer [75-76]. Rectangular transducers have linear waves parallel to each other; on the other hand, convex transducers have wider waves with increased depth [78].

Figure 7: The beam spread varies with the diameter and frequency of US. (A) Simulated images of beam spread at 1, 5, and 9 MHz frequencies from a 0.75-inch diameter transducer, (B) images of beam spread at 50 kHz frequency from transducers of 3, 7, and 10 mm diameter. Adapted from the NDT resource center webpage [75].

The position of the drug depot also plays an essential role in effective payload release. The closer is the position of the depot to the transducer, the higher is the effect of US Kennedy *et al.* found that less energy was required to get iron oxide release from subcutaneously implanted hydrogels in the chicken carcass tissue models than in the scintillation vials [68]. When tested in scintillation vials, a portion of the US energy gave kinetic energy to the particles to bounce in PBS [68].

2.3.3 Pulse number and pulse ratio

Pulse number and pulse ratio are found to be influencing parameters for drug delivery. Introducing several stimulations in a fixed time frame instead of one continuous stimulation was found to be more effective in releasing payloads [29, 43, 65]. The seal-healing Ca^{2+} crosslinked alginate hydrogel used by Huebsch *et al.* was found to be effective in the *in vivo* tumor models up to 2 weeks for repeated irradiation because the scaffold reverted its structure when not irradiated [6].

Pulse ratio is often expressed as a duty cycle where a 20% duty cycle or pulse ratio 1:4 means keeping the machine on for one unit and off for four units. 100% duty cycle refers to continuous stimulation whereas 10% duty cycle is the least exposure to irradiation [66]. Wang *et al.* was able to increase the release rate of diclofenac from alginate microcapsules by 16% compared to non-US by using six pulses (**Figure. 8**) [70].

Figure 8: Release of diclofenac from alginate microcapsules is a function of pulse number and ultrasound power. US was applied at 1.2 MHz frequency, 1 W, 5 W, 9W intensities with 30 sec period. Adapted from Wang *et al.* [70].

2.3.4 Biomaterial construction

Design of a drug carrier plays a role to enable more control over the payload release. Different formulations of scaffolds and nanocarriers were proposed to get the spatiotemporal on-demand release of payloads. Coated hydrogel film [58], hydrogel micro-composite with nanocarriers and liposomes [61, 67-68, 70], coated nanoparticles [69], micelles [64-65, 71], liposome nanobubble conjugate [57] are some reported examples where encapsulated drugs underwent prolonged therapeutic delivery for several days to months. Wu *et al.* found the higher release of BSA (66

kDa) with the US when the amount of NIPAM in the NIPAM/MBAm hydrogel was increased to 3 g from 1 g due to high-temperature coefficients of denser gels aided more pore channel formation in the gel matrix than less dense gels [74]. On the other hand, Jiang *et al.* reported lower polymer concentration of cellulose in DMAc/LiCl hydrogel film aided increased release of the mimosa drug than higher concentrations of cellulose [72]. This group suggested the enhancement of the release is due to the mimosa-water and mimosa-cellulose hydrogel bond breaking by the US (**Figure. 9**). Thereby the 0.5 wt% of cellulose was less rigid than 2 wt% that can be disrupted easily with the same amount of energy [72].

Figure 9: Plot of mimosa release concentrations with time when subject to varying cellulose concentrations. 30 W ultrasound was irradiated at 43 kHz frequency for various exposure time. Adapted from Jiang *et al.* [72]

Kearney et al. showed that the increased surface-to-volume ratio of the hydrogels increases the ultrasound dependent release of nanoparticles rather than degradation-based release from the scaffold. The cumulative release of PEGlyted gold nanoparticles (AuNP) from Ca^{2+} crosslinked alginate microbeads of $d \sim 250 \mu\text{m}$

(**Figure 10B**) were higher than alginate hydrogels (**Figure 10A**). Increased surface-to-volume ratio provided PEG-AuNPs less distance to get liberated from the matrix when subjected to US [67].

Figure 10: Cumulative release of PEG-AuNPs from (A) alginate hydrogels and (B) alginate microbeads using 9.6 mW cm^{-2} US for 2.5 min h^{-1} up to 5 hrs following overnight storage at 37°C . TEM images are showing that the media only had isolated NPs after the US but no gel fragments. Adapted from Kearney *et al.* [67].

Concentration and composition of coatings on nanoparticles can help to tune the release kinetics. Kennedy *et al.* synthesized weak and strong hydrogel capsules by using 50 mM CaCl_2 and 100 mM BaCl_2 crosslinkers respectively that released their payloads selectively at 20% and 80% pressure amplitude of a 20 kHz signal (equivalent to 1.8 and 7.4 W/cm^2 energy intensity respectively) ultrasound (**Figure 11**) [68].

Figure 11: Alginate capsules of varying strength released AuNP particles at different US power. (A) microscopic images of weak and strong capsules made with 50 mM CaCl_2 and 100 mM BaCl_2 crosslinkers respectively. (B) Percent AuNP release with time when stimulated at 20% (i) and 80% (ii) ultrasonic power amplitude. Adapted from Kennedy *et al.* [68].

On the other hand, Moncion *et al.* reported that double emulsion perfluorocarbon (PFC) microbubbles in fibrin scaffold required more acoustic thresholds than its single emulsion formulations [79]. Beyond a specific US amplitude, PFC within these emulsions can undergo a phase transition from liquid to gas. The power required to initiate cavitation varied for single and double emulsion formulations [79].

2.3.5 Effect of microbubbles

The presence of microbubbles in the scaffold influences the rate of cavitation. PFC microbubbles have been used for DNA and gene therapy with ultrasound [53-55, 57, 60]. Epstein-Barash *et al.* incorporated perfluorobutane microbubbles and dye-containing liposomes in the hydrogels [61]. The increased concentration of the PFC microbubbles in the hydrogels increased cavitation, reduced US intensity and duration yielding less tissue injury [61]. Type of microbubbles determines the power required to initiate nucleation sites. Moncion *et al.* used two types of PFC microbubble emulsions, perfluoropentane (PFP) and perfluorohexane (PFH) in the fibrin hydrogels.

Hydrogels with PFP and PFH were found to have different threshold pressures (2.5 MPa and 3 MPa respectively) for acoustic droplet vaporization and initial cavitation when ultrasonically irradiated [79].

2.4. Complex profiles

2.4.1 Existing strategies

US-based treatment mostly offers burst release immediately after irradiation [28, 35-36]. After a few hours of treatment, the effect of the drug in the area of interest diminishes by body's hepatic and renal clearance [57, 61]. The depletion of drug from the depot in a sustained manner provides an opportunity for the cancer cell to develop adaptive resistance [5]. There is evidence that complex drug release profiles are advantageous over continuous release for tumor size reduction [11, 80]. Pulsatile doses (several on/off ultrasound pulses) spreads out the US energy into short segments which avoids burst release of drugs and excessive blood vessel extravasation [6, 57, 69-70]. The most important advantage of applying pulsatile dose on tumor tissue is avoiding adaptive resistance of cancer cells and increase the cytotoxicity of anticancer drugs [5]. The US-responsive C12 methylene chains coated pHEMA/PEGDMA hydrogels described by Kwok *et al.* was found to be released insulin for five days in therapeutic range at 1.1 MHz US pulses (for 5 min twice a day) and maintained minimum leaching rate when not stimulated (**Figure 12A**) [58].

Figure 12: (A) Release of insulin from methylene chains-coated pHEMA/PEGDMA matrices when exposed to 1.1 MHz in the US for 5 min. Adapted from Kwok *et al.* [58]. (B) Trypan blue release from liposome-microbubble conjugate in hydrogels *in vivo* when subjected to US of 6 W/cm² intensity for 1 min. Adapted from Epstein-Barash *et al.* [61].

Epstein-Barash *et al.* was able to get triggered release of trypan blue dye for 2 weeks by injecting hydrogels (**Figure 12B**) with dye-containing liposomes and microbubbles in vivo and using a short pulse with a relatively low-intensity (six pulses, 10 sec on and 1 sec off, 48 h interval) [61]. Huebsch *et al.* presented a high concentration mitoxantrone dose of 28.8 ug/min to the MDB-MB-231 and MCF7 cell lines for a short time of 2.5 min/12 h and repeated for five days. This profile was able to keep the lowest viability of cell (less than 10%) compared to the sustained release profile of the same integrated dose. (**Figure 13**) This group achieved significantly low tumor volume on Nu/J mice models on both short-term (8 days) and 50% survival after long-term (180 days) experiments of 2.5 min/d pulsatile dose for [6].

Figure 13: Effect of mitoxantrone dose profiles on the viability of cancer cells. (A) schematic representation of sustained vs. pulsatile dose having the same integrated dosing amount. (B) Fluorescence micrographs MDA-MB-231 breast cancer cells exposed to a sustained mitoxantrone dose (Center) or a lower baseline drug dose with a periodic high dose, applied for 10 min once every 12 h (right). (C) Cell viability in MDA-MB-231 (blue) and MCF7 (red) breast cancer cell lines treated with sustained or pulsatile exposure to mitoxantrone. Adapted from Huebsch *et al.* [6].

Wu *et. al.* was able to get on-demand triggered release of large and small molecules BSA (66 kDa) and dextrans (3-5 kDa) from NIPAM/MBAm hydrogels (**Figure 14A**) when stimulated with 2 US pulses of 3 min duration (1 MHz, 30% duty cycle at 3 W/cm²) [74]. Wang *et al.* tested the anti-inflammatory effect of diclofenac loaded alginate microcapsules in the carrageenan-induced rat paw edema models with the US. The short US pulses of high intensity 9 W for 60 min in a pulsatile manner (**Figure 14B**) (two irradiations of 3 pulses, 2 min on, 4 min off with an interval of 24 min) was able to lower the inflammatory inhibition by 81% [70].

Figure 14: Cumulative release of (A) BSA (left) and dextran (right) from NIPAM/MBAm hydrogels [74] and (B) diclofenac from alginate microcapsules [70] when subjected to pulsatile US irradiations. Adapted from Wu *et al.* [74] and Wang *et al.* [70].

The PSPLBC loaded paclitaxel drug to melanoma tumor model in C57BL/6 mice described by Chandan *et al.* resulted in 100% survival rates after 2 weeks that incorporated 5 doses of the drug on every 3rd day with immediate administration of US (2 W/cm², 50% duty cycle, 60 s) [57]. Kearney *et al.* was able to get a switchable PEGlyted AuNP release on demand from alginate hydrogels over multiple days (on day 1 and day 5) with five 2.5 min/h US pulses of 9.6 mW/cm² intensity. During the incubation time, the group reported baseline diffusive release of 1-4% AuNP (**Figure 15**) whereas the US-induced 200-fold and 5000-fold higher release respectively on day 1 and day 5 [67].

Figure 15: Cumulative release of PEG-AuNPs from alginate microbeads using 9.6 mW cm⁻² US for 2.5 min h⁻¹ up to 5 hrs following five days of storage at 37⁰C. Adapted from Kearney *et al.* [67].

Kennedy *et al.* also reported higher retention of AuNP payload in the alginate capsules when not stimulated.100% The release was obtained on day seven after 100 sec US stimulation (20% amplitude of a 20 kHz signal) which exhibited a similar release profile as day 1 [68]. This group demonstrated a sequential release of iron oxide particles from weak and strong capsules (50 mM CaCl₂ and 100 mM BaCl₂ respectively) loaded in 2 wt.% alginate hydrogels in tissue model by 20% and 80% amplitude of a 20 kHz signal in for 10 sec [68].

Figure 16: (A) Percent AuNP release vs. time for when weak and strong capsules were exposed to ultrasound for 200 s on day seven at 20% amplitude followed by at 80% amplitude for 100 s five minutes later. (B) Images of the release of iron oxide loaded capsules integrated into Ca^{2+} crosslinked alginate hydrogels when subjected to US stimulation under the chicken carcass model at 37°C . Adapted from Kennedy *et al.* [68].

Instead of degradation based sustained drug release profile, a pulsatile profile can be optimal for cancer tumor treatment whereas sequential release can be useful for tissue engineering application like bone regeneration, wound healing, and vascularization, etc. that require presentation of multiple agents to functionalize properly.

2.4.2 Prospected strategies

Using tunable parameters such as frequency, intensity, exposure time, gel construction several treatment strategies can be designed.

a. Scaffold with strong capsules

There is evidence that sequential administration of 5-fluorouracil (5FU) and irinotecan can be more cytotoxic to pancreatic cancer treatment [10, 12]. A system can be developed to release two anti-cancer drugs from a single scaffold sequentially on different days (**Figure 17A**). For example, A hydrogel can be designed to release 5-fluorouracil at first by diffusion for few days while retaining the irinotecan loaded strong microcapsules crosslinked by BaCl_2 as suggested by Kennedy *et al.* [68]. When 5FU is depleted US can disrupt the microcapsules to release irinotecan with precise temporal control.

b. Scaffold with differently sized PEGlyted AuNPs

A depot can be designed to release multiple agents selectively by tuning the size of the NP containing microbeads (**Figure. 17B**). Smaller PEGlyted AuNP in alginate microbeads were more responsive to the US than bigger AuNPs [67]. For example, 100 nm, 60 nm, and 30 nm diameters of AuNP can be incorporated with multiple agents where the different intensity of US can selectively release different agents.

c. Two compartment scaffold

Two-compartment scaffold with different release kinetics can be fabricated. A concentric cylindrical two-compartment structure can be useful for cancer treatment where the outside matrix can be made with low polymer concentration for pro-apoptotic drug release with low-intensity US (**Figure 17C**). The inner compartment

made with high polymer concentration that can retain the drug long-term and will release on-demand high intensity focused on the US.

Figure 17: Prospected strategies for improving the release profiles by US stimulation.

(A) Scaffold with strong capsules, (B) Scaffold with differently sized PEGlyted AuNPs, (C) Two compartment scaffold, (D) Scaffold with strong capsules, (E) Scaffold with microbubbles and strong capsules.

d. Scaffold with strong capsules

A scaffold with twice as much strength than 2 wt % Ca^{2+} crosslinked alginate hydrogels responsive to the US may provide prolonged release of growth factors (**Figure. 17 D**). For bone regeneration, wound healing and vascularization, a scaffold with GF 1 can help the nascent cell to grow, where GF 2 released from stronger capsules by the US will aid the regeneration process.

e. Scaffold with microbubbles and strong capsules

A release profile can be tailored by incorporating microbubbles with drug-containing nanocarriers. For example, a strong nanocarrier (liposome, micelles,

microbeads) can be ruptured by cavitating microbubbles (**Figure 17E**). Microbubbles also used for DNA and gene therapies on-demand with US-irradiation [53-55, 57, 60-61].

Drug delivery from a depot by ultrasonic stimulation largely depends on the degradation of the polymer matrix. Uncontrolled leaching by any unpredicted burst release may cause clinical toxicity to patients. Hence, depot should have robust structural integrity to maintain low baseline release when not stimulated.

2.5. References

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CHAPTER 3

Manuscript 1: Ultrasonic generation of pulsatile and sequential delivery profiles from calcium-crosslinked alginate hydrogels

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Tania Emi¹, Kendra Michaud², Emma Orton², Grace Santilli¹, Catherine Linh², Meaghan O'Connell², Fatima Issa² and Stephen Kennedy^{1,2,*}

¹Department of Chemical Engineering, University of Rhode Island, Kingston, RI 02881

²Department of Electrical, Computer, and Biomedical Engineering, University of Rhode Island, Kingston, RI 02881

*Corresponding Author: Stephen M. Kennedy, Ph.D. Email: smkennedy@uri.edu

3.1. Abstract

Control over of biological processes can potentially be therapeutically regulated through localized biomolecular deliveries. While implantable hydrogels can provide localized deliveries, they do not traditionally provide the temporally complex delivery profiles required to regulate complex biological processes. Ionically crosslinked alginate hydrogels have been shown to release encapsulated payloads in response to a remotely applied ultrasonic stimulus, thus potentially enabling more temporally complex delivery profiles. However, thorough characterizations of how different types of molecular payloads are retained and ultrasonically released need to be performed. Additionally, the impact of potentially disruptive ultrasonic stimulations on hydrogel structure and temperature need to be characterized to better understand what range of ultrasonic signals can be used to trigger release. Finally, experiments need to be performed demonstrating that ultrasonic stimulation can be used to generate release profiles demonstrated to have potential therapeutic benefits (e.g., pulsatile and sequential anticancer delivery profiles). This work addresses the above-described needs and underscores the potential of using ultrasonically responsive polymeric hydrogels for improving drug delivery treatment strategies.

3.2. Keywords

Ultrasound; on-demand release; cancer therapy; drug delivery; biomaterials.

3.3. Introduction

Biological systems conduct themselves with high degrees of spatial and temporal complexity. Many of the biological processes that underlie injury and disease constantly change in space and time and must be regulated with spatiotemporal

precision for proper therapeutic outcome. For instance, wound healing and tissue engineering applications involve complex cell instructions where the spatial gradient, timing, and sequence of bioactive molecules are vital to orchestrating proper tissue regeneration [1-5]. Regeneration of tissues requires the generation of vascular networks. This involves initializing angiogenic sprouting from nearby existing vasculature (through the establishment of pro-angiogenic signaling factor gradients emanating away from the regeneration site). After these sprouts have invaded the site of regeneration, they must mature into larger, blood-perfusing vessels (through localized presentations of pro-maturation signaling factors) [1-2]. Regenerating tissues also demands the establishment of tissue-specific cells types, requiring recruitment of progenitor cells to the injury site (through the establishment of recruitment factor gradients emanating away from the injury site). Then, these recruited progenitors must be directed to proliferate and differentiate (through localized presentations of proliferation and differentiation signaling factors) [3, 6-9]. Beyond regenerative therapies, cancer treatment strategies often aim to present anticancer therapeutics locally at tumor sites to minimize off-target side effects and polymeric hydrogel materials can be used to provide this localized delivery [10]. However, there is growing evidence that the localized and sustained deliveries provided by hydrogels are not optimal and may be particularly not well-suited for preventing adaptive resistance. Many emerging tumor treatment strategies thus involve more temporally dynamic, pulsatile delivery schedules [11-13] and even sequences of multiple anticancer therapeutics [14-21].

Polymeric hydrogels have shown the ability to release bioactive payloads in response to externally applied stimuli such as changes in pH [22], light [23], temperature [24], electric field [25], magnetic field [26-31], ultrasound [32-35]. These stimuli-responsive polymeric hydrogel systems could thus provide the localized, on-demand, complex molecular deliveries needed to direct spatiotemporally complex biological processes. Ultrasonically responsive hydrogels are of particular interest in that ultrasound (US) has been extensively used in a wide variety of therapeutic applications owing to its safety, relatively high spatiotemporal precision and ability to penetrate tissues [36-38]. Ca^{2+} crosslinked hydrogels respond to ultrasound stimulation reversibly (i.e., they can self-heal due to the calcium ions re-crosslinking the network after US-stimulation) which gives enhanced release when stimulated and minimal baseline release when not stimulated [33, 39]. However, repeated and/or too-intense of US stimulus can irreversibly damage the hydrogel scaffold. This is undesirable in that (i) alteration of the hydrogel structure can impact baseline release kinetics and (ii) loss of hydrogel integrity could prohibit subsequent ultrasonically triggered release events. Additionally, repeated and/or too-intense of US stimulus can render sensitive payloads bio-inactive. While several previous studies have demonstrated ultrasonically triggered release from hydrogels—with some systems even demonstrating the ability to produce sequential release profiles by leveraging multiple ultrasonically triggerable compartments [40-42]—it remains unclear (i) how the intensity and duration of ultrasonic stimulation impacts release for a diverse range of molecules, (ii) how the intensity and duration of ultrasonic stimulation impacts temperature increase and hydrogel integrity, and (iii) if pulsatile and multi-payload

delivery profiles can be produced through ultrasonic stimulation of bulk hydrogel materials. The work presented here endeavored to elucidate these issues.

3.4. Results

3.4.1. Impact of ultrasonic intensity and duration on molecular release from Ca^{+2} crosslinked hydrogels

To investigate how different ultrasonic exposures impacted release from calcium-crosslinked hydrogels, 1 wt% alginate hydrogels with 30 mM calcium crosslinker were fabricated and pre-loaded with a variety of different molecules (dextrans of various size and charge, chemotherapeutic agents, and different protein signaling factors) (**Figure 1a** (i): hydrogels loaded with FITC-labeled dextran). This hydrogel formulation was previously reported to be capable of ultrasonically triggered drug delivery [33, 39, 42]. Immediately after making the hydrogels, they were cut into small cylinders and were transferred to scintillation vials with 5 mL Dulbecco's phosphate buffered saline (DPBS) and subjected to ultrasound stimulation at various amplitudes for various durations (**Figure 1a** (ii)-(vi)). Immediately following ultrasonic stimulation, samples were collected to ascertain the amount of the molecular release (**Figure 1a** (vii)) (see Materials and Methods for more details).

We hypothesized that the size and charge of molecules would have an impact on ultrasonically stimulated release from these alginate hydrogels. To test this hypothesis, we studied the release of four different model macromolecules (FITC dextrans) when subjected to US stimulation of 0%, 20% and 40% amplitude for durations of 1 and 5 min. Two sizes were used: relatively small dextrans of 3-6 kDa and larger dextrans of 70 kDa. Additionally, to investigate the impact of charge, uncharged dextrans were

used as well as DEAE-modified cationic dextrans. It was thought that the positively charged side groups on the DEAE dextrans would electrostatically interact with the negatively charged groups presented by the alginate hydrogel matrix, thus impacting release.

Figure 1: Ultrasonic amplitude, ultrasonic duration, molecular size, and molecular charge influence the amount of molecular release from Ca^{+2} crosslinked hydrogels. (a) FITC-Dextran-loaded Ca^{2+} crosslinked alginate hydrogel fabrication: casting gel on a sigma-coated glass plate (i), cutting into 8-mm-diameter cylindrical gels using a biopsy punch (ii), putting a gel in a scintillation vials with 5 mL DPBS (iii), gels before, during, and after ultrasound stimulation (iv-vi), and 1 mL

sample collection for quantifying molecular release (vii). (b) – (e) FITC-dextran release vs. ultrasonic amplitude for gels exposed to 1-min (blue) and 5-min (red) of ultrasonic stimulation. Asterisks (*) indicate levels of statistical differences for release compared to controls (0% amplitude) unless otherwise specified with lines. N = 4.

Indeed, cationic dextrans released at levels generally lower (**Figure 1d** and **1e**) than their neutrally charged counterparts (**Figure 1b** and **1c**). In some instances, the size of the dextran impacted release as well. For example, ultrasonic stimulation did not yield higher amounts of release than controls (0% amplitude) for hydrogels loaded with larger cationic dextran, except when stimulated at 40% amplitude for 5 minutes (**Figure 1e**). However, smaller cationic dextrans did release with statistical significance at 20% amplitude for 5 minutes, 40% amplitude for 1 minute, and 40% amplitude for 5 minutes (**Figure 1d**). For neutrally charged dextrans, size seemed to particularly impact baseline, unstimulated release. For instance, higher amounts of smaller neutral dextran released under 0% ultrasonic amplitude (**Figure 1b**, blue and red bars at 0%) than larger neutral dextrans (**Figure 1c**, blue and red bars at 0%). These results are consistent with the notion that larger, more electrostatically interactive molecules exhibit less mobility in and through the negatively charged hydrogel matrix and thus exit the matrix at reduced rates.

As expected, the amplitude and duration of ultrasonic stimulation also influenced release. Generally, for increasing ultrasonic amplitudes, there was an upward trend in the amount of molecular release (**Figure 1b** and **1c**: both blue and red bars trended upwards at increasing ultrasonic amplitudes). In fact, nearly all the drug contained in each hydrogel (80.4 $\mu\text{g/gel}$) was released when stimulating neutral dextran-loaded

hydrogels with 40% ultrasonic amplitude for 5 minutes (**Figure 1b** and **1c**, red bars at 40%). However, this trend was less pronounced when examining the cationic dextrans. The smaller cationic dextran maintained only a modest upward trend as ultrasonic amplitude was increased (**Figure 1d**). This upward trend was not exhibited well for the larger cationic dextran (**Figure 1e**). Thus, for larger molecules that exhibit the opposite charge as the polymer matrix, there seems less ability to ultrasonically select the amount of molecular release by stimulating at specific amplitudes and durations. While the larger cationic dextran did release from the hydrogels with statistical significance when stimulated at 40% ultrasonic amplitude for 5 minutes, these degrees of ultrasonic stimulation may be disruptive to both the hydrogel structure and the payload itself (see Section 2.2).

While the use of dextrans as model drugs was useful for understanding general trends in how molecular weight and charge impacted ultrasonically triggered delivery, we wanted to perform experiments characterizing the release of actual therapeutic agents. Therefore, we investigated the release of small molecular weight chemotherapeutics (mitoxantrone, irinotecan, and 5-fluorouracil) from these hydrogels under the ultrasonic stimulus. Note that when using different chemotherapeutic agents, different amounts of agent needed to be loaded in the hydrogels in order for the amount released to be detectable and quantifiable via absorbance spectroscopy (40.2 $\mu\text{g/gel}$, 201.1 $\mu\text{g/gel}$, and 80.4 $\mu\text{g/gel}$ of chemotherapeutic for mitoxantrone, irinotecan, and 5FU respectively). Thus, these data are plotted as percent release on their y-axis rather than the amount released in weight for easier comparisons. As with the model dextrans, stronger US amplitudes generally resulted in a higher degree of

drug release (**Figure 2**, bars trend upwards for increasing amounts of ultrasonic amplitude). Also, increasing the duration of ultrasonic stimulation increased the amount of release (**Figure 2**, red bars are generally higher than blue bars at the same ultrasonic amplitude).

Figure 2: The amplitude and duration of ultrasonic stimulation impact the amount of chemotherapeutic released. Release vs. ultrasonic amplitude for mitoxantrone (a), irinotecan (b), and 5-fluorouracil (c). Asterisks (*) indicate statistically significant release compared to controls (0% amplitude) unless otherwise specified with lines. N = 4.

The relative size and charge of these chemotherapeutics likely impacted release characteristics to some degree, though some inconsistencies were observed. Though all three-chemotherapeutics tested here were relatively small molecules, they did exhibit different charges: mitoxantrone (444.5 Da, +2 charge), irinotecan (586.7 Da, +1 charge), and 5FU (130.1 Da, neutral charge). Irinotecan diffused out of the gels at higher levels than mitoxantrone (**Figures 2a** and **2b**: red and blue bars at 0% are higher for irinotecan than mitoxantrone). This seemed reasonable in that irinotecan had a lower positive charge density and would have a lower electrostatic affinity to the negatively charged alginate matrix. However, 5FU (which is neutral and the smallest

chemotherapeutic examined here) did not diffuse out of the gels at lower amounts than irinotecan as initially expected (**Figure 2b** and **2c**: bars at 0% US amplitude were, in fact, lower for 5FU than irinotecan). Note, however, that release kinetics are also based on parameters such as polarity. In fact, 5FU is polar and water-soluble (~1 g/L) whereas irinotecan is less water-soluble (~0.1 g/L). Thus, 5FU may present more opportunities to hydrogen-bond with alginate (which presents an abundance of electronegative groups) compared to the less polar irinotecan. But, for all three chemotherapeutics, application of ultrasound greatly enhanced release. 75% - 90% of chemotherapeutic could be released when stimulated at 40% ultrasonic amplitude for 5 minutes (though with the caveat that this type of ultrasonic stimulation may be disruptive to the gel and possibly even the drug molecule itself).

Figure 3: The amplitude and duration of ultrasonic stimulation impact the amount of signaling factor released. Release vs. ultrasonic intensity for VEGF (a) and PDGF (b). Asterisks (*) indicate statistically significant release compared to controls (0% amplitude) unless otherwise specified with lines. N = 4.

To examine how ultrasonic stimulation impacted signaling factor release, calcium-crosslinked alginate gels were loaded with 6100 pg/gel of vascular

endothelial growth factor (VEGF, a pro-angiogenic factor) and platelet-derived growth factor (PDGF, a pro-maturation factor) and stimulated at various ultrasonic amplitudes for different amounts of time. Despite being loaded with the same amount of protein, VEGF generally released in higher amounts than PDGF (**Figure 3**, comparing parts a to b). This is likely due to VEGF having a lower affinity to the alginate contained in the hydrogel's matrix compared to PDGF's alginate affinity. As was seen with dextrans and chemotherapeutics, longer ultrasonic durations and stronger amplitudes resulted in higher levels of release (**Figure 3**, upward trends at higher amplitudes and red bars being higher than blue bars at the same amplitude).

3.4.2. Erosion and heating of scaffold due to ultrasound stimulation

While the data described in the previous section underscores the ability to ultrasonically regulate molecular release, application of ultrasonic stimulation can potentially irreversibly damage the hydrogel structure (prohibiting subsequent deliveries from the hydrogel) and/or reduce the bioactivity of sensitive therapeutic payloads (like protein signaling factors due to thermally induced conformational alterations). To investigate the range of ultrasonic stimulations that do not overly erode or heat the hydrogel structure, experiments were conducted that quantified the degree of hydrogel erosion and rise in temperature when exposed to different amplitudes and durations of ultrasound. As expected, with higher ultrasonic amplitudes and longer durations of exposure, hydrogel erosion was more pronounced (**Figure 4a**: photos of gels under different US conditions that were loaded with blue mitoxantrone). Quantifications of erosion (percent weight loss before/after ultrasound) revealed that the maximum US exposure (40% amplitude for 5 minutes) significantly

eroded these gels (**Figure 4b**). However, all other US conditions yielded erosions lower than 50%. In terms of gel heating, all ultrasonic stimulations resulted in heating, as expected. However, it was desirable to keep temperature increases to under 8°C (i.e., as to not increase temperatures from body temperature (37°C) to temperatures that may impact protein conformation (45°C)). Indeed, both the 1-minute at 20% US amplitude and 5-minute at 20% US amplitude conditions heated samples less than 8°C (**Figure 4c**). When taking both gel erosion and heating into account, the 1-minute 20% of US exposures appeared to be the safest to use. However, these 1-minute, 20% US amplitude exposures did not yield statistically significant enhancements in release for cationic dextrans (**Figures 1d** and **1e**, blue bars at 20%), mitoxantrone (**Figure 2a**, blue bar at 20%), or VEGF and PDGF (**Figure 3**, blue bars at 20%).

Figure 4: Ultrasound erodes and heats the gels in manners that are dependent on the intensity and duration of the applied ultrasound. (a) Photographs of mitoxantrone-loaded hydrogels after exposure to the US at the indicated amplitudes and durations. (b) Percent weight loss vs US intensity for gels stimulated for 1 minute (light blue) and 5 minutes (light purple). (c) Temperature elevation vs US intensity for gels stimulated for 1 minute (yellow) and 5 minutes (orange). Asterisks (*) indicate

statistically significant differences compared to controls (0% amplitude) unless otherwise specified with lines. N = 4.

3.4.3. Triggered release delivery by pulsing the ultrasound stimulation

Even though 1-minute ultrasound exposures at 20% amplitude did not statistically enhance releases for a many of molecules tested here, we hypothesized that repeated 1-min, 20% US exposures could enhance release while helping to preserve hydrogel structure and maintain relatively low temperatures. That is, the mechanism of ultrasonic release from these calcium-crosslinked alginate hydrogels is thought to be due to the displacement of calcium crosslinks (due to ultrasonically driven cavitation within the matrix) [33, 39, 41]. This displacement allows the matrix to unravel, and in the process, liberate drugs trapped within the matrix. If ultrasonically stimulated mildly enough, the matrix may remain relatively intact with enough free calcium crosslinkers present to re-crosslink the matrix upon termination of the ultrasonic stimulus (though some drug may be liberated from the matrix during stimulation). If the ultrasonic stimulation is of sufficient duration and intensity, the calcium crosslinked, and the alginate polymer may become so dispersed that the matrix is not able to re-crosslink. So, if a gel is (i) initially stimulated with the US that does not overly disrupt the matrix but produces some drug release, then (ii) left to recover with the US is turned off, and then (iii) subsequently stimulated with US and allowed to recover, these pauses in ultrasonic stimulation may enable appreciable amounts of accumulated release while providing time for the matrix to recover and for heat to dissipate between subsequent ultrasonic stimulations.

To test this hypothesis, we exposed calcium-crosslinked alginate gels to 1-minute US pulses at 20% amplitude in pulse trains with varying pulse numbers over a 1-hour period. Specifically, gels were exposed to controls (no pulses), one 1-min pulse, two 1-min pulses, three 1-min pulses, four 1-min pulses, and four 1-min pulses (Figure 5a, moving left to right).

Figure 5: Pulsed ultrasound can enhance drug delivery while helping to control gel erosion and heating. (a) Photos of hydrogels (bottom row) after exposure to the illustrated ultrasonic pulse trains (top row). (b) Percent weight loss of hydrogels vs. pulse number. (c) Temperature rise vs. pulse number. (d) Mitoxantrone release vs. pulse number. Asterisks (*) indicate statistically significant differences when compared controls unless otherwise specified with lines. N = 4.

Additionally, gels were exposed to one 4-min pulse (Figure 5a, rightmost illustration) which is equivalent to four 1-min pulses with no delay time between them. This was done to directly compare the same total exposure of ultrasonic

stimulation with and without pauses (i.e. pulsed ultrasound vs. continuous). While increasing the number of ultrasonic pulses slightly increased the degree of erosion (**Figure 5b**: increasing values for 1, 2, 3, and 4 1-min pulses, though the 3 and 4 pulse conditions were statistically indifferent), temperature elevation did not appreciably increase for higher pulse numbers (**Figure 5c**: similar values for 1, 2, 3, and 4 1-min pulses). And, the amount of drug released did increase for increasing pulse numbers (**Figure 5d**: increasing values for 1, 2, 3, and 4 1-min pulses). Thus, increasing the number of ultrasonic pulses may provide a means to increase drug release while helping to minimize temperature increase and, to some extent, help maintain gel structure (compared to increasing the amplitude or duration of the US signal, which was shown to dramatically increase heating and gel erosion (**Figure 4b** and **4c**)).

Also, of note was that one continuous 4-minute pulse eroded and heated the gel much more than 4 individual 1-minute pulses separated by 14 minutes (**Figures 5a** and **5b**: comparing 1,4-min bars to 4, 4-min bars). While the highly eroded gel naturally yielded a higher degree of drug release (**Figure 5c**, comparing 1,4-min bars to 4, 4-min bars), these results suggest that pausing between shorter US pulses can help maintain the structure and temperature of the gel while still producing statistically significant amounts of drug release.

3.4.4. Ultrasonically generating pulsatile temporal delivery profiles

As discussed in the introduction, many emerging drug delivery strategies involve generating localized delivery profiles that are more temporally complex. For example, there is growing evidence that pulsatile chemotherapeutic delivery profiles are more effective in destroying tumor cells than sustained deliveries [11, 19, 33, 43]. Because

of this potential, we aimed to demonstrate that ultrasonic stimulation could be used to generate pulsatile delivery profiles from these gels by periodically turning on and off the ultrasonic signal. To mimic a pulsatile mitoxantrone delivery profile that was previously demonstrated to enhance melanoma cell destruction [43], we exposed mitoxantrone-loaded, calcium-crosslinked alginate hydrogels to a 1-hour period of pulsed US stimulation one time a day for 3 days. Specifically, for a 1-hour period each day (for 3 days total), gels were stimulated with various US pulse trains composed of 1-minute pulses at 20% amplitude: (i) 0 pulses (control), (ii) one 1-minute pulse (and 59 minutes of no stimulation), (iii) two 1-minute pulses (with 29 minutes of no stimulation following each pulse), and (iv) three 1-minute pulses (with 19 minutes of no stimulation following each pulse).

Figure 6: Ultrasound can be used to generate pulsatile chemotherapeutic delivery profiles over a 3-day period, though gel structure is not maintained, and delivery rates change vs. time. (a) Photos of hydrogels immediately after ultrasonic exposures

on the indicated days. (b-d) The rate of mitoxantrone release vs time when stimulated with pulsed ultrasound for 1 hour each day for control and one 1-min pulse (b), two 1-min pulses (c), and three 1-min pulses (d). Asterisks (*) indicate the degree of statistical differences relative to controls. n.s. indicates that no statistical significance was found compared to controls. N = 4.

Photographs were taken immediately following each 1-hour stimulation period to assess the relative degree of gel erosion (**Figure 6a**). As expected, US exposure regimens that involved a higher number of pulses generally resulted in higher degrees of gel erosion. For US exposures of two and three 1-minute US exposures, gels exhibited high degrees of erosion by the 3rd day of the experiment (**Figure 6a**, rightmost column, bottom two rows: gels began to fragment). While exposures to single 1-minute US pulses allowed the gels to remain relatively intact (**Figure 6a**, rightmost column, 2nd from top row), these mild ultrasonic stimulations did not produce pulsed mitoxantrone release rates that were statistically higher than controls (**Figure 6b**, red and black curves are statistically similar). The two and three 1-minute pulsed US exposures did yield statistically significant mitoxantrone release rates during these 1-hour “on” periods (**Figures 6c and 6d**) but did so in an inconsistent manner vs. time. That is, mitoxantrone release rates during 1-hour “on” periods tended to increase over time, particularly on the final day of the experiments (**Figure 6c and 6d**: the pulse heights from 48-49 hours were higher than from 0-1 and 24-25 hours). We attributed this to the gel being more highly eroded from repeated ultrasonic exposures, having more surface area by day 3, and possibly being more fragile.

Nonetheless, statistically significant pulsatile delivery rates were achieved, and the gels were not completely annihilated after these 3-day experiments.

To achieve more consistent pulsatile mitoxantrone delivery rates during subsequent “on” periods and to help maintain gel structure, we explored the idea of modifying US exposures vs. time. Specifically, we reduced the number of US pulses used during the 1-hour US exposure period on day 2. That is, we used 3, 1-minute pulses on day 1 (from 0-1 hours), and then reduced the US pulse number to 2, 1-minute pulses on days 2 and 3 (from 24-25 hours and from 48-49 hours). This strategy resulted in more mild gel erosion over time upon visual inspection (**Figure 7a**, bottom row) and provided more uniform mitoxantrone pulse rates during 1-hour “on” periods on subsequent days (**Figure. 7b**, pulse heights of the blue curve are of similar heights at times 0, 24, and 48 hours).

Figure 7: Pulsatile delivery profiles with consistent release rates vs. time can be achieved by altering the US pulse number over time. (a) Photos of hydrogels immediately after ultrasonic exposure on each day for control gels and those exposed to the indicated pulsed US regimen. (b) Release rate vs. time when stimulated with 3, 1-minute pulses on day 1 and 2, 1-minute pulses on days 2 and 3.

1-min; 2, 1-min; and 2, 1-min ultrasound pulses on day 1, 2, and 3, respectively. Asterisks (*) indicates levels of statistical differences relative to controls. N = 4.

3.4.5. Sequential 5-fluorouracil and irinotecan delivery on melanoma cells in vitro

Some treatment strategies involve the sequential delivery of two or more therapeutics. For example, sequential delivery of 5FU and irinotecan is commonly used for treating colorectal cancer, though there is some debate as to which therapeutic should be delivered first: 5FU followed by irinotecan vs. irinotecan followed by 5FU [17-18, 21]. Though particular sequences may be used to optimize therapeutic outcome, other parameters likely impact outcome as well: the duration of each therapeutic delivery, the temporal profile of each therapeutic delivery, the relative dose of each therapeutic, the spacing between each delivery, etc. For example, we conducted in vitro experiments on melanoma cells by exposing them to different delivery schedules involving both 5FU and irinotecan. B16F10 mouse melanoma cells were plated, allowed to grow for 36 hours, and then exposed to different delivery schedules of 5FU and irinotecan for 36 hours (**Figure 8a**, schedules s1 – s4). B16F10 populations were monitored in real time using an xCELLigence system (which uses the electrical impedance of a well-plates surface to quantify cell population vs. time). xCELLigence data from these experiments are included as cell index (a measure of cell population) vs. time in **Figure 8b**. For the four delivery schedules used here (s1 – s4), all resulted in statistically similar B16f10 populations except for schedule s3 (**Figure 8b**, s3 purple curve resulted in statistically smaller populations at +36 hours). So, for these particular 5FU and irinotecan doses, durations, and timings on this particular cell type in vitro, schedule s3 (5FU followed by irinotecan) was most

effective in reducing melanoma cell populations. Critically, it is unclear what sequence would be optimal when testing a more diverse array of drug types, timings, doses, and sequences on different types of cancers in vivo. Therefore, material systems are urgently needed where these types of parameters can be readily controlled and modified from experiment to experiment. Ultrasonically responsive hydrogels could provide a material system for performing such optimizations in vivo if they can be tailored to generate multi-therapeutic, sequential delivery profiles.

Figure 8: 5-fluorouracil and irinotecan delivery sequence had an impact on melanoma cell survival. (a) A timeline describing the in vitro cytotoxicity experiment where B16F10 mouse melanoma cells were seeded and allowed to grow for 36 hours before treatment and exposed to several treatment schedules s1-s4 for 36 hours. (b) Normalized cell index (B16-F10 cell population) vs. time when exposed to s1 (red

curve), s2 (blue curve), s3 (purple curve, blue marker), s4 (purple curve, red marker).

** indicates a statistically significant difference with $p < 0.01$. $N = 4$.

3.4.6. Generation of the sequential 5-fluorouracil and irinotecan deliveries

To demonstrate the ability to produce sequential delivery profiles from these ultrasonically responsive hydrogels, we aimed to mimic the delivery profile which reduced melanoma cell populations most efficiently in the above-described studies: schedule s3 (initial 5FU delivery followed by irinotecan delivery starting at 18 hours). So, one criterion of our design demanded that 5FU exit the gels rapidly at early time points. While 5FU did not diffusively release as much as irinotecan (comparing **Figures 2b** and **2c** at 0% US amplitude), a non-trivial amount of 5FU still did released when no ultrasonic stimulation was applied (**Figure 2c**, ~10% release in 1 to 5 minutes). We believed that, over the course of 18 hours, this amount of release could be therapeutically relevant. Thus, our strategy was to design the gel to initially release 5FU via diffusion with no ultrasonic stimulation and then ultrasonically trigger irinotecan release at 18 hours. Additionally, the initial release of 5FU could be further enhanced by the method of loading 5FU into the gel. That is, instead of incorporating 5FU into the matrix during gel fabrication, 5FU could be loaded by soaking the gel in concentrated 5FU to the gel after gel fabrication. This would potentially limit how well 5FU incorporated into the gel and help it release more readily from the matrix.

To achieve delayed, ultrasonically triggered irinotecan delivery, it was desirable to have the gel release as little irinotecan as possible when no ultrasound was applied for the first 18 hours and then release as much as possible when ultrasonically stimulated (at 18 hours). However, examination of **Figure 2b** reveals that irinotecan

releases at high rates, even when not ultrasonically stimulated ($> 40\%$ release in 5 minutes = 80 mg in 5 minutes = 16 mg/min). To enhance unstimulated irinotecan retention, we pursued two strategies. First, we believed that reducing the hydrogel's mesh size (by increasing the polymer and crosslinker concentrations from 1.0 wt% alginate with 30 mM Ca^{2+} to 1.6 wt% alginate w/ith 50 mM Ca^{2+}) would serve to better entrap and impede the release of irinotecan. This, of course, could also lead to lower amounts of release when ultrasonically stimulated. However, ultrasonically stimulated delivery of irinotecan could be very efficient (**Figure 2b**, 40% US amplitude for 5 min results in 90-100% release). So, we hoped that ultrasonic stimulation would still produce strong amounts of irinotecan release even with higher polymer and crosslinker concentrations. Second, we believed that rinsing the gels could help reduce the amount of diffusive irinotecan release at early time points. The studies presented in **Figure 2** were performed immediately after gel formation and did not include a rinsing step. If they were rinsed, some of the less well-incorporated irinotecan could be removed, thereby reducing initial, diffusive release.

Figure 9: Ultrasonically responsive gels can be used to generate a triggered release of irinotecan after an initial burst release of 5-fluorouracil. (a) Cumulative

release of 5-fluorouracil (blue) and irinotecan (red) vs time when ultrasonically stimulated for 2-minutes at 40% US amplitude at 18 hours. Control gels (black) were not subjected to ultrasonic stimulation at 18 hours. (b) The 18-hour average rate of 5-fluorouracil and irinotecan release vs. time for the same conditions presented in part (a). Asterisk (*) indicates levels of statistical differences relative to controls. N = 4.

Taking this all together, we produced 1.6 wt% alginate, 50 mM calcium-crosslinked gels (60% polymer and crosslinker) that were loaded with 200 mg/gel of irinotecan and 0 mg of 5FU. Gels were rinsed for 5 minutes in 5 mL of DPBS to remove excess irinotecan that did not incorporate well into the hydrogel matrix. Then, gels were soaked in 100 mL of concentrated 5FU (2 mg/mL) for 30 minutes to load the gels with 5FU in a manner that would be conducive to rapid, burst release. When placed in experimental media at time 0, indeed, gels released 5FU rapidly with low levels of irinotecan release (**Figure 9a**: 5FU curve (blue) rises to ~ 90 mg in the first 2 hours whereas the irinotecan curve (red) does not rise to detectable levels). For the 18 hours prior to ultrasonic stimulation, 5FU reached ~100 mg of cumulative release whereas irinotecan reached only ~10 mg (**Figure 9a**: values of blue and red curves at 18 hours, respectively). Immediately after being stimulated with an ultrasonic signal at 40% US amplitude for 2 minutes, irinotecan released reached over 25 mg of cumulative release (**Figure 9a**, jump in the red curve just after 18 hours). However, because the gel still had 5FU loaded in it, 5FU levels also increased in response to ultrasonic stimulation (**Figure 9a**, jump in a blue curve just after 18 hours). For the remaining 18 hours of the experiments, very little changes in 5FU and irinotecan release were observed (**Figure 9a**: flatline curves from just after US stimulation to 36

hours). When looking at time-average release rates for the 18 hours prior to and following the US, as designed, release rates of 5FU were higher before the US and fell to lower levels after US (Figure 9b, the blue curve is at higher values before 18 hours). And, irinotecan release rates were lower before the US and increased after the US (Figure 9b: red curve is at higher values after ultrasound).

3.5. Discussion

The ultrasonically responsive Ca^{2+} crosslinked hydrogels explored here were capable of producing various delivery profiles that may provide benefits, particularly in cancer treatment strategies. The pulsatile release profiles demonstrated here (one, 1-hour “on” period of enhanced mitoxantrone delivery per day for 3 days) were demonstrated to reduce melanoma cell populations more effectively than the more constant delivery profiles provided by traditional hydrogel materials [33, 43]. It is thought that pulsatile deliveries can also help combat adaptive resistance in tumors [11-13, 19]. Critically, the specific pulsatile delivery parameters (i.e., pulse height (dose), pulse width (duration), duty cycle (the relatively time when the delivery is high vs. low), and pulse number (how many periods of enhanced delivery per day)) that lead to optimized tumor cell destruction likely depend on the specific therapeutics used, the type of tumor, and other patient specifics. Though this presents a wide parametric space for optimization, ultrasonically responsive gels such as those presented here could help streamline these optimizations. That is, delivery parameters such as pulse height, width, and duty cycle can be flexibly regulated by altering the timing, duration, amplitude, and pulse number of the ultrasonic signal. The studies presented here have uncovered some additional considerations moving forward.

Namely, that undesirable temperature increases and gel erosion occur at longer and higher-amplitude ultrasonic stimulations and that erosion can impact release kinetics vs. time (higher release rates vs. time). However, we presented strategies for dealing with these issues (i.e., by pulsing the ultrasonic signal itself and reducing ultrasonic stimulations vs. time).

While the pulsatile deliver profiles generated in these studies (**Figure 7b**) are very similar to those demonstrated to improve melanoma cell destruction [43], it remains unclear with what level of flexibility ultrasonically responsive gels systems can produce a diverse range of pulsatile delivery profiles while preserving gel integrity and temperature. For example, studies still need to be performed to demonstrate how many subsequent pulses can be generated before the gel is either depleted of drug or erodes to the point of disused. The range of delivery rates achievable during ultrasonic stimulation has also not been identified. While we believe that increasing US parameters such as amplitude, duration, and US pulse number will increase the rates of delivery, increasing these parameters also generates more heat and erodes the gels (as demonstrated here). Note, that while generation and heat and gel erosion can limit subsequent releases and render some more sensitive payloads bio-inactive, ultrasonic stimulation can have a complimentary effect on damaging tumors, as demonstrated previously by Heubsch et al. [33]. The studies conducted here have shown that 8 to 10 $\mu\text{g}/\text{min}$ mitoxantrone pulses can be achieved while preserving gel structures for 3 days, but we have not explored pulse numbers beyond 3, ultrasonic amplitudes and durations beyond 20% and 1-minute, or the pulsatile delivery of other anticancer payloads. Some fine-tuning can likely be achieved. Nonetheless, we believe these

studies underscore the potential of these ultrasonically responsive gels to provide pulsatile delivery strategies and reinforce the need for continued exploration of their potential.

In addition to pulsatile anticancer deliveries, these ultrasonically responsive gels were demonstrated to produce a particular sequence of 5FU and irinotecan deliveries that were shown to reduce melanoma cells populations in vitro (i.e., 18 hours of heightened 5FU delivery followed by 18 hours of heightened irinotecan delivery) (**Figures 8 and 9**). While a specific drug-loading strategy was used to attain this specific sequence (i.e., irinotecan was loaded during gel fabrication and rinsed for enhanced retention whereas 5FU was loaded just prior to the start of the experiment for enhanced burst release), similar strategies can be employed for achieving different sequences. For example, if—as some believe it to be more beneficial [17, 21, 44]—a sequence of irinotecan followed by 5FU is desired, then 5FU can be incorporated during gel formation and irinotecan can be added just prior to experimentation. Future work on gel system demonstrated here includes optimizing parameters for achieving specific delivery rates at different times. For example, characterizations are needed to determine how hydrogel formulation (polymer and crosslinker concentrations), the ultrasonic stimulation parameters (US amplitude, US pulse width, US pulse number and delay between pulses), and the manner of loading various payloads impact the delivery rates of baseline, unstimulated irinotecan release rates and ultrasonically triggered irinotecan release rates. Additionally, future work needs to explore how changing these parameters can be used to control the timing of the sequence release. For instance, in these studies, delayed irinotecan release occurred at 18 hours. Being

able to flexibly control this time point will be critical to optimizing therapies. Ultrasonically responsive gel systems may provide unique advantages for optimizing the timing of deliveries in that triggered deliveries directly correspond to the timing of ultrasonic signal application. Finally, some cancer treatment strategies utilize sequences of different therapeutics [14-16]. Continued exploration of these ultrasonically responsive gels is needed to determine if they are suitable for coordinating sequential delivery of other therapeutic agents in cancer treatment and beyond.

Finally, while these hydrogels demonstrated promise for producing sequential deliveries in cancer treatments, they may also be of use in treating injuries and diseases for which sequential biological process must be controlled. The ability to generate sequential delivery profiles would be of great utility in tissue engineering applications, where sequences of biological events (such as recruitment, proliferation, and differentiation) can be directed through sequential delivery of recruitment, proliferation, and differentiation signaling factors [3, 6-9]. In addition to sequencing these factors (to establish tissue-specific cell types at the site of regeneration), vascularizing regenerated tissue is also critical. Generation of new vasculature also involves an important sequence of biological events: (i) generation of angiogenic sprouts from nearby, existing vasculature (which can be induced by projecting a pro-angiogenic signal gradients—such as VEGF—emanating away from the regeneration site) and (ii) maturation of the newly established network of angiogenic sprouts into larger, blood-perfusing vessels (which can directed through localized deliveries of pro-maturation factors—such as PDGF) [1-2]. Thus, calcium-crosslinked alginate gels

may enable the establishment of VEGF gradients through diffusive release of VEGF followed by delayed, ultrasonically triggered release of PDGF. This may help optimize deliver strategies for regenerating vasculature in tissue engineering applications. In fact, we demonstrated VEGF releases in modest amounts under no ultrasonic stimulation (**Figure 3a**, 200 pg in 5 minutes at 0% US amplitude). This may aid in generating initial burst release of VEGF. PDGF tends to release in lower quantities when not ultrasonically stimulated (**Figure 3b**, ~50 pg in 5 minutes at 0% US amplitude). However, when ultrasonically stimulated, PDGF release can be substantially enhanced (for example, **Figure 3b**: ~200 pg in 1 minute at 40% US amplitude). These attributes may help retain PDGF when not ultrasonically stimulated and enable its triggered release when stimulated.

3.6. Materials and Methods

3.6.1 Materials

B16F10 mouse melanoma cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Sodium alginate (Protanal LF20/40) of high molecular weight (~ 250 kDa) was donated by FMC BioPolymers (Philadelphia, PA). Trypan Blue, Dulbecco's modified eagle's medium (DMEM), bovine serum albumin (BSA), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA solutions, morpholineethanesulfonic acid (MES) hydrate, Dulbecco's phosphate buffered saline (DPBS), Sigmacote, activated charcoal, calcium sulfate dihydrate, irinotecan hydrochloride, 5-fluorouracil, mitoxantrone hydrochloride, fluorescein isothiocyanate (FITC) dextran (3-6 kDa and 70 kDa), fluorescein isothiocyanate diethylaminoethyl (FITC-DEAE) dextran (3-6 kDa and 70 kDa) and rhodamine

dextran (10 kDa) were all purchased from Sigma-Aldrich (St. Louis, MO). Vascular endothelial growth factor (VEGF), Platelet-derived growth factor (PDGF), Bone morphogenic protein-2 (BMP-2), stromal cell-derived factor-1 alpha (SDF-1 α), VEGF, PDGF, BMP-1, and SDF-1 α DuoSet ELISA kits were purchased from R&D Systems, Inc (Minneapolis, MN). 16-well xCELLigence e-plates were purchased from ACEA Biosciences, Inc. (San Diego, CA).

3.6.2 Calcium Crosslinked Hydrogel Fabrication

To make ultrasonically responsive hydrogels, alginate was purified through dialysis (3500 MW cut off, Spectrum Laboratories, Compton, CA), activated charcoal treatment, filtration, and lyophilization. In a previously described manner [33], alginate was dissolved in MES buffer (pH = 6.5) to make a 2.5 wt % alginate solution. 2 mL of 2.5 wt % alginate solution was mixed with 2 mL solution of a molecules of interest (drug, protein, or dextran dissolved in MES buffer). This 4 mL solution of alginate and “drug” was quickly mixed with 1 mL of 21 mg/mL CaSO₄ in water slurry by linking two 5 mL syringes (Becton, Dickinson and Company, Franklin Lakes, NJ) together with a LuerLock Connector (W. W. Granger Inc., Lake Forest, IL) and rapidly transferring the mixture back and forth between syringes. Hydrogels were cast by quickly discharging the contents of the LuerLock syringes on glass plates and immediately placing a second glass plate on top of the gel mixture with 2-mm spacers in between the two plates. After gelation (30 min), individual 8 mm diameter cylindrical gels were cut using a biopsy punch. The resulting Ca²⁺ crosslinked 1 wt % alginate, 30 mM calcium-crosslinked hydrogels had 8 x 2 mm cylindrical structures.

3.6.3 *Release Studies*

The Ca²⁺ crosslinked hydrogels were loaded with a drug, protein or dextran during the gel fabrication process as described above. Immediately after cutting the hydrogels, they were transferred to 5 mL of DPBS contained in Sigmacote-treated scintillation vials (to prevent molecular adsorption to the walls of the vial) and simulated with a 20 kHz ultrasonicator using a standard 1/8" microtip (5.4" L × 0.5" dia, titanium alloy, 0.25 lbs.) sonication probe (QSONICA, Newtown, CT) at various ultrasonic amplitudes (%) and durations: amplitudes ranging from 0% to 40% for durations between 1 and 5 minutes. Previous analyses revealed that 20% US amplitudes corresponded to ultrasonic powers of 0.714 ± 0.02 W and 40% US amplitudes corresponded to 2.09 ± 0.05 W. Immediately after stimulation, 1 mL samples were taken from the scintillation vials for later analysis to quantify the amount of molecular release. In time-course experiments where multiple samples were acquired to plot release vs. time, 1 mL samples were removed from the vials and saved for later analysis, and the remaining 4 mL was removed and discarded. A fresh 5 mL of DPBS was added for the next time point. The concentration of FITC-dextran release was quantified using fluorescence ex/em at 495 nm and 525 nm in a BioTek Cytation3 microplate reader against a standard curve. The concentrations of mitoxantrone, 5-fluorouracil and irinotecan release samples were quantified by measuring optical absorbance at 610 nm, 350 nm, and 370 nm, respectively, on the plate reader against a standard curve. ELISA was used to measure the concentration of signaling factor release (VEGF and PDGF).

3.6.4 Sequential Release Studies

1.6 wt% alginate hydrogels were crosslinked with 50 mM CaSO₄ solutions and loaded with irinotecan at 100 µg/gel using methods described in the previous sections. Immediately after cutting the 8-mm-diameter cylindrical hydrogels, they were rinsed for 5 min with 5 mL of DPBS to remove unincorporated irinotecan. Then each gel was soaked with 100 µL of 2 mg/mL 5FU for 30 min to load then with 5FU in a manner that would facilitate rapid 5FU release. After soaking, gels were transferred into new scintillation vials. The experiments began when 5 mL of DPBS was added to the scintillation vials. At 18 hours, gels were subjected to ultrasound stimulation at 40% US amplitude for 2 min. Samples were collected at various time points and analyzed for irinotecan and 5FU release using a plate reader as described in the previous section.

3.6.5 Cell Culture and Maintenance

B16-F10 mouse melanoma cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO₂. B16-F10s were routinely split to avoid confluence over 70%, roughly every other day, by trypsinizing (5 min with 0.25% w/v trypsin and 0.5 mM EDTA), collecting, centrifuging, washing, and re-seeding on 75 cm² flasks.

3.6.6 In Vitro Melanoma Cell Survival Studies

The effect of sequential 5-fluorouracil and irinotecan exposure was compared with delivering 5-fluorouracil (5FU) and irinotecan to B16F10 mouse melanoma cells in vitro on an xCELLigence real-time cell index system. B16F10s were seeded at 500 cells per cm² on 16-well xCELLigence e-plates and allowed to grow in DMEM for 36

h. Then, B16F10s were exposed to various combinations of 5-fluorouracil and irinotecan (**Figure 8a**, schedules S1 to S4) for 36 hours. For delivery schedules that involved changing the drug vs. time (i.e., schedules S3 and S4 for sequential deliveries of 5FU and irinotecan), cells were rinsed 3 times with fresh DMEM immediately after removing the first drug (at the 18-hour mark) as to remove any residual drug before adding the other drug. Real-time cell index data (indicating cell population levels) were collected using xCELLigence software for analysis and plotting. Cell index values were normalized and set to 1 at time 0 when treatment began. Each condition S1 through S4 was repeated in 4 separate wells ($N = 4$) to compute means and standard deviation. Note that a control condition is not included (i.e., one where no chemotherapeutic is delivered). This was not included because cell index values saturated the xCELLigence system when no anticancer agent was present to impede B16F10 growth (i.e., B16F10s reached confluency during the experiment, saturating the cell index signal).

3.6.7 Data Representation and Statistical Analysis

All quantitative data presented here are represented by means $\pm$ standard deviation. For all statistical analyses, one-way Analysis of Variance (ANOVA) was used with Tukey's post-hoc tests for multiple comparisons (using Kaleidagraph software, version 4.5.2) with p-values of less than 0.05 being our benchmark for statistical significance. *, **, ***, and **** indicates statistical significance of $p < 0.05$, 0.01, 0.001, and 0.0001 respectively. n.s. indicates no statistical significance. ($p > 0.05$).

3.7. Conclusions

The work presented here first characterized how different molecular payloads were released when subjected to ultrasonic signals of various amplitudes and durations. While stronger amplitudes and longer durations enhanced molecular delivery to higher degrees, they also eroded the hydrogels and increased hydrogel temperatures, often to ranges that could damage sensitive protein payloads. Ultrasonically induced gel erosion also altered the release characteristics (generating higher amounts of release vs. time) and sometimes prohibited subsequent ultrasonic triggering of molecular release (i.e., when the gels became completely eroded). Erosion and heating could be minimized by using lower ultrasonic amplitudes for shorter durations, but these stimulations did not yield statistically significant payload deliveries. This work went on to demonstrate that pulsing of the ultrasonic signal (i.e., by only stimulating in short, 1-minute ultrasonic bursts, and pausing between bursts) could be used to reduce erosion and temperature increase while still generating statistically significant amounts of payload release. This work also demonstrated that both pulsatile and sequential cancer therapeutic delivery profiles could be achieved. Pulsatile deliveries (i.e., 1-hour periods of enhanced delivery rates of $\sim 8 \mu\text{g/hr}$ per day for 3 days) were generated by pulsing ultrasonic stimulations for short periods of time over the course of 3-day experiments. Of note was that the pulsatile mitoxantrone delivery profiles achieved here were previously shown to improve the destruction of melanoma cells in vitro [43]. In vitro experiments conducted here demonstrated that a sequential delivery profile of 5FU (for 18 hours) followed by irinotecan (at 18 hours) reduced melanoma cell populations compared to irinotecan delivery alone, 5FU

delivery alone, and a sequence of irinotecan followed by 5FU. These calcium-crosslinked alginate gels were demonstrated to reproduce a similar sequential profile of initial 5FU deliveries followed by ultrasonically triggered delivery of irinotecan at 18 hours. In sum, these characterizations and demonstrations will pave the way to developing new strategies for generating temporally complex drug delivery profiles (e.g., pulsatile, delayed, and sequential deliveries), thus leading to improved therapeutic strategies where temporally complex biological processes must be controlled.

3.8. Author Contributions

Conceptualization, T.E. and S.K.; Methodology, S.K. and T.E.; Formal Analysis, T.E.; Investigation, T.E., K.M., E.O., G.S., C.L., M.O. and F.I.; Resources, S.K.; Data Curation, T.E.; Writing-Original Draft Preparation, T.E.; Writing-Review & Editing, S.K. and T.E.; Supervision, S.K. and T.E.; Project Administration, S.K.; Funding Acquisition, S.K.

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CHAPTER 4

Manuscript 2: Pulsatile Chemotherapeutic Delivery Profiles Using Magnetically Responsive Hydrogels

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Tania T. Emi,[†] Tanner Barnes,[‡] Emma Orton,[‡] Anne Reisch,[‡] Anita E. Tolouei,[†] S. Zahra M. Madani,[†] and Stephen M. Kennedy^{*,†,‡}

[†]Department of Chemical Engineering, University of Rhode Island, Kingston, RI 02881

[‡]Department of Electrical, Computer, and Biomedical Engineering, University of Rhode Island, Kingston, RI 02881

*Corresponding Author: Stephen M. Kennedy, Ph.D. Email: smkenedy@uri.edu

4.1. Abstract

Pulsatile chemotherapeutic delivery profiles may provide a number of advantages by maximizing the anti-cancer toxicity of chemotherapeutics, reducing off-target side effects, and combating adaptive resistance. While these temporally dynamic deliveries have shown some promise, they have yet to be clinically deployed from implantable hydrogels, whose localized deliveries could further enhance therapeutic outcomes. Here, several pulsatile chemotherapeutic delivery profiles were tested on melanoma cell survival *in vitro* and compared to constant (flatline) delivery profiles of the same integrated dose. Results indicated that pulsatile delivery profiles were more efficient at killing melanoma cells than flatline deliveries. Furthermore, results suggested that parameters like the duration of drug “on” periods (pulse width), delivery rates during those periods (pulse heights), and the number/frequency of pulses could be used to optimize delivery profiles. Optimization of pulsatile profiles at tumor sites *in vivo* would require hydrogel materials capable of producing a wide variety of pulsatile profiles (e.g., of different pulse heights, pulse widths, and pulse numbers). This work goes on to demonstrate that magnetically responsive, biphasic ferrogels are capable of producing pulsatile mitoxantrone delivery profiles similar to those tested *in vitro*. Pulse parameters such as the timing and rate of delivery during “on” periods could be remotely regulated through the use of simple, hand-held magnets. The timing of pulses was controlled simply by deciding when and for how long to magnetically stimulate. The rate of release during pulse “on” periods was a function of the magnetic stimulation frequency. These findings add to the growing evidence that pulsatile chemotherapeutic delivery profiles may be therapeutically

beneficial and suggest that magnetically responsive hydrogels could provide useful tools for optimizing and clinically deploying pulsatile chemotherapeutic delivery profiles.

4.2. Keywords

Chemotherapy, cancer therapy, responsive hydrogels, smart hydrogels, ferrogels, on-demand drug delivery.

4.3. Introduction

Cancer is a widespread family of diseases, causing nearly half a million deaths in the United States in 2016. It is estimated that roughly 40% of American people will be diagnosed with cancer at some point in their life.¹ This motivates the need to develop new cancer treatment strategies. Traditional cancer treatment strategies involve the systemic delivery of chemotherapeutics. However, especially for solid tumors which comprise nearly 85% of cancer cases,^{2,3} systemic chemotherapeutic deliveries can have difficulties maintaining drug concentrations at the tumor site and are plagued by off-target side effects.^{4,5} Localized deliveries can be achieved from implantable biomaterials and can circumvent some of the aforementioned problems associated with systemic chemotherapeutic deliveries.⁴⁻¹⁵ In fact, several biomaterial-based chemotherapeutic treatments are on the market (e.g., Gliadel[®] and Zoladex[®]).¹³⁻¹⁵

While localized chemotherapeutic deliveries from hydrogel implants have yielded promising outcomes, a limitation in their use resides in the fact that the therapeutic concentrations at tumor sites cannot be altered vs. time after implantation. This prevents clinicians from altering the course of therapy in responses to updates in patient prognosis. Additionally, there is a growing evidence suggesting that the

sustained delivery profiles produced by traditional chemotherapeutic-eluting biomaterials (i.e., relatively constant chemotherapeutic concentration vs. time) are not optimal. For instance, cancer chronotherapies utilize pulsed chemotherapeutic deliveries in order to expose cancer cells to higher drug concentrations when they are most susceptible to that drug (e.g., when metabolically active) but when the rest of the body is less susceptible.¹⁷ This approach is based on the fact that tumor cells can exhibit an accelerated metabolic cycle whereas the rest of the body adheres to a slower, circadian cycle.¹⁸⁻²⁰ Pulsatile deliveries can also be useful in combating adaptive resistance—a major hurdle in cancer treatment.^{21,22} This adaptive resistance may be particularly problematic when using a hydrogel-based approach since they provide cancer cells with an exposure profile that is highly amenable to building resistance (i.e., localized and sustained concentrations). In fact, it has been shown that that adaptive resistance can be reduced when chemotherapeutic deliveries are paused and then resumed—a so-called “drug holiday”.²¹ Taken altogether, these findings suggest that more pulsatile (i.e., periodically on/off) delivery profiles from implantable materials could be advantageous in cancer treatment strategies. While hydrogel materials are highly versatile²³ and can provide localized deliveries,⁴ they do not inherently provide pulsatile delivery capabilities.

Stimuli-responsive hydrogels⁶ may provide the on-demand control needed to produce localized, pulsatile chemotherapeutic delivery profiles. This class of hydrogels can potentially produce higher delivery rates (establishing higher localized chemotherapeutic concentrations) when subjected to externally applied stimuli (e.g., electrical fields,²⁴ magnetic fields,²⁵⁻³² and ultrasonic signals)³³⁻³⁵ while only

producing baseline levels of release when the stimuli is off. Macroporous alginate ferrogels are of particular interest due to their ability to (i) produce triggered-release profiles *in vivo* when exposed to the benign magnetic fields emanating from common hand-held magnetics,^{25,26} (ii) impede fibrous capsule formation,³⁶ and (iii) generate temporally complex delivery profiles, even over the course of days to weeks.³⁷ While showing promise in a number of drug delivery applications, these macroporous ferrogels have not been adapted to deliver the types of pulsatile chemotherapeutic delivery profiles needed to enhance anticancer activity. For example, a common chemotherapeutic installment for treating acute myeloid leukemia involves three days of mitoxantrone delivery,³⁸ and chronotherapies often involve one chemotherapeutic pulsation per day.³⁹ However, ferrogels have been limited to pulsatile chemotherapeutic deliveries over the course of hours, not days (i.e., 2 min periods of magnetically enhanced release every half-hour for 3 h total).²⁵ This study therefore aimed to (i) investigate the impact of different pulsatile chemotherapeutic delivery profiles on cancer cells *in vitro* over time scales more pertinent to cancer treatment (i.e., 1 pulse per day for 3 days) and (ii) demonstrate the ability to magnetically reproduce multiday, pulsatile delivery profiles using macroporous ferrogels. In pursuit of these aims, this work also addresses other issues arising from attempting to extend pulsatile delivery profiles from hours to days. Namely, this work investigates the use of magnetic field frequency as a means to (i) explicitly regulate the rate of chemotherapeutic release during pulsation and (ii) maintain control over drug delivery rates over time as drug becomes depleted from the gel.

4.4. Materials and Methods

4.4.1 Materials

B16-F10 mouse melanoma cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). LIVE/DEAD cell imaging kits were purchased from Invitrogen (Carlsbad, CA). Sodium alginate (Protanal LF20/40) of high molecular weight (≈ 250 kDa) was donated by FMC BioPolymers (Philadelphia, PA). Trypan Blue, MES hydrate, adipic acid dihydrazide (AAD), 1-hydroxybenzotriazole (HOBT), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), iron (II, III) oxide powder, sodium chloride, activated charcoal, irinotecan hydrochloride, 5-fluorouracil, mitoxantrone, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA solutions were all purchased from Sigma-Aldrich (St. Louis, MO). 16-well xCELLigence e-plates were purchased from ACEA Biosciences, Inc. (San Diego, CA).

4.4.2 Cell culture and maintenance

B16-F10 mouse melanoma cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO₂. B16-F10s were routinely split to avoid confluence over 70%, roughly every other day, by trypsinizing (5 min with 0.25% w/v trypsin and 0.5 mM EDTA), collecting, centrifuging, washing, and re-seeding on 75 cm² flasks.

4.4.3 In vitro melanoma cell survival studies

Cancer cell survival after flatline (constant) mitoxantrone delivery exposures was compared to those after pulsatile deliveries using B16-F10 mouse melanoma cells *in*

vitro. B16-F10s were seeded at 500 cells per cm² on 6 well-plates and allowed to grow in DMEM for 3 days before treatment (**Figure 1A**, $t = -3$ d through 0). Then, for 3 days (**Figure 1A**, $t = 0$ d through 3 d), B16-F10s were exposed to various mitoxantrone concentrations vs. time (**Figure 1B**, schedules s0 – s4). Each of these delivery schedules (s1 – s4) utilized the same integrated amount of mitoxantrone (666 ng mL⁻¹ day⁻¹). Varying mitoxantrone concentration vs. time was achieved by exchanging fresh DMEM with measured concentrations of mitoxantrone in DMEM. When transitioning from mitoxantrone-containing media to mitoxantrone-free media, cells were rinsed 3 times in fresh media in order to remove any residual mitoxantrone. After 3 days of treatment, cells were washed 3 times with DMEM so that no residual mitoxantrone was left and allowed to grow for a day in fresh DMEM (**Figure 1A**, “recovery” $t = 3$ d through 4 d).

Cell viability was quantified on day 3 and day 4 using a LIVE/DEAD staining assay. Also, culture population was monitored in real-time using 16-well xCELLigence e-plates. E-plates wells (similar in size as 96-well plates) were plated on day -3 at 500 cells per cm² and allowed to grow for 3 days (days -3 through 0) before mitoxantrone treatment for 3 more days (days 0 through 3) following the same timeline as provided in **Figure 1A**. Mitoxantrone concentrations were altered as described above. xCELLigence software was used to collect real-time cell index data for later analysis and plotting. Because cell index values—which represents cell population vs. time based on measured impedance—can be variable from experiment to experiment, cell index values were normalized so that values were set to 1 across all conditions at time 0, when mitoxantrone treatment began.

Figure 1. (A) A timeline describing the *in vitro* cytotoxicity experiment where B16-F10 cells were seeded and allowed to grow for 3 days before treatment, exposed to mitoxantrone treatment profiles s0 – s4 for 3 days, and allowed to recover for a day. (B) Delivery schedules (s0 – s4) were used on B16-F10 cells where the integrated dose was maintained at 666 ng mL⁻¹ day⁻¹.

4.4.4 Biphasic ferrogel fabrication

To make magnetically responsive hydrogels, alginate was purified through dialysis (3500 MW cutoff, Spectrum Labs, Compton, CA), activated charcoal treatment, filtration, and lyophilization. In a manner previously described,^{26,37} alginate was dissolved in MES buffer (pH = 6.5) with AAD, and iron oxide powder to form a solution containing 7 wt % iron oxide, 1 wt % alginate, 2.5 mM AAD crosslinker. Crosslinking was initiated using EDC and the hydrogels were cast between two glass plates spaced 2-mm apart with a magnet placed on top as to pull the iron oxide to one side of the gel, achieving a biphasic structure (**Figure 2A**). Here, biphasic ferrogel structures were used due to their demonstrated ability to provide higher levels of drug

delivery under similar magnetic stimulation conditions when compared to monophasic designs.²⁶

Figure 2. A schematic of the biphasic ferrogel fabrication process. (A) Gels were cast between two glass plates with a magnet on top, (B) allowed to swell in DI water after gel formation, (C) frozen at -20°C to form ice crystals, and (D) lyophilized to evaporate ice crystals, leaving pores. (E) Photograph of a completed macroporous biphasic ferrogel.

After gelation (40 minutes), individual 8-mm diameter cylindrical gels were cut using a biopsy punch and were rinsed in deionized water for 3 days (exchanging liquid 3-times daily) (**Figure 2B**). This removed residual reagents from the gel and allowed the gel to swell fully. Gels were then frozen at -20°C (**Figure 2C**) and lyophilized (**Figure 2D**). The resulting structure was an 8 x 2 mm cylindrical dehydrated, macroporous, biphasic ferrogel (**Figure 2E**).

4.4.5 Electron microscopy imaging of ferrogels

Structural analyses of freeze-dried ferrogels were performed using Scanning Electron Microscopy (SEM) with a Zeiss SIGMA VP Field Emission-Scanning Electron Microscope (FE-SEM). Backscattered electron imaging was done using identical conditions at an accelerator voltage of 20 keV and a chamber pressure of

5×10^{-6} torr. No sputter coating was applied to the ferrogels for imaging.

4.4.6 Biphasic ferrogel release studies

Lyophilized ferrogels were loaded with known amounts of mitoxantrone by adding precise volumes of drug solutions to the lyophilized gels. It was determined that ferrogels would absorb no more than 65 μL of liquid. Thus, solutions containing 125 μg of mitoxantrone per 65 μL of PBS were prepared and added dropwise to the iron-oxide-free side of ferrogels (i.e., the white side of the biphasic gel, **Figure 2E**) and allowed to soak overnight while sealed in a scintillation vial. This amount of mitoxantrone loading (125 μg per gel) represents an experimentally optimized loading that does not saturate the ferrogel with mitoxantrone (see Supporting Information, Section 1, **Figure S1**). This lack of saturation reduces the amount of diffusive mitoxantrone release when not magnetically stimulated and enables therapeutically relevant release rates when stimulated (single-digit micrograms per hour). To remove unincorporated mitoxantrone, ferrogels were then soaked in 5 mL of PBS for 1 hour. It was found that this method of loading resulted in over 80% of the original 125 μg of mitoxantrone to be taken up by the ferrogels and that subsequent rinsing removed very little beyond that (**Figure S2**). That is, after loading, an average of 103.3 μg of mitoxantrone was taken up by the ferrogels, and rinsing reduced this amount to an average of 101.4 μg . This amount of mitoxantrone represents the drug contained in the gels prior to release studies and is used to compute the amount of drug remaining (%) vs time. Immediately following rinse, gels were placed in 1 mL of PBS and the release study was initiated. Ferrogel-containing scintillation vials were placed on top of a custom magnet stimulation system (see next subsection) and exposed to various

magnetic stimulation signals (or no signal for control experiments). Samples were taken periodically by fully removing the 1 mL of PBS, reserving it for later analysis, and replacing it with a fresh 1 mL of PBS. The concentration of mitoxantrone contained in collected samples was quantified using BioTek Cytation3 microplate reader to measure optical absorbance at 610 nm for mitoxantrone against a standard curve.

4.4.7 Custom magnetic stimulation system

To expose ferrogels to a wide variety of magnetic stimulation frequencies, a custom magnetic stimulation system was designed and built (**Figure 3**). This system consisted of an electric motor whose speed could be controlled through a computer interface. This electric motor drove a crankshaft which drove four balanced, in-line pistons. Each piston held a single 1.27 x 1.27 cm cylindrical neodymium magnet (K&J Magnetics, Inc., Pipersville, PA) whose vertical position cyclically raised and lowered as the electric motor ran. A scintillation vial was held on a platform just above each piston in a manner that held ferrogel samples close to the magnets at maximum height but far enough away to avoid physical contact with the magnet. Thus, four ferrogels could be simultaneously exposed to cyclic magnetic gradients at frequencies prescribed by the motor speed (between 0.01 and 20 Hz). Ferrogel samples contained within their scintillation vials were exposed to between 0 and 5.6 kGauss at piston minimum and maximum positions, respectively, as measured by Hall-effect sensors. Three movies are provided in Supporting Information that shows this device in use at 0.1 Hz, 1 Hz, and 10 Hz (MovieS1.mov, MovieS2.mov, MovieS3.mov, respectively).

Figure 3. Schematic (top) and photograph (bottom) of the custom magnetic stimulation setup used in these studies.

4.4.8 Data representation and statistical analyses

All quantitative data presented here are represented by means $\pm$ standard deviation, unless otherwise specified. For all statistical analyses, Analysis of Variance (ANOVA) was used with Tukey's post-hoc tests for multiple comparisons (using Kaleidagraph software) and p -values of less than 0.05 being our benchmark for statistical significance. *, ** and *** indicates statistical significance of $p < 0.05$, 0.01, and 0.001, respectively. n.s. indicates no statistical significance. ($p > 0.05$).

4.5. Results

4.5.1 *Continuous vs. pulsatile chemotherapeutic deliveries on tumor cells in vitro*

To determine if pulsatile chemotherapeutic deliveries were more toxic to cancer cells than continuous (flatline) deliveries, B16-F10 mouse melanoma cells were exposed to various mitoxantrone concentration profiles vs. time over the course of 3 days (see **Figure 1**). Despite using the same integrated doses of mitoxantrone ($666 \text{ ng mL}^{-1} \text{ day}^{-1}$), it was determined that pulsatile delivery schedules could result in lower numbers of live melanoma cells than continuous profiles (**Figure 4A**). Specifically, immediately after mitoxantrone treatment (**Figure 1A**, day 3), all three pulsatile delivery schedules tested (schedules s2 – s4) resulted in significantly fewer live melanoma cells than the continuous schedule s1 (**Figure 4B**, left). However, when given a full day to recover after mitoxantrone treatment (**Figure 1A**, day 4), cells exposed to schedule s4 recovered somewhat (**Figure 4B**, comparing blue and red bars for s4). Notably though, pulsatile schedules s2 and s3 remained at low levels (**Figure 4B**, red and blue bars for s2 and s3 remained low).

Pulsatile delivery profiles may have resulted in lower melanoma cell survival than the constant profile due to (i) improved prevention of the cells developing resistance to the drug, but also simply due to (ii) the use of temporary and periodically higher dosing. That is, while the same amount of total drug was used in schedules s1–s4, pulsatile delivery schedules required that higher concentrations be delivered during “on” phases of the delivery profile to match the total integrated dose of the continuous profile. Thus, during these “on” phases, cells were exposed to more toxic concentrations of mitoxantrone. Notably, however, when very toxic/high

concentrations were used but for very short “on” periods (i.e., schedule s4: 192 $\mu\text{g/mL}$ but only held for 15 min per “on” period), melanoma cell elimination became less effective (**Figure 1B**, s4).

Figure 4. Pulsatile temporal delivery profiles enhance the toxicity of mitoxantrone exposure when compared to constant delivery profiles. (A) Fluorescence microscopy images of B16-F10 cells after LIVE/DEAD (green/red) staining on day 4 after 3 days of exposure to schedules s0 – s4 and a day of recovery. (B) Quantification of live cells after the indicated delivery schedules immediately after treatment (left, blue) and after a day of recovery (right, red). N = 6. (C) Left: Normalized cell index (melanoma

cell population) vs. time when exposed to no mitoxantrone (dashed black), constant mitoxantrone concentration (s1, solid black), and a pulsed mitoxantrone profile (s3, solid green). Right: Zoomed-in index vs. time for cells exposed to pulsatile schedule s3. Blue rectangles indicate where the mitoxantrone pulses are “on” for the s3 condition. N = 4.

This could be attributed to there not being a sufficient amount of time for the drug’s toxicity to manifest. In the case of this chemotherapeutic, mitoxantrone molecules must be able to internalize and access the cell nucleus where it can disrupt DNA synthesis and repair mechanisms. In fact, mitoxantrone is known to rapidly absorb to tissues³⁸ with absorption half-lives reported to be on the order of 10 min.⁴⁰ Thus, for fleeting drug exposures on the order of that time scale (e.g., 15 min), only a fraction of mitoxantrone would be expected to absorb and have a therapeutic impact on the cells. Of the four mitoxantrone schedules tested, the pulsatile schedule s3 (24 $\mu\text{g/mL}$ held for 2 h per “on” period) yielded the most significant reduction in melanoma cell survival (**Figure 4B**, right). This may represent a more effective balance of increased mitoxantrone concentrations being held for a sufficiently long period of time (i.e., about 12 half-lives) to reach and interact with intercellular targets.

To test how the number/frequency of pulses impacted melanoma cell survival, experiments were conducted where B16-F10s were seeded, allowed to grow, exposed to different mitoxantrone pulsed profiles at the same integrated dose, and left to recover after treatment, all while being monitored for cell index in real time (**Figure 5A**). Here, pulsatile delivery profiles were either a single pulse (**Figure 5B**, p1), two pulses (p2), or three pulses (p3), and compared to a constant (flatline) delivery profile

(p0). Results indicate significant differences between the number of live melanoma cells remaining after pulsed mitoxantrone treatments with different numbers of 2-h pulses (**Figure 5C**). In particular, the two-pulse delivery profile (**Figure 5C**, schedule p2, blue curve) appeared to be the most effective in eliminating melanoma cells. While these effects are the subject of ongoing investigations, schedule p2 may present an effective combination of sufficiently high pulsed dosing repeated enough to be most toxic to melanomas.

Figure 5. For pulsatile mitoxantrone deliveries, the number of pulses can have an impact on how many melanoma cells survive treatment. (A) A timeline describing the in vitro experiments was conducted. (B) Schematics describing the different pulses

mitoxantrone delivery schedules used (0–3 pulses, schedules p0–p3, respectively). (C) Normalized cells index for melanoma cells vs time during mitoxantrone treatment (blue shaded region) and after treatment for cells exposed to schedules p0 (black), p1 (red), p2 (blue), and p3 (green). ** indicates statistically significant differences with $p < 0.01$ as computed using one-way ANOVA with Tukey's post hoc tests for multiple comparisons. $N = 4$.

Taken together, these data indicate that pulsatile delivery profiles may provide advantages over constant (flatline) delivery profiles. Systemic delivery of pulsatile profiles may be problematic, however, since periodically high concentrations of chemotherapeutics may impose undesirable side effects. These issues could be reduced if deliveries were more localized, for instance, by using a drug-releasing hydrogel implanted at the tumor site. However, traditional hydrogels do not provide pulsatile delivery profiles. This work will therefore investigate if pulsatile deliveries can be administered over the course of several days (mimicking the deliveries here) using magnetically responsive biphasic ferrogels.

4.5.2 Biphasic ferrogels for magnetically controlled drug delivery profiles

In order to produce hydrogels capable of generating pulsatile deliveries similar to those investigated in the previous section, biphasic ferrogels^{26,36,37} were fabricated. These cylindrical gels consisted of (i) an iron-oxide-laden region and (ii) a soft and deformable porous alginate region (**Figure 6A**). The particular ferrogel formulation used here (7 wt % iron oxide, 1 wt% alginate, 2.5 mM AAD crosslinker, freeze-dried at -20°C) was previously optimized to provide maximal deformation and drug delivery when exposed to hand-held magnets.²⁶ After lyophilization, these ferrogels' alginate

regions could absorb concentrated solutions of mitoxantrone (**Figure 6B**, i & ii, mitoxantrone is dark blue). Ferrogels were capable of releasing loaded mitoxantrone earnestly when magnetically compressed with a hand-held magnet and returned to their original shape between compressions (**Figure 6B**, iii).

Figure 6. Magnetically responsive biphasic ferrogels were porous in structure and capable of magnetically triggered drug delivery. (A) (i) Photograph of a whole biphasic ferrogel (left) and its cross-section (right). SEM images of the iron-oxide-laden region (ii), porous alginate region (iii), and the transition between the two regions (iv). Elemental mapping data show iron (yellow) and carbon (blue). (B) Photographs of a flipped and lyophilized ferrogel prior to drug loading (i), a ferrogel during loading (ii), and a loaded ferrogel being repeatedly stimulated with a hand-held magnet (iii).

4.5.3 Generation of pulsatile mitoxantrone profiles from biphasic ferrogels

Biphasic ferrogels were loaded with mitoxantrone and stimulated with magnetic signals, periodically, in order to generate pulsatile delivery profiles. Specifically, the strategy adopted here was to (i) magnetically stimulate at 1 Hz (i.e., 1 magnetic compression per second) during “on” periods in order to generate temporarily higher mitoxantrone release rates and (ii) not magnetically stimulate during “off” periods in order to generate lower mitoxantrone release rates (**Figure 7A**).

Figure 7. Biphasic ferrogels generate pulsatile delivery profiles when periodically stimulated with hand-held magnets. (A) Schematic of the 1 Hz magnetic frequency

stimulation profile used. (B) Mitoxantrone release rate vs. time for magnetically stimulated ferrogels (blue) compared to unstimulated controls (black). (C) Percent of mitoxantrone remaining in ferrogels vs. time for stimulated (blue) and unstimulated (black) ferrogels. $N = 4$.

This magnetic stimulation profile did result in periodically higher mitoxantrone release rates as compared to control ferrogels that were not magnetically stimulated (**Figure 7B**, comparing the height of blue and black curves during “on” periods). Note that even control ferrogels exhibited slightly increased release rates during “on” periods, even though no magnetic stimulation was applied during these times.

This was attributed to the agitation associated with removing and adding fresh media during sample collection and reestablished concentration gradients across the perimeter of the gels when fresh media was added. Nevertheless, magnetic stimulation still resulted in statistically significant increases in mitoxantrone release rate during each “on” periods compared to controls (**Figure 7B**, $p < 0.01$ at 0, 24, and 48 hours). While these data show promise, one observed issue was that the magnetically triggered “on” release rates were not consistently high on subsequent days. That is, the release rate achieved through magnetic stimulation decreased each day, despite being stimulated with the same stimulations on each day (**Figure 7B**, blue curve, descending pulse height at 0, 24, and 48 hours). This was attributed to the depletion of mitoxantrone remaining in the gel over time (**Figure 7C**). As time progressed, less mitoxantrone was available to be magnetically squeezed out of the gel, therefore generating lower release rates when magnetically stimulated. This depletion issue will be addressed in the following subsection. The other observed issue was that the release

rates during “on” periods were not explicitly controlled. That is, magnetic stimulation generally enhanced release rates, but the degree of this enhancement was not dictated by the magnetic stimuli. This will be addressed in the next subsection.

4.5.4 Regulating chemotherapeutic release rate using the frequency of magnetic stimulation

The ability to remotely regulate the release rate of chemotherapeutics during “on” periods would be very desirable as it would enhance the ability to control the release characteristics after implantation. It was hypothesized that stimulating at higher frequencies would increase the rate of release. That is, when compressed more times within a given window of time, more drug would be convectively purged from the ferrogel. Therefore, the frequency of magnetic stimulation could potentially be used as a way to remotely regulate the rate of release. In order to test this hypothesis, biphasic ferrogels were loaded with mitoxantrone and stimulated for 10 minutes at different magnetic frequencies. It was determined that increasing frequencies from 0.1 to 10 Hz did increase the amount of mitoxantrone released (**Figure 8**).

Figure 8. The rate of release can be regulated by stimulating at different frequencies. Percent of drug released after 10 minutes of magnetic stimulation at the indicated magnetic stimulations: no magnetic stimulation control (black), 0.1 Hz (yellow), 1 Hz (blue) and 10 Hz (green). N = 4.

Based on these results, it was also hypothesized that the frequency of magnetic stimulation could be used to generate different release rates during “on” phases of a pulsatile delivery schedule. In other words, it was thought that frequency could be used to remotely regulate pulse “height.” In order to test this, experiments similar to those presented in **Figure 7** were conducted but using frequencies of 0.1, 1, and 10 Hz used during “on” periods (**Figure 9A**). During the first “on” period, different magnetic stimulation frequencies resulted in statistically different release rates (**Figure 9B**, day 1 results). However, during subsequent “on” periods, the use of different frequencies to generate different release rates became progressively less effective (**Figure 9B**, day 2 and day 3 results). In fact, by the time the day 3 “on” period was magnetically triggered, there was no statistical difference between any condition (**Figure 9B**, day 3

results). This effect was attributed to, again, depletion of available mitoxantrone in the gels. As time progressed, less mitoxantrone was available for release (**Figure 9C**), making it more difficult to magnetically purge drug for all stimulation frequencies.

During the first “on” period, different magnetic stimulation frequencies resulted in statistically different release rates (**Figure 9B**, day 1 results). However, during subsequent “on” periods, the use of different frequencies to generate different release rates became progressively less effective (**Figure 9B**, day 2 and day 3 results). In fact, by the time the day 3 “on” period was magnetically triggered, there was no statistical difference between any condition (**Figure 9B**, day 3 results). This effect was attributed to, again, depletion of available mitoxantrone in the gels. As time progressed, less mitoxantrone was available for release (**Figure 9C**), making it more difficult to magnetically purge drug for all stimulation frequencies.

Figure 9. Stimulation at different frequencies yielded different release rates during “on” periods initially, but significant differences were not achieved at later time points. (A) Mean mitoxantrone release rate vs. time for ferrogels exposed to no stimulation (black), 0.1 Hz (red), 1 Hz (blue), and 10 Hz (green) at the indicated times

(red, blue, and green shaded regions, respectively). (B) Mean and standard deviation of release rates during “on” periods for days 1 (left), 2 (middle), and 3 (right) for the same conditions shown in (B) Mean and standard deviation of release rates during “on” periods for days 1 (left), 2 (middle), and 3 (right) for the same conditions shown in part (A). (C) Percent of mitoxantrone remaining in gels vs. time for the same conditions shown in part (A). N = 4.

4.5.5 Pulsatile delivery schedules with consistent “on” period release rates vs. time

Because mitoxantrone could be more efficiently released when using higher stimulation frequencies, it was thought that higher stimulation frequencies could be used to compensate for reduced release rates as time progressed due to drug depletion. Specifically, the strategy was to use progressively higher stimulation frequencies in order to maintain more consistent release rates as the drug became more difficult to magnetically purge from the gel (due to there being a less available drug). Therefore, experiments were conducted where subsequent “on” periods used stimulation frequencies of 0.08 Hz, 0.8 Hz, and 8 Hz (**Figure 10A**). This progressive magnetic stimulation profile resulted in pulsatile mitoxantrone delivery profiles with consistent pulse heights (**Figure 10B**). These “on” period release rates were statistically similar on days 1 – 3 and each higher than controls (**Figure 10C**). Likewise, the amount of drug remaining in the gels more consistently dropped during subsequent stimulations (**Figure 10D**, drops during times shaded in gray).

Figure 10. “On” period release rates could be more consistent vs. time when progressively higher stimulation frequencies were used. (A) Schematic of the magnetic stimulation profile used. (B) Release rate vs. time when subjected to the magnetic stimulation profile described in part (A). (C) Release rates during “on” periods on days 1, 2, and 3 for unstimulated control gels (black) and gels exposed to the progressive stimulation profile (green). (D) Percent of mitoxantrone remaining in the gels vs. time. N = 4.

4.5.6 Extending the duration of Pulsatile deliveries from Ferrogels beyond Three Days.

While the 3-day pulsatile profiles used in these in vitro studies (**Figure 1B**) and those generated magnetically from ferrogels (**Figure 10B**) were based on (i) existing mitoxantrone chemotherapies (e.g., a recommended treatment for acute myeloid leukemia involves 3-day mitoxantrone delivery installments)³⁸ and (ii) the fact that chronotherapies often involve one chemotherapeutic pulse per day,³⁹ other emerging therapies could require pulsed delivery profiles extending beyond 3 days. Thus, to investigate ferrogels' abilities to produce pulsatile mitoxantrone delivery profiles for durations longer than 3 days, a magnetic stimulation profile was tested that used progressively higher magnetic stimulation frequencies on subsequent days over the course of 8 days (**Figure 11A**). Note, however, that the magnetic stimulation setup (**Figure 3**) permitted only stimulations up to 10 Hz (requiring the electric motor to run at 600 rpm). Thus, on days 4 through 8, magnetic stimulation frequency was maxed out at 10 Hz. Nonetheless, magnetically triggered pulse heights were statistically higher than control gels through day 7 and statistically indifferent from each other through day 5 (**Figure 11B**). Note that elsewhere, increased mitoxantrone release rates have been delivered from biphasic ferrogels at frequencies up to 40 Hz using electromagnets (without moving parts) to generate these higher frequencies.³⁷ Thus, it may be possible to compensate for drug depletion for longer durations than achieved here by continuing to progressively increase stimulation frequency beyond 10 Hz. Also note that though the day-8 magnetically triggered pulse did not meet our benchmark for being statistically higher than controls, it did exhibit a modestly low p-

value ($p = 0.057$). On day 8, some of the ferrogels began losing their structural integrity after being so aggressively stimulated on subsequent days (5 days straight at 10 Hz).

Figure 11. Pulsatile mitoxantrone delivery schedules from ferrogels can be extended beyond 3 days. (A) Table detailing the magnetic stimulation profile used. On each day, 1 h of magnetic stimulation was performed at the indicated frequency. (B) Mitoxantrone release rate vs time when subjected to the magnetic stimulation profile described in part A (blue) compared to controls (black). (C) Percent of mitoxantrone remaining in the gels vs time for magnetically stimulated gels (blue) compared to controls (black). Asterisks indicate levels of statistical differences for given pulse rates compared to controls (see Section 2.8). † indicates that magnetically triggered release rate is statistically lower than triggered rates achieved on other days ($0.03 < p < 0.05$). $N = 4$.

This loss of structure likely enabled more drug release from some of the gels, thus leading to higher standard deviations in the release data and thus a lack of statistically

significant differences when compared to controls. Future ferrogel designs will have to be more robust to facilitate the delivery of prolonged pulsed profiles (i.e., those lasting weeks), though gels held up amply during 3-day pulsatile deliveries, which were shown to be very effective against melanoma cells *in vitro* (**Figure 4**).

4.6. Discussion

These studies demonstrate that pulsatile delivery schedules could provide enhancements in the anti-cancer activity of chemotherapeutics and that magnetically responsive hydrogels could be used to locally deliver these types of pulsatile schedules. The findings here that pulsatile delivery profiles are more effective in eliminating cancer cells than constant profiles of the same integrated dose are consistent with (i) other studies that have found that short bursts of high mitoxantrone concentrations are more effective in destroying breast cancer cells,³³ (ii) findings that cancer cells respond to drug exposures more dynamically than once thought,^{18,41} and (iii) indications that dynamical drug exposures can have significant impact on cellular responses.⁴² If delivered *in vivo*, pulsatile drug scheduling may also enjoy some of the added benefits associated with chronotherapies. For example, pulsing drug concentrations may be a more effective means to deliver toxins when tumor cells are most susceptible to the drug and while off-target tissues are less susceptible.¹⁷⁻²⁰ Also, turning drug concentrations on and off may be useful in combatting adaptive resistance.²¹ While the work presented here adds to the growing evidence that pulsatile delivery schedules are beneficial, it is important to note that the specific delivery profiles examined here do not represent full optimizations. Namely, more complete optimizations will require testing a wider range of pulsatile profiles with

different “on” period delivery rates (pulse heights), “on” period durations (pulse widths), and frequencies of pulsing. Optimizations will also need to be tested *in vivo*, through systemic delivery of these pulsatile profiles would likely pose problems since periodic overdosing could exacerbate off-target side effects. This motivates the need for implantable drug-delivery materials capable of delivering pulsatile profiles locally at tumor sites. This, in turn, requires hydrogels that can generate a wide variety of different pulsatile delivery schedules (i.e., various pulse widths/heights and frequencies) so that delivery schedules can be experimentally optimized.

The magnetically responsive hydrogels developed here were capable of producing pulsatile mitoxantrone delivery profiles similar to those tested on melanoma cells *in vitro* (i.e., pulsed over the course of 3 days). Critically, pulsatile delivery parameters such as the timing and delivery rates of pulses could be remotely controlled using magnetic fields emanating from simple hand-held magnets. Specifically, the timing and duration of “on” periods were controlled simply by choosing when and for how long to magnetically stimulate. Rates of delivery during “on” periods were also capable of being remotely regulated by applying different magnetic stimulation frequencies. Applying different stimulation frequencies allowed for different “on” rates initially or could be used to maintain more consistent release rates as the gels became depleted of the drug over time. While the use of these magnetically responsive ferrogels could provide the above-outlined clinical advantages, devices must first be commercially developed to magnetically stimulate implanted ferrogels over a range of frequencies. This could be achieved using simple electromagnets, which have been demonstrated to efficiently regulate mitoxantrone release rates from biphasic ferrogels

at stimulation frequencies up to 500 Hz.³⁷

Elsewhere, magnetically compressible ferrogels were shown to be capable of delivering molecular payloads after implantation *in vivo*.²⁵ In fact, their cyclic magnetic compressions have actually been shown to resist fibrous capsule formation.³⁶ Previous studies have demonstrated the ability to magnetically generate pulsatile mitoxantrone deliveries from ferrogels. Zhao et al.²⁵ demonstrated that magnetic fields could be used to periodically enhance release rate from magnetically compressible ferrogels when stimulated for 2 min every half hour for 3 h. This resulted in significantly enhanced amounts of release after 3 h compared to controls. The work presented here builds upon the work of Zhao et al. by extending the timeframes of pulsatile release to durations thought to be relevant to chemotherapies and chronotherapies (e.g., days)^{19–22} and demonstrating that specific pulsatile deliveries profiles produced by ferrogels have beneficial impact on destroying tumor cell populations. Additionally, by extending these timeframes, magnetic stimulation strategies had to be developed to maintain delivery rates to compensate for drug depletion over time. Finally, this work builds upon previous work by devising strategies for controlling the degree of enhanced delivery rate during magnetic stimulation using the frequency of magnetic stimulation to remotely regulate release rates. Taken altogether, the studies presented here (combined with their *in vivo* capabilities demonstrated elsewhere) suggest that these magnetically responsive hydrogels could be used to deliver more temporally complex and effective chemotherapeutic delivery profiles to tumor sites in future studies with the degrees of on-demand control needed to (i) experimentally optimize delivery profiles and (ii)

clinically alter the course of therapies according to up-to-date prognoses.

4.7. Conclusions

These studies demonstrate that pulsatile delivery profiles of a chemotherapeutic (mitoxantrone) are more effective at eliminating melanoma cells than constant (flatline) deliveries of the same integrated dose *in vitro*. Some pulsatile profiles worked better than others (i.e., schedule s3 was most effective: 24 $\mu\text{g/mL}$ for 2 h during “on” periods, 22 h “off” periods, repeated for 3 days). However, a more complete optimization of delivery profiles will require testing a broader range of delivery profiles *in vivo*. This work has also demonstrated that a magnetically responsive, biphasic ferrogel can be used to generate pulsatile mitoxantrone delivery profiles similar to those tested on melanoma cells *in vitro*. The timing of mitoxantrone pulses could be regulated by choosing when to apply magnetic stimuli (i.e., from simple hand-held magnets). The rate of delivery during magnetic stimulation could be regulated by stimulating at different magnetic field frequencies. Thus, these materials could potentially streamline the optimization of pulsatile deliveries by enabling the production of a wide variety of pulsatile profiles directly to tumor sites through on-demand, magnetically triggered stimulations. Finally, these materials could also provide powerful tools for clinically deploying optimized pulsatile delivery profiles at tumor sites, while retaining the real-time control needed to alter the course of therapies on-the-fly.

4.8. Associated Content

The Supporting Information is available free of charge on the ACS Publications website at DOI: Movies of custom magnetic stimulation setup.

4.9. Funding Sources

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CHAPTER 5

Manuscript 3: Magnetically responsive biomaterial system enables on-demand, sequential delivery of biomolecules for variety of biomedical applications

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Anita E. Tolouei,^a Tania T. Emi,^a Zahra M. Madani,^a and Stephen Kennedy^{b*}

^a Department of Chemical Engineering, University of Rhode Island, Kingston, RI 02881

^b Department of Electrical, Computer, and Biomedical Engineering, University of Rhode Island, Kingston, RI 02881

*Corresponding Author: Stephen M. Kennedy, Ph.D. Email: smkennedy@uri.edu

5.1. Abstract

Sequential protein release is required in regulating many biological processes that underlie injury and disease. A magnetically responsive dual-compartment biomaterial was therefore designed and successfully applied to provide on-demand sequential release of proteins relevant to specific therapies that would benefit from sequential release. The composition of this biomaterial system consists of a gelatin outer compartment and a ferrogel inner compartment. Three pairs of relevant proteins were incorporated in the biomaterial system: (1) Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF, a dendritic cell recruitment factor) and Heat Shock Protein 27 (HSP27, as a model cancer antigen) for use in cancer immunotherapy; (2) Vascular Endothelial Growth Factor (VEGF, an angiogenic sprouting factor) and Platelet Derived Growth Factor (PDGF, a factor that aids in maturing vascular sprouts) for tissue vascularization; and, (3) Stromal cell Derived Factor-1 α (SDF-1 α , a bone progenitor cell recruitment factor) and bone morphogenetic protein 2 (BMP2, a osteo-differentiation factor) for bone regeneration. It was demonstrated that proteins loaded in the outer compartment (GM-CSF, VEGF, SDF-1 α) released rapidly within the first 24 to 100 hours and that the amount released was dependent on how much protein was loaded in the compartment. timing and rate of release of these proteins can be controlled via magnetic stimulation. Proteins loaded in the inner ferrogel (HSP27, PDGF, BMP2) could be magnetically triggered to provide delayed enhancements in release rate where the timing (between days 1 and 8) and rate of release (0.2 to 1 ng/hr) were externally controlled through the temporal profile of magnet application and the frequency of that application. This biomaterial system can be used to

investigate how the timing and sequence of protein deliveries impacts the biological processes that underlie cancer immunotherapy, tissue vascularization, and bone regeneration (and potentially in many other therapeutic areas) and can be used to experimentally optimize deliveries in these therapies.

5.2. Introduction

Hydrogels have been commonly used as biomaterials in drug delivery and tissue engineering applications due to their biocompatibility and versatility. [1]–[3] These drug delivery materials can potentially control important biological processes that need to be regulated to treat injury and disease. However, most biologics are sequential in nature and require sequential presentations of bio-instructive factors for proper regulation. For example, cancer is the second most common cause of death in the United States and accounts for nearly 1 of every 4 deaths. [4] This emphasizes the necessity of finding effective cancer treatment strategies. One promising cancer treatment strategy is biomaterials-based immunotherapy in which the immune system of the patient's own body is reprogramed in order to initiate an immunological attack against cancer cells. [5]–[7] In this approach, first, dendritic cells (DCs) need to be recruited to the biomaterial by releasing a DC recruitment factor (Factor I) (**Fig 1.A.i, ii**). Once a large number of DCs are resident in the biomaterial (**Fig 1.A.iii**), they can become activated when presented with a cancer antigen (Factor II) (**Fig 1.A.iv**). Activated DCs would then migrate out of the biomaterial towards lymph node (**Fig 1.A.v**), triggering an immunological attack against cancer. Therefore, sequential release of recruitment factor followed by release of activating factor could potentially

improve control over regulating the biological processes pertinent to biomaterial-based cancer immunotherapies.

Beyond cancer treatments, regulation of vascular growth can be of great potential in the treatment of different cardiovascular diseases (CVDs), which are the most common cause of death in the United States and worldwide. [8] It has been reported that CVD was the main cause of more than 50% of deaths in 2010. [9] Additionally, regeneration of tissues after surgery or injury often involves regulating the growth of new vascular networks. [10]–[12] Pericyte cells play a key role in vessel formation and presentation of several growth factors can regulate this process.[13] For example, growth of new blood vessels can be initiated by an initial presentation of angiogenic factors (Factor I) which instructs pericyte cells to detach from the endothelium of nearby vasculature. This detachment destabilizes the endothelium and allows small vascular sprouts to grow away from the existing blood vessel (**Fig 1.B.i, ii**). These nascent sprouts are thin, unorganized, and not mature enough to efficiently perfuse blood through them (**Fig 1.B.iii**). Hence, an additional maturation factor (Factor II) is subsequently released to recruit pericyte cells back to neovessels (**Fig 1.B.iv**), which in turn helps neovessels mature into a thicker and more interconnected network (**Fig 1.B.v**).

Finally, sequential delivery of bio-instructive factors can be of potential value in regenerating bone tissues. Each year more than 6 million bone fractures occur in the United States, leading to approximately 900,000 patient hospitalizations.[14] Biomaterial scaffolds can be promising substitutes for traditional autogenic and allogenic grafting since they can decrease the problems associated with donor site

sensitivity, morbidity, and limited availability of these grafts.[15], [16] Bone regeneration also is naturally regulated by a sequence of growth factor presentations.[17] First, osteoprogenitor cells need to be recruited to the scaffold by releasing a bone progenitor recruitment factor (Factor I) (**Fig 1.C.i, ii**). After establishing a population of these progenitor cells in the biomaterial (**Fig 1.C.iii**), they can be differentiated down the osteogenic lineage by exposing them to an osteo-differentiation factor (Factor II) (**Fig 1.C.iv**). Differentiated bone cells would then start secreting their own robust bone matrix, which is a vital step in regenerating new bone tissues (**Fig 1.C.v**). Therefore, in order to better regulate these regenerative processes, sequential delivery of bone progenitor recruitment and differentiation factors is necessary.

Figure 1. Sequential delivery of multiple factors is essential for improved outcome in multiple therapies. A) Schematic showing how sequenced deliveries could be

beneficial for biomaterial-based cancer immunotherapy B) tissue vascularization and C) bone regeneration.

In previous studies, hydrogels were demonstrated to have sequentially protein release capabilities using formulations containing phases with different degradation rates. However, the timing between these two deliveries was not capable of being regulated after implantation or injection of these biomaterials. [18]–[20] Additionally, these delivery profiles can more aptly be described as dual deliveries with different rates and not sequential release per se (i.e., on burst release followed by a second, delayed burst release).

Figure 2. New Biomaterial system is designed to improve sequential delivery of several factors in an on-demand manner. A) Schematic of the multi-compartment biomaterial system shows how delivery of multiple factors can be accomplished in a controlled sequenced manner B) Desired cumulative release of factor I and II. C) Table shows different therapies and factors mediating these processes.

The biomaterial system presented here was specifically designed to provide sequential delivery profiles where there is an initial burst release of one factor followed by a magnetically triggered, delayed release of a second factor. This was achieved by composing the biomaterial with two compartments (**Fig 2.A.i, ii**). Compartment 1, initially releases Factor I and has a functionality to maintain recruited cells (**Fig 2.A.iii, iv**). Compartment 2 is capable of releasing Factor II in an on-demand manner when remotely stimulated with a magnetic field (**Fig 2.A.v**). This study aimed to demonstrate the ability to generate these sequential delivery profiles for specific recruitment factors (GM-CSF, VEGF, SDF-1 α) followed by magnetically triggered delivery of programming factors (HSP27, PDGF, BMP2) (**Fig 2.B**), which are relevant to regulating sequential biologies in cancer immunotherapy, generation of new vascular networks, and in regenerating bone tissues, respectively (**Fig 2.C**).

5.3. Experimental

5.3.1 Materials

High guluronate content sodium alginate (Protonal LF 20/40) with molecular weight of ~ 250 kDa was provided by Pronova Biopolymers (Olso, Norway). Other chemicals such as Adipic Acid Dihydrazide (AAD), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), compound 2-(N-morpholino)ethanesulfonic acid (MES), 1-hydroxybenzotriazole (HOBt), iron (II,III) oxide powder (< 5 micron), Phosphate Buffered Saline (PBS), Sigmacote®, and Bovine Serum Albumin (BSA) were all purchased from Sigma-Aldrich (St. Louis, MO). Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Heat shock Protein 27 (HSP27), Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), Stromal Cell-

derived Factor 1- α (SDF-1 α), Bone Morphogenetic Protein 2, and all Enzyme-linked Immunosorbent Assay (ELISA) kits and kit reagents were purchased from R&D Systems (Minneapolis, MN). Lyophilized gelatin sponges (GelFoam™ sponge sheets) were purchased from Pfizer (Groton, CT).

5.3.2 Fabrication and characterization of biomaterial system

The outer compartments of these two-compartment biomaterial systems were made from GelFoam™ gelatin sponges. The 2 x 12 x 7 mm GelFoam™ lyophilized sheets provided by the manufacturer were shaped into hollow cylinders with biopsy punches (2 mm thick, 8 mm outer diameter-OD, 4 mm inner diameter-ID). In order to image the gel's porous structures, they were sputter-coated with gold for 30 seconds and imaged under the Scanning Electron Microscopy (SEM) on a Zeiss SIGMA VP Field Emission-SEM and Energy-dispersive X-ray Spectroscopy (EDS) for elemental mapping.

The inner compartment ferrogels were made of alginate according in a similar manner to the biphasic ferrogels described elsewhere. [21]–[23] Briefly, alginate (at 1% wt) was dissolved in MES buffer (100 mM MES and 500 mM NaCl at pH = 6.0) with AAD and HOBt. This mixture was then mixed with iron Oxide particles ($\text{Fe}_3\text{O}_4 < 5 \mu\text{m}$) and 100 mg/mL EDC. Next, the mixture was cast between two sigmacote-treated glass plates separated by 2 mm spacers. During casting (~ 30 minutes), a magnet was placed on top of the glass plate to pull the iron oxides to the top of the ferrogel, resulting in a biphasic design. Ferrogels were then cut into 4 x 2 mm (diameter- thickness) disks using 4-mm biopsy punches. Gels were then washed in deionized water for 3 days with water being changed 2 to 3 times a day, this was done

in order to remove residual reagents and let them swell. Next, to achieve a porous structure, gels were frozen overnight at -20 °C and lyophilized. For SEM imaging, ferrogels were cut with a sharp razor, exposing their cross section and prepped for SEM imaging following the same protocol as described above for gelatin outer compartments.

5.3.3 Magnetic stimulation of ferrogels

A crankshaft set up was used to magnetically stimulate the Ferrogels. This apparatus was used for exposing ferrogels to frequencies (up to 14 Hz) but for relatively short periods of time (see Emi et al. for a more thorough description [23]). This setup involving the use of 0.5”x0.5” cylindrical neodymium magnets (K&J Magnetics, Pipersville, PA) consisted of a crankshaft driven by a programmable electric motor. The crankshaft was connected via cams to four pistons (much like a car engine) that would move up and down in a sinusoidal manner as the crankshaft was turned by the electric motor. Each piston contained a neodymium magnet and a scintillation vial containing a ferrogel could be suspended directly above each of the four pistons. Thus, when the piston was in the up position, a ferrogel would be exposed to a strong magnetic field (measured to be 5 kGauss). When the piston was in the down position, the magnetic field was weak (measured to be < 10 Gauss). Thus, the speed of the motor dictated the magnetic stimulation frequency. It was determined that this apparatus could magnetically stimulate ferrogels at frequencies up to 14 Hz.

5.3.4 Protein release studies

In order to load proteins to these gels, concentrated solutions of proteins in PBS were made. It was determined that each outer gelatin compartment had the capacity to

absorb 40 μ L of solution which was used as the basis to calculate protein loading concentrations (i.e., loading of 1000 ng protein would require preparation of a solution containing 1000 ng GM-CSF in 40 μ L PBS). Sigmacote-treated scintillation vials (to prevent protein adsorption to the surface of the vials) were used as containers for these gels. Protein carrying solutions were added dropwise to these outer compartments for loading. Vials were capped, and gels were left at room temperature overnight for full absorption of the protein into the gels. Release studies from the outer compartment began the next day when gels were submerged in PBS with 1% BSA ($t = 0$). 1 ml samples were collected periodically and replaced with fresh media each time. Collected samples were stored in 1.5 mL low-adsorption tubes in the freezer. After collecting all samples, samples were thawed and quantified for protein content (i.e., GM-CSF, VEGF, SDF-1 α) using ELISA.

Inner compartment fabrication resulted in lyophilized alginate biphasic gels with a layer of iron-oxide-free porous alginate on one side of the gel and an iron-oxide saturated layer with smaller pores on the other side of the gel. The loading of these ferrogels and release studies from them were similar to those described for the outer gelatin compartments above. However, the absorption capacity of these ferrogels were 20 μ l. Ferrogels were placed in scintillation vials with their iron-oxide free region facing up. Next, a 20 μ l solution containing protein was added to them dropwise. Vials were capped and left in order for gels to absorb the proteins overnight. Ferrogels were flipped over so that their iron-oxide-saturated regions faced upwards (so that a magnet applied under the vial would deform the ferrogel in a downward motion) and then rinsed in PBS with 1% BSA for 3 days to remove proteins that were not well

integrated in the gel structure. Samples were then taken at different times and fresh media was replaced at each time point. During these time course release experiments, ferrogels were magnetically stimulated at a number of time points and at various frequencies, depending on the experiment. ELISA was performed on the collected samples to quantify the concentration of the released proteins from ferrogels (HSP27, PDGF, BMP2).

5.3.5 Data representation and statistical analysis

Analysis of variance (ANOVA) with post-hoc Tukey HSD (Honestly Significant Difference) test was performed in order to determine statistically significant differences when multiple conditions and comparison were made. The numerical values presented in the graphs were represent means $\pm$ standard deviations from 4 independent replicates (N = 4). P-values less than 0.05 were considered significant.

5.4. Results and Discussion

5.4.1 Characterization of the two-compartment biomaterial system

The two-compartment biomaterial system was made of an outer gelatin compartment and an inner biphasic ferrogel nested within the outer gelatin compartment (**Fig 3. A**). The gelatin outer compartment was highly porous (**Fig 3. B**), which would be desirable for allowing fast diffusive release of load proteins (due to high surface area) and potentially efficient penetration of recruited cells. Additionally, by virtue of being made from gelatin, this outer compartment contained cell-binding integrins needed to have recruited cells attach the scaffold and proliferate within the scaffold. The inner biphasic ferrogel compartment contained a Fe₃O₄ saturated region on the one side of the gel and a highly porous Fe₃O₄-free region on the opposite side

of the gel (**Fig 3. C. i**). This biphasic structure was capable of deforming in the presence of magnetic field (**Fig 3. C. ii**).

Figure 3. Dual Compartment biomaterial system with porous outer compartment and magnetically responsive inner compartment. A) Photographs of the whole system B) Characterization of the outer compartment with SEM imaging. C) i. SEM with elemental mapping (bottom) differentiates between Iron (yellow) and carbon (blue) parts of the gel. ii. Ferrogels before (top) and after (bottom) stimulating with magnet.

5.4.2 Release characteristics of the outer gelatin scaffold

The outer compartment's porous gelatin scaffold was capable of initially releasing proteins (proteins described herein as “Factor I”) in a rapid manner. Specifically, DC recruitment factor (GM-CSF) released mostly within the first 24 hours (**Fig 4. A**). The total amount of GM-CSF release was dependent on the amount loaded in the outer compartment (**Fig 4. A**: higher loadings resulted in higher amounts of cumulative release). This rapid and adjustable initial release of DC recruitment factor may be useful in rapidly recruiting DCs to the scaffold for biomaterials-based cancer immunotherapy applications. Similarly, pro-angiogenic factors (VEGF) rapidly

released from the gelatin scaffold, depleting from the gel within the first 5 hours (**Fig 4. B**). VEGF cumulative release was also dependent on the amount loaded into the scaffold (**Fig 4. B**: higher loadings plateaued at higher levels of cumulative release). This rapid release of VEGF may be useful in rapidly initiating vascular sprouting into the scaffold in applications that demand neo-vascularization (e.g., treating CVDs, wound healing and tissue engineering applications). Finally, bone progenitor recruitment factor (SDF-1a) released in a relatively rapid manner, plateauing 7 days (**Figure 4. C**). Again, the final cumulative release values were a function of the amount of protein loaded (**Figure 4. C**: higher loadings yielded higher plateau values). This relatively rapid release profile may enable rapid recruitment of bone progenitor cells to the scaffold for bone regeneration applications.

Figure 4. The outer compartment can rapidly release initial factors pertinent to cancer immunotherapy, tissue vascularization, and bone regeneration. A) Top: Cumulative release versus time of GM-CSF, Bottom: Zoomed in initial release B) VEGF, and C)

SDF-1 α from outer compartment when loaded 1000 ng (green) and 250 ng (blue) of proteins (Colors represent different protein concentrations).

5.4.3 Release characteristics of the inner ferrogel

The inner compartment provides magnetically triggered, delayed, and on-demand release of proteins that can be used to direct the behavior of (i.e., program) cells recruited to the outer compartment. For example, a model cancer antigen's (HSP27's) release rate drastically increase when ferrogels inner compartments are magnetically stimulated on days 0 (**Figure 5. A.** inset, slope of green curve increases in the grey shaded region of the curve compared to blue curve which is the control) or on day 3 (slope of green curve increases in the yellow shaded region compared to the blue curve which is control), depending on when the magnetic stimulation is applied (i.e., on day 0 or 3, respectively). Note that the magnetic stimulation used in these studies was at 5 Hz for 10 minutes and 1 Hz for 50 minutes in 1-hour cycle for the entire duration of 4 hours using the piston stimulation technique. These on-demand, delayed enhancements in release rate could be used to optimize the time point at which recruited DCs are earnestly presented with cancer antigen for biomaterials-based cancer immunotherapy applications.

Figure 5. Timing of the delivery from ferrogels can be controlled by stimulating at different time points. A) Percent Cumulative release of HSP27 when stimulated on days 0 (dashed) and 3 (solid). B) PDGF, and C) BMP2.

Likewise, the rate of pro-vascular-maturation factor (PDGF) release could be greatly enhanced when these ferrogel inner compartments were magnetically stimulated (**Figure 5. B**). And again, the time at which magnetic stimulation was applied enabled this enhanced release to occur at specific times (**Figure 5. B**: magnetic stimulation on days 0 (grey shaded), 3 (yellow shaded), yielded enhancements in release rates on days 0 and 3 respectively). These magnetically triggered delays in pro-vascular-maturation factors could help optimize the time allotted for inducing vascular sprouting before re-stabilizing the nascent vascular network.

Finally, the rate of osteo-differentiation factor (BMP2) release could also be enhanced when these ferrogels were magnetically stimulated (**Figure 5. C**: increased slope of the green curve in the grey and yellow shaded region). Though the specific timing of this magnetically triggered enhancement in release rate was not demonstrated as was with PEGF and HSP27, it is likely that enhancements in release would be generated at different time points upon magnetic stimulation at different

time points. The ability to magnetically trigger enhancements in osteo-differentiation factors to recruited bone progenitors may allow for optimizations in how much time is provided to build bone progenitor populations in the scaffold prior to instructing them to differentiate.

5.4.4 Strategies for magnetically controlling the rate of delayed release

While the delayed release capabilities outlined above may be of use in improving the timing of deliveries in cancer immunotherapies, re-vascularization therapies, and bone regeneration, the rate of release when magnetically stimulated is also a critical parameter for optimization. Here, strategies were explored for using alterations in the magnetic stimulation profile to regulate the release rate during magnetic stimulation. A previous study demonstrated that periodically turning on and off sinusoidal magnetic stimulations could actually improve triggered release rates compared to continuous sinusoidal magnetic stimulation. [24] Here, this principle was investigated further by adjusting parameters associated with pulsing the magnetic stimulation regiment. Namely, ferrogel inner compartments were exposed to pulsed magnetic stimulation regiments where the frequency and duration of magnetic stimulation pulses were changed vs. time.

It was demonstrated that applying different pulsed magnetic stimulation regiments could be used to control the rate of protein release during magnetic stimulation. For example, after 3 days of diffusive release, ferrogels containing HSP27 were magnetically stimulated using one of two different pulsed regiments: either a regiment switching between (i) 10 minutes of magnetic stimulation at 5 Hz followed by 50 minutes at 1 Hz, repeated over 4 hours (**Figure 6.A.i: Mag-a**), or (ii) 10

minutes' stimulation at 10 Hz followed by 20 minutes at 2 Hz, repeated over 4 hours (Figure 6. A.i: Mag-b). These two pulsed stimulation profiles resulted in statistically higher release rates on day 3 compared to controls (Figure 6. A. ii and iii). While these two pulsed stimulation profiles produced slightly different stimulated release rates on day 3 from each other, there was no statistically significant difference between them (Figure 6. A. iii: comparing Mag-a to Mag-b release rates). Statistical differences may be achieved in future studies by fine-tuning the material makeup of the ferrogel as well and the magnetic stimulation profile (e.g., different frequencies at different times for different durations and different magnetic field intensities).

Figure 6. Rate of the delivery of proteins from ferrogels can be controlled by applying various frequencies. A,B,C. i) Illustration of the magnetic stimulation used. A,B,C. ii)

Cumulative release versus time for proteins when not stimulated (grey when stimulated with magnetic profile-a (red) and magnetic profile-b (blue) A,B,C. iii) Release rate from 72 to 76 hours for gels stimulated with nothing, magnetic profile-a and magnetic profile-b.

The use of different pulsed magnetic stimulation regimens was more effective in regulating the rate of PDGF release. For example, PDGF-loaded inner compartment ferrogels were magnetically stimulated on day 3 using either a pulsed regiment that switched between (i) 10 minutes of magnetic stimulation at 5 Hz followed by 50 minutes at 1 Hz, repeated over 4 hours (**Figure 6.B.i: Mag-a**), or (ii) 30 minutes' stimulation at 5 Hz followed by 30 minutes at 10 Hz, repeated over 4 hours (**Figure 6.B.i: Mag-b**). Results indicated that both pulsed stimulation profiles enhanced PDGF release rates on day 3 compared to controls (**Figure 6.B. ii and iii**). Additionally, one magnetic stimulation profile enhanced release rates compared to the other stimulation profile (**Figure 6.B. iii: Mag-a is statistically higher than Mag-b**).

Based on the differences in magnetically stimulated release rates between HSP27 and BMP2, it is apparent that different proteins have different stimulated release characteristics and that these differences are likely influenced by the interactions between the proteins and the ferrogel's alginate matrices. For example, BMP2 interacts highly with alginate (i.e., BMP2 is heparin-binding and alginate is heparin-mimicking). In fact, when loaded with BMP2, these inner compartment ferrogels had difficulty in releasing BMP2 at rates that were statistically different than controls. For instance, when loaded with BMP2 and stimulated on day 3 using either a pulsed regiment that switched between (i) 10 minutes of magnetic stimulation at 5 Hz

followed by 50 minutes at 1 Hz, repeated over 4 hours (**Figure 6.C.i: Mag-a**), or (ii) 10 minutes' stimulation at 14 Hz followed by 20 minutes at 2 Hz, repeated over 4 hours (**Figure 6. C.i: Mag-b**), there were no observed differences in BMP2 release rates compared to controls (**Figure 6.C. ii and iii**). Again, however, it may be possible in future studies to demonstrate magnetically triggered release rates that are statistically higher than controls when optimizing the material makeup of the ferrogel and the magnetic stimulation profile (e.g., different frequencies at different times for different durations and different magnetic field intensities).

Finally, we were able to produce a dual compartment biomaterial system that can initially release factor I through diffusion and delay the release of factor two and only appreciably release it when stimulated with magnetic field. Cumulative release of GM-CSF from the outer compartment (**Figure 7.A. grey curve**) accompanied with delayed release of HSP27 (**Figure 7.A. Green curve**) was produced with this dual compartment system. Nonetheless, the rate of HSP27 release on day 3 was compared with control set where there was no magnetic stimulation (**Figure 7. A. blue curve**, Bottom graph shows the comparison between rate of release of HSP27). This sequential delivery enables initial recruitment of the dendritic cells followed by presenting them with cancer antigen when stimulated with magnetic field which leads to larger population of activated dendritic cells. For tissue vascularization purposes, this dual compartment system also can produce rapid initial diffusive release of VEGF (**Figure 7. B. Grey curve**) from the outer compartment and have burst release of PDGF when stimulated with magnetic field (**Figure 7. B. Green curve**). Rate of release of PDGF on day 3 was significantly increased when stimulated with magnetic

pulse (**Figure 7. B. Bottom Graph. Control vs. Freq A**). Furthermore, this biomaterial system was able to release SDF-1 α initially (**Figure 7. C. Grey curve**) and the stimulation of the inner ferrogel on day 3 increased not only the release of SDF-1 α from the outer compartment but also increased the release of BMP-2 from the ferrogel (**Figure 7. C. Green curve**). However, the rate of release of the BMP-2 was not significantly any different than control (**Figure 7. C. Bottom Graph**). Which indicates the high affinity of the BMP-2 to the alginate that explained early on in the paper.

Figure 7. Developed biomaterial system can initially release factor I in fast diffusive manner and delay the release of a second factor until stimulated with magnetic field. Cumulative release of A) GM-CSF, B) VEGF and C) SDF-1 α vs. time (grey curve) with delayed of factor II, A) HSP27, B) PDGF, and C) BMP-2 on day 3 when stimulated with magnetic pulse for 4 hours at frequency A.

Ferrogel deformation under the magnetic field causes a forced outward flow of fluid surrounding the biomaterial which not only increases the rate of release of drug

from the ferrogel but also it increases the release of factor I loaded in the outer compartment as well. Some applications can benefit from this effect for example in cancer immunotherapy and bone regeneration, where we need sustained release of initial factor to recruit cells, usually after the first 24 hours the rate of release from the outer compartment drops significantly, this forced flow in later days increase the rate of release in later days in order to maintain the gradient of initial factor to continue cell recruitment. However, in some other applications like in tissue vascularization or wound healing [24] limiting the release of initial factor in later time points is a necessity. Therefore, the biomaterial system's flexibility in modifying it can come in handy to increase the affinity of the certain proteins to the binding polymer. Subsequently to prevent unwanted release of drug from the outer compartment at later days.

These two-compartment biomaterial systems may provide improvements in a wide range of biomedical applications, including biomaterial-based cancer immunotherapies, therapies involving regeneration of vascular networks, and in bone regeneration. For example, the sequential deliveries of DC recruitment factors followed by flexibly timed and delayed delivery of cancer antigen may enable specific improvements in biomaterial-based cancer immunotherapies. While biomaterial-based cancer immunotherapy has shown promising results (90% mouse survival after 3 months after being challenged with xerographic melanoma if biomaterial cancer vaccines were implanted 14 days prior to cancer challenge),[25] when the biomaterial vaccine was implanted in mice with existing melanomas (well-developed 13-day-old tumors) mouse survival rates were less promising.[26] A potential area of

improvement for these strategies lies in the timing of DC recruitment and the presentation of cancer antigen to recruited DCs. In the biomaterial melanoma vaccine described above, [25], [26] DCs are presented with activation factors (cancer antigen and danger signal) as they enter the scaffold. This may result in (i) diffusive loss of activation factors while DCs are in transit during recruitment and (ii) activation of DCs being distributed over time (i.e., not all DCs arrive in the scaffold and are presented with activation factors at the same time). Both of these may result in a less intense anti-tumor response from the host. The two-compartment magnetically responsive biomaterial system presented could enable recruitment of DCs to the outer compartment and then delivery of activation factors to recruited DCs only after a strong population of DCs has been recruited to the scaffold. In this manner, a strong population of DCs can be activated, enabling a high number of activated DCs to home to the lymph nodes, initiating a potent anti-cancer immunogenic response. Moreover, the biomaterial system described here could enable (i) optimizations in regard to the timing of recruited DC activation and (ii) could provide repeated/subsequent release of activation factors through subsequent magnetic stimulations (i.e., injection-less boosters).

The biomaterial system described here may also provide improvements for therapies where new vasculature needs to be developed. Due to the biology outlined in **Fig 1. B**, sequential release of pro-angiogenic (VEGF) and pro-maturation (PDGF) factors is necessary for growth of new vasculature. In light of this, Richardson et al. [27] created a biomaterial capable of delivering both VEGF and PDGF, ostensibly in sequence, by composing a scaffold with different degradation rates. While delivery of

PDGF alone improved vessel maturation (as measured by the distribution in vessel diameter) compared to VEGF delivery alone, combined release of VEGF + PDGF did not improve average vessel diameter with statistical significance over PDGF delivery alone. This may have been due to the specific temporal profiles of VEGF and PDGF delivery from the system. The differential degradation approach adopted by Richardson et al. yielded VEGF and PDGF release profiles that were more of different rates as opposed to sequential per se. That is, there was not a drastic (albeit slight) increase in PDGF release at later time points (i.e., dramatically delayed PDGF increases in release rate, as observed here in using magnetically responsive ferrogels). This lack of sequential delivery may have not properly coordinated the sequence of pericyte detachment, vascular sprouting, vascular invasion, pericyte re-recruitment and attachment, and sprout maturation required to generate mature vasculature. The delivery profiles achieved by the two-compartment biomaterial system described here may more properly coordinate these sequential biological events. Furthermore, the on-demand capabilities of this two-compartment system may enable critical optimization in the timing allowed for sprouting and sprout infiltration prior to initiating vascular maturation.

Finally, the two-compartment magnetically responsive biomaterial system described here might be beneficial for optimizing deliveries in treating bone defects, injuries, and diseases. Regenerating bone requires coordinated sequence recruitment, proliferation, and differentiation events, which can each potentially be coordinated through protein deliveries. In an attempt to coordinate these events, Lee et al. [28] developed a biomaterial that contained two factors with the goal of enhancing the

number of calcium phosphate matrix-producing osteoblasts: TGF β (to enhance the population of bone progenitors resident in the regenerative scaffold) and BMP2 (to differentiate those bone progenitors into osteoblasts). While this strategy demonstrated that BMP2-loaded scaffolds yielded significantly higher amounts of bone-matrix than controls, dual-loaded BMP2/TGF β scaffolds did not produce more bone matrix than only BMP2-loaded scaffolds (and may in fact have yielded less bone matrix). Again, this result may have been due to the timing of how and when these proteins were presented to bone progenitors as they entered the scaffold from surrounding tissues. For example, while TGF β is known to enhance cell proliferation, it is also known that differentiated cells do not proliferate well. [28] Thus, if cells are instructed to osteo-differentiate by being presented with BMP-2 upon entering the scaffold, they may not proliferate well despite receiving instruction to proliferate through TGF β presentation. This set of simultaneous and conflicting instructions may have limited the number of osteo-differentiate cells in the Lee et al. studies. However, if presented in sequence (i.e., first to proliferate to a strong population while retaining their stem-like ability to proliferate through TGF β signaling; and then to differentiate down the osteogenic lineage through BMP2 signaling), it may be possible to generate a more robust population of bone-matrix-producing osteoblasts. The dual-compartment material system presented here may afford coordination of this sequence and furthermore be capable of optimizing the timing of this sequence for maximized bone matrix production.

5.5. Conclusions

In this study, a biomaterial system was developed and its ability to sequentially release two different proteins pertinent to three therapies was demonstrated. This system was capable of rapidly releasing initial factors (GM-CSF, VEGF, or SDF-1 α) from a porous gelatin outer compartment that was designed to facilitate cell infiltration. The amount of this delivery depended on the amount of the factor that was loaded into the outer compartment. Additionally, this biomaterial system contained a magnetically responsive ferrogel inner compartment, which was able to produce enhanced delivery of its payload (HSP27, PDGF, or BMP2) at a time dictated by an externally applied magnetic signal. It was also demonstrated that the rates of these delayed and magnetically triggered protein deliveries could potential be regulated by altering the frequencies and/or temporal profile of the magnetic stimulation itself.

5.6. Acknowledgements

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CHAPTER 6

CONCLUSION

6.1. Importance and Summary

This doctoral research is focused on two broad applications of hydrogel-based biomaterials—cancer research and tissue engineering. The primary goal of this dissertation was to develop biomaterial systems that can produce controlled drug delivery profiles critical to improving the quality of therapeutic treatments in these areas. This research aimed to i) investigate the impact of dynamic and sequential release profiles of anticancer agents to B16-F10 melanoma cancer cells, ii) develop stimuli-responsive hydrogels systems to release therapeutic molecules when subjected to external ultrasonic or magnetic stimulation, iii) explicitly characterize the biomaterials to improve our understanding of their performance (and limitations of their performance), thus expanding their utility in cancer therapies, tissue engineering strategies, and beyond.

In *Chapter 1*, the motivation behind this work, present knowledge and problem in this field were discussed. Then the principal objectives were mentioned to address this problem.

Chapter 2 contained the elaborate discussion on current ultrasound-based drug delivery systems. The principal mechanisms underlying drug release from biomaterials by ultrasound were investigated as a function of various tunable properties. For instance, the rate of payload release by ultrasound stimulation is a function of ultrasound intensity, duration, pulse number, etc. Therefore, several

strategies were suggested to remotely control the deliveries from biomaterial scaffolds by exploiting those parameters.

Chapter 3 presented ionically crosslinked hydrogels that can release various molecular payloads when subjected to ultrasound stimulations. These studies demonstrated that release rates were a function of molecular size, polarity, charge, hydrogel properties (polymer and crosslinker concentrations), and stimulation properties (intensity, timing, duration, pulse number). These characterizations were used to design studies and demonstrate the ability to i) deliver mitoxantrone in a pulsatile manner, and ii) sequentially deliver 5-fluorouracil and irinotecan, both while retaining the hydrogel integrity and maintaining moderate temperatures.

In *Chapter 4*, the importance of pulsatile chemotherapeutic deliveries was investigated. It was demonstrated that pulsatile delivery profiles significantly reduced B16-F10 melanoma cell populations compared to the more consistent delivery profiles produced by traditional hydrogel materials. Then, a magnetically responsive biphasic ferrogel was used to produce these optimized pulsatile delivery profiles. Furthermore, it was demonstrated that the applied magnetic field frequency could be used to regulate the characteristics (timing and rates) of the pulsatile delivery profile.

In *Chapter 5*, multiple sequential cytokine deliveries were investigated that are pertinent to regulating biological processes in tissue engineering and cancer immunotherapy applications. These sequential delivery profiles were demonstrated with a low frequency, benign magnetic field. A two-compartment hydrogels system was developed where the outer gelatin compartment released payload in a burst profile and inner ferrogel compartment released another payload in an on-demand, delayed

manner. These studies included sequential release profiles of a) GM-CSF and HSP-27 (for cancer immunotherapies), b) VEGF and PDGF (for vascular regeneration), and c) SDF-1 α and BMP-2 (for bone regeneration applications).

6.2. Future Directions

The stimuli-responsive biomaterial systems presented in this dissertation have a strong potential in improving cancer treatment and tissue engineering strategies by enabling pulsatile and sequential deliveries. However, future work is necessary to optimize and translate the use of these materials fully. Further optimizations will include testing a broad range of pulsatile and sequential delivery profiles (different rates, stimulation duration, total treatment duration, the timing of adding other agents, the timing of stimulation off periods). Additionally, work must be performed on other cancer cells (other than melanomas), 3D tumor spheroids (the studies here only involved 2D cultures), and combinations of other anticancer agents (irinotecan, 5FU, leucovorin, oxaliplatin, etc.).

Successful application of implantable biomaterial constructs *in vivo* will also be necessary for moving forwards using these materials in cancer and tissue engineering treatments. Beyond the clinically applicable aspects of this work, the use of these stimuli-responsive materials *in vivo* may also provide a great deal of investigative utility. Being able to easily alter the timing and sequence of multiple therapeutics between experiments may greatly streamline experiments designed to enhance our knowledge of cancer and regenerative biology.

Beyond therapeutic applications, porous hydrogels have potential utility in wastewater treatment and remediation. There is a strong need for a low-cost

wastewater treatment that are portable and reusable. Polymeric hydrogels made with graphene oxide nanoparticles have the potential to sequester cationic contaminants. Hydrogels saturated with contaminants can be transported and purged using chemical-free desorption, potentially through stimulation if the materials were endowed with stimuli-responsive capabilities. Electroconductive hydrogels, for instance, can be employed to selectively sequester, transport and reuse contaminants based on their polarity, magnitude, and frequency of the electronic signal used to excite the material. Thus, these materials have broad potential in critical therapeutic applications, but they also have potentially fruitful applications beyond medicine. Such broad potential utility strongly warrants future investigations on the use of stimuli-responsive hydrogel materials.

APPENDICES

Appendix A

Supporting Information

Manuscript 2-Pulsatile Chemotherapeutic Delivery Profiles Using Magnetically Responsive Hydrogels

Tania T. Emi,[†] Tanner Barnes,[‡] Emma Orton, [‡] Anne Reisch, [‡] Anita E. Tolouei, [†] Zahra M. Madani, [†] and Stephen M. Kennedy*,^{†,‡}

[†]Department of Chemical Engineering, University of Rhode Island, Kingston, RI 02881

[‡]Department of Electrical, Computer, and Biomedical Engineering, University of Rhode Island, Kingston, RI 02881

*Corresponding author. E-mail: smkennedy@uri.edu

S.1. Loading mitoxantrone in ferrogels and rinsing

In order to assess how much mitoxantrone was incorporated into ferrogels after loading, how saturated the gels were, and how much mitoxantrone remained after rinsing, supplemental experiments were conducted. Ferrogels were loaded with various amounts of mitoxantrone (25, 50, 125, 250, and 500 μg) by adding concentration solutions (65 μL) of mitoxantrone dropwise to the iron-oxide-free, alginate region of ferrogels (similarly as described in Section 2.6). First, to determine the relative degree of mitoxantrone saturation in these ferrogels, mitoxantrone-loaded ferrogels were allowed to soak in 1 mL release media until they equilibrated, taking 5 days. For various amounts of mitoxantrone loading, the amount released from day 0 to equilibrium could be quantified vs. time (**Figure S1A**), including the 125 μg loading we adopted in our pulsatile release studies (**Figure S1A**, red curve). From these data, the amount of mitoxantrone remaining in the ferrogels could be plotted vs. time (**Figure S1B**), which could also be normalized by the substrate's mass (i.e., the dry weight of the ferrogels, **Figure S1C**). Thus, at equilibrium, the normalized amount of mitoxantrone bound to the gel could be plotted for various amounts of mitoxantrone loading (a partial isotherm curve, **Figure S1D**). It can be seen that the 125 μg loading (**Figure S1D**, red point) we adopted in our pulsatile S2 release studies is not in the saturation region of this isotherm curve, and thus represents a loading that leaves additional binding sites for mitoxantrone if it were to be loaded with higher amounts of mitoxantrone.

Figure S1. The 125 μg of mitoxantrone used in our pulsed delivery studies does not represent a loading that saturated ferrogels with mitoxantrone. (A) Mitoxantrone diffusively released over 5 days with no media changes at the indicated mitoxantrone loadings. (B) Mitoxantrone remaining in ferrogels vs. time for 5 days. (C) Mitoxantrone remaining in ferrogels vs. time normalized by the dry mass of ferrogels. (D) Mitoxantrone to ferrogels at equilibrium vs. loading concentration normalized by ferrogel dry mass. In parts A-D, red curves represent the loading used in pulsed delivery studies (125 micrograms per gel). N = 4.

In order to quantify the amount of mitoxantrone loaded in ferrogels, experiments were conducted to load ferrogels with various amounts of mitoxantrone (including 125 μg per gel) and then digest them so that the amount recovered could be sampled (**Figure S2A**). Results demonstrated that over 80% of the 125 micrograms of

mitoxantrone was recovered after gel digestion (**Figure S2B**). Additionally, similar experiments were conducted to quantify how much S3 additional mitoxantrone was lost in the 1-hour rinse (**Figure S2C**). Results indicated that very little mitoxantrone was rinsed off during the 1-hour rinse. For ferrogels loaded with 125 micrograms of mitoxantrone, an average of 101.4 micrograms remained in the gels prior to experimentation.

Figure S2. When loaded with 125 μg of mitoxantrone, over 80% of the drug integrated into the gels and very little in addition to that was rinsed off during 1-hour rinses. (A) Schematic of procedure for quantifying amount of mitoxantrone loading. (B) Percent of mitoxantrone recovered after loading vs. amount loaded. (C) Schematic of procedure for quantifying amount of mitoxantrone rinsed off during 1-hour rinses. (D) Amount of mitoxantrone recovered after loading (light bars) and amount remaining after 1-hour rinses (dark bars). For parts A-D, red data represent data obtained using 125 micrograms of loading. $N = 3$.

S.2. Movies of magnetic stimulation system.

<< MovieS1.mov >>

Movie S1. Movie of the custom magnetic stimulation system operating at 0.1 Hz.

<< MovieS2.mov >>

Movie S2. Movie of the custom magnetic stimulation system operating at 1 Hz.

<< MovieS3.mov >>

Movie S3. Movie of the custom magnetic stimulation system operating at 10 Hz

APPENDICES

Appendix B

Manuscript 4: Understanding the influence of magnetic compression frequencies on ferrogels for therapeutic deliveries

Manuscript in preparation for Journal Macromolecular Bioscience

Tania T. Emi ¹, and Stephen Kennedy ^{1, 2, *}

¹ Department of Chemical Engineering, University of Rhode Island, Kingston, RI 02881

^{2*} Department of Electrical, Computer, and Biomedical Engineering, University of Rhode Island, Kingston, RI 02881

*Corresponding Author: Stephen M. Kennedy, Ph.D. Email: smkenedy@uri.edu

S.1. Abstract

Biphasic ferrogel has shown promise in drug delivery and tissue engineering applications. Its biocompatibility, and porous structure enable better drug molecule and cell retention. The release rates of therapeutic molecules can be tuned by various parameters to achieve temporal profiles. Magnetic stimulation frequency is one of those parameters used for regulating release rates. However, when ferrogels are exposed to repeated pulsations, their responses vary depending on magnetic stimulation frequencies. The purpose of this study was to investigate the effect of mechanical properties and rheology of ferrogels on therapeutic molecule delivery application when exposed to various dynamic compression frequencies. Here, we dynamically compressed magnetically responsive biphasic alginate ferrogels at 0.04 mm/mm constant strain from 0.01 Hz to 3 Hz compression cycle frequencies. Finally, experiments were performed to study the release rates of various model macromolecules (dextrans), chemotherapeutics, and protein signaling factors from ferrogels when exposed magnetic stimulation frequencies from 0.1 Hz to 14 Hz. Therefore, this letter highlights the impact of dynamic compressions on the viscoelastic behavior of ferrogels to optimize a suitable operating region for real-time control in the clinical application.

S.2. Introduction

Implantable magnetically responsive hydrogels have shown potential for biomedical applications, such as cancer chemotherapy, wound healing, muscle and bone regeneration applications [1-7]. The biological system precisely operates by a sequence of instructions for a tissue regeneration event (vascularization, injury,

fracture healing) [8-11]. These ferrogels has reported hosting cells by carrying cell recruitment and differentiation factors in the scaffold [1, 3]. Additionally, for cancer therapy, ferrogels showed encouraging results by providing dynamic temporal delivery profiles [4, 6]. Ferrogels are soft that can undergo large deformations when subjected to magnetic stimulation and return to its original shape when magnetic stimulation reverts [3-4]. The main advantage of these ferrogels is they can establish a local gradient of molecules by triggering the payload release in repeated on/off cycles in an on-demand manner. They respond to an alternating magnetic field in such a way amenable for better payload release. Their macroporous structure allows for transport of molecules from pores to the gel matrix when equilibrates then finally release out from the ferrogel scaffold. The rate of payload release from the ferrogels was reported to be regulated by tuning the magnetic field frequencies. Depending upon the size, charge, polarity, and affinity of the molecular payloads some frequencies that can result in greater release rates than other frequencies [4]. For example, low-affinity drug molecules can be released at higher rates at 50 Hz frequency than at 200 Hz frequency [4].

Ferrogels can be compressed for therapeutic applications using a simple hand-held magnet or by a graded magnetic field non-thermally which is critical for sensitive biologic payloads. Although ferrogels convectively purge out molecular payloads, it is not well understood why the release rate varies depending on the frequencies. For instance, some lower frequencies might result in higher release rates when compared to the higher frequencies [4]. Therefore, we hypothesized that the compression frequencies could potentially influence the rheology of ferrogels. While there are few

viscoelastic properties for hydrogels found in the literature, very few experimental results are available for ferrogels. Additionally, there is growing evidence that anticancer agents delivered from ferrogels by periodic pulsations can result in significant tumor growth reduction [6, 12-14]. Therefore, in-depth understanding of the viscoelastic behavior of ferrogels is essential for selecting the magnetic stimulation parameters (frequency, the amplitude of deformation, duration) that will not compromise the structural integrity. Besides, the temperature of the freeze-dried ferrogels was reported to influence the pore size [1]. While pore size of ferrogels is a critical parameter for payload retention and transport, the lack of therapeutic molecule release data from ferrogels freeze dried at different temperatures limit its selection for drug and cell delivery applications. Therefore, this letter investigated i) the mechanical properties of ferrogels freeze dried at two different temperature (-20°C and -80°C), ii) how frequency can impact their rheology, and finally, iii) presented magnetically therapeutic molecule release experiments for strategic implementation.

S.3. Results and Discussion

We fabricated ferrogels using 1 wt% alginate polymer, crosslinked with 2.5 mM AAD (adipic acid dihydrazide) and 7 wt% iron oxide (Fe_2O_3) particles previously optimized for maximum deformation [3-4]. Two types of biphasic ferrogels were fabricated by using -20°C and -80°C freeze drying temperatures. The ferrogels consist of iron oxide-laden region transitioned to deformable and macroporous alginate region (**Figure S1A**, top photographs and bottom SEM images). Their SEM images revealed higher pore size of 275 μm for -20°C ferrogels than 175 μm for -80°C ferrogels (**Figure S1B** (i), blue and green bars). Higher pore interconnectivity of 71% and 63%

resulted that were consistent with literature reported that -20°C and -80°C ferrogels respectively tested by water wicking method (**Figure S1B (ii)**). The Young's modulus of ferrogels showed that -20°C ferrogels were softer and had less toughness than -80°C ferrogels (**Figure S1C**). It was because of the smaller pore size of the -80°C ferrogels, they potentially were stiffer than the -20°C ferrogels.

Figure S1: -20°C and -80°C ferrogels shown different morphological and mechanical properties. A) photographs and SEM images of ferrogels with elemental mapping. B) Pore size calculated by ImageJ software (i) and pore interconnectivity measured by water wicking test (ii). C) Mechanical properties of ferrogels, Young's modulus (i), and toughness (ii). Asterisk (*) indicates statistical differences where p -value < 0.05 calculated by student t. test.

To determine the viscoelastic behavior of the ferrogels, we conducted dynamic compression testing of submerged ferrogels at a constant strain. -20°C and -80°C ferrogels were hydrated overnight with PBS. Fully swollen ferrogels were subjected to

sinusoidal mechanical compressions at 0.04 mm/mm constant strain from 0.01 Hz to 3 Hz compression frequencies while submerged in the 20 mL of PBS buffer solution. Note that, 0.04 mm/mm strain amplitude was chosen to remain in the linear viscoelastic region. Also, at above 5 Hz frequency, the mechanical compression testing instrument was not being able to maintain the constant strain. Therefore, to maintain consistency, 0.01 Hz to 3 Hz cyclic frequency region was chosen. At lower frequencies of 0.01 Hz to 1 Hz, ferrogels underwent constant strain and stress that started to take a downward trend at above 1 Hz (**Figure S2A**). -80°C ferrogels were found to hold more stress than -20°C ferrogels as predicted from its Young's moduli (**Figure S2B**, blue and green lines). The phase lag between the strain and stress seemed to have a decreasing trend (**Figure S2C**) which is consistent with the simulated results found elsewhere [15]. 75° phase lag existed at 0.01 Hz frequency, which started to go lower and maintained a uniform lag of 60° beyond 0.5 Hz frequencies for both types of ferrogels. It was because ferrogels behaved more viscous at lower frequencies but started to gain its elasticity at higher frequencies. Two kinds of ferrogels shown uniform storage moduli of 15 kPa and 18 kPa for -20°C and -80°C ferrogels respectively at frequencies below 1 Hz and it increased to > 20 kPa beyond 1 Hz (**Figure S2D**, blue and green lines at ~1 Hz). The reason for this might be ferrogels were forced to equilibrate at a faster rate with increasing compression frequencies. The viscous property of the -80°C ferrogels was also seemed to reduce with the increase of frequencies as expected (**Figure S2E**). While -80°C ferrogels behaved more elastic with increasing frequencies, the -20°C ferrogels had shown a slight increase in its viscous behavior at frequencies beyond 1 Hz. The rate of convectively purging of

liquids from larger pores of -20°C ferrogels and then reverting to their original shape was slower compared to purging liquids from smaller pores of -80°C ferrogels.

Figure S2: Rheology of ferrogels changed when subjected to cyclic compressions,

A) strain vs. frequency, B) stress vs. frequency, C) phase lag vs. frequency, D) storage

moduli vs. frequency, E) loss moduli vs. frequency, and F) damping vs. frequency. Blue and green lines represent data for -20°C and -80°C ferrogels respectively.

However, the damping or loss of energy seemed to be had similar trends for both ferrogels (**Figure S2F**, blue and green lines at above 1 Hz). They maintained a constant value up to 0.05 Hz frequency and went to a downward direction beyond that.

Freeze dried ferrogels were subjected drug release tests at various magnetic frequencies to determine the rate of releases at different frequencies. Ferrogels were loaded with dextrans, chemotherapeutic agents or protein signaling factors to investigate their release characteristics as a function of ferrogel freezing temperature and magnetic frequencies. Ferrogels were reported to remotely trigger therapeutic molecule release [3, 5-6]. We hypothesized that different freezing temperature might result in different release rates. To test this hypothesis, we at first studied the release of four different model macromolecules (FITC dextrans): relatively small dextrans of 3-6 kDa and larger dextrans of 70 kDa. Additionally, to investigate the impact of charge, uncharged dextrans were used as well as Diethylaminoethyl (DEAE) cationic dextrans. Then we tested for small molecular weight chemotherapeutics (mitoxantrone, irinotecan, and 5-fluorouracil), large molecular weight proteins (Vascular Endothelial Growth Factor (VEGF) and Platelet-Derived Growth Factor (PDGF)). 64 μ L of dextran, drug or protein solutions containing 125 μ g/gel drug or dextran or 65 ng/gel protein were loaded to a ferrogel. This volume and concentration of molecules were chosen because they were previously found this author's previous work to be optimum for a ferrogel without saturating it [6]. Gels were rinsed with 1 mL of PBS buffer for 1 h to reduce the unincorporated molecules that will result for

diffusive release without any stimulations. Ferrogels were then exposed to a graded magnetic field for 10 min at magnetic frequencies from 0.05 Hz to 14 Hz. This region of frequencies was chosen because our custom magnetic system operated in this region.

Figure S3: Ferrogels released model macromolecules when exposed to magnetic stimulation. FITC dextran release rate vs frequency plots (A) small neutral (3-6 kDa dextran), (B) large neutral (70 kDa dextran), (C) small cationic (DEAE 3-6 kDa dextran), (D) large cationic (DEAE 70 kDa dextran). Asterisks (*) indicate levels of statistical differences for release rates compared to -20°C and -80°C ferrogels. Dashed lines indicate non-stimulated release rates.

Small and large molecular weight dextrans of neutral charge seemed to have higher release rates at higher frequencies than non-stimulated diffusive release, where release rates were higher from -80°C ferrogels than from -20°C ferrogels (**Figure S3A and B**, green and blue lines). Small-cationic dextrans had lower release rates compared to neutral dextrans where frequencies being less influential (**Figure S3A and C**). Release rates of large-cationic dextran were very low which were almost the same as the non-stimulated diffusive release (**Figure S3D**, solid and dashed lines). The cationic dextrans electrostatically interacted with the negative charges of polymer alginate matrix. Thus, cationic dextrans released at a lower rate compared to their neutral counterparts. The freezing temperature of ferrogels did not impact the release rates of cationic dextrans (**Figure S3D**). This was because of the higher electrostatic affinity to the polymer matrix might overcome the external force of the magnetic field. Whereas, neutral dextran molecules transported throughout the matrix shown to have greater mobility when stimulated.

Release experiments with four model macromolecules helped to understand the release characteristics of actual therapeutic agents. Next, we loaded the ferrogels with mitoxantrone, irinotecan, and 5-fluorouracil (**Figure S4A** (i)-(iii)). All of those have lower molecular weight with various charges and polarity: 5FU (130.1 Da, neutral charge), irinotecan (586.7 Da, +1 charge), and mitoxantrone (444.5 Da, +2 charge). We, therefore, hypothesized that smaller and polar drug molecule would release at a faster rate from ferrogels. Indeed, 5FU being the lowest of these three anticancer agents, showed less sensitivity to magnetic stimulations and resulted in slightly higher release rates than non-stimulated diffusive release for any frequencies (**Figure S4A**

(i), solid and dashed lines). Because of its smaller size and water solubility, the convective transport from the molecules become easier. However, release rates from -80°C ferrogels shown to have a downward trend with increasing frequencies (**Figure S4A** (i), solid green and blue lines above and below 5 Hz). Mitoxantrone has shown to get triggered release at higher frequencies (1 Hz to 14 Hz) which is consistent with the ferrogels dynamic mechanical properties of being progressively elastic at higher frequencies.

-20°C and -80°C ferrogels had shown oppositely higher and lower release rates at 1 Hz and 14 Hz frequencies (**Figure S4A** (iii)). At 1 Hz, -20°C and -80°C ferrogels released at ~20 µg/hr and ~40 µg/hr rates respectively, whereas, at 14 Hz, they released at ~50 µg/hr and ~30 µg/hr rates respectively. Irinotecan, on the other hand, has relatively higher molecular weight than others shown higher release rates from -20°C ferrogels than from -80°C ferrogels while being less sensitive to the magnetic field frequencies (**Figure S4A** (ii)). The bigger pore size of the -20°C ferrogels might attribute to the higher release rates. The irinotecan release rates were generally higher from stimulated gels than their non-stimulated counterparts at any frequencies (**Figure S4A** (ii), solid and dashed lines).

To examine how the frequency of magnetic stimulations effects on the VEGF and PDGF release, ferrogels were loaded with 65 ng/gel VEGF or PDGF. Tests were conducted using the same manner used for dextrans and anticancer agents. Both VEGF and PDGF resulted in significantly higher release rates from -20°C ferrogels. The bigger pores might also facilitate greater release rates from -20°C ferrogels.

Figure S4: Magnetic stimulation also triggered the release of chemotherapeutics and proteins from ferrogels. (A) Release rate vs. frequency plots of chemotherapeutics: 5-fluorouracil (i), irinotecan (ii), and mitoxantrone (iii). (B) Release rate vs. frequency plots of proteins: VEGF (i), and PDGF (ii). Asterisks (*)

indicate levels of statistical differences for release rates compared to -20°C and -80°C ferrogels. Dashed lines indicate non-stimulated release rates.

The study presented in this letter focused on the impact of various magnetic stimulation frequencies on the viscoelastic properties of the ferrogels and diverse payload release. The evidence of effective temporal release profiles on cancer therapy and tissue engineering applications motivates to investigate the viscoelastic behavior of ferrogels [11, 16-20]. Therefore, the potential use of understanding the rheology of ferrogels are critical for finding out the optimal region of operation. For example, a release strategy can be developed where, burst release occurs at frequencies > 1 Hz, whereas a delayed release might happen by more slow/passive stimulations at frequencies < 0.05 Hz. Besides, for a multi-drug sequential release, a two-compartment ferrogel consisted of -80°C ferrogel as outer compartment and -20°C ferrogel as inner compartment can be designed from optimizing the release rates of chemotherapeutics found in this work. It can provide release of one molecule significantly higher at 1 Hz from the outer compartment and other molecules from the inner compartment at 14 Hz frequency on a user-defined time. Another potential use of the ferrogels was reported for muscle regeneration. These ferrogels can be effective to regenerate 2-2.5% of the injured muscle tissue by two weeks without any bioagents [2]. The viscoelastic properties of ferrogels can optimize this application of ferrogels by varying their thickness, size, crosslinker and polymer concentrations, and maximum deformations, etc.

S.4. Conclusions

This study investigated the mechanical and viscoelastic properties of ferrogels freeze dried at -20°C and -80°C . Then this paper went on to demonstrate the triggered release of various molecules (model macromolecules, chemotherapeutics, and protein signaling factors) in the presence of external magnetic stimulation. Finally, release rates were found to be a function of frequencies and freezing temperature of ferrogels which can potentially be used for selective therapeutic molecule deliveries.

S.5. Experimental

S.5.1 Ferrogel fabrication, imaging, and porosity

Magnetically responsive hydrogels were fabricated from purified alginate through dialysis (3500 MW cutoff, Spectrum Labs, Compton, CA), activated charcoal treatment, filtration, and lyophilization. In a previously described manner, alginate was dissolved in MES buffer ($\text{pH} = 6.5$) with AAD, and iron oxide powder to form a solution containing 7 wt % iron oxide, 1 wt % alginate, 2.5 mM AAD crosslinker [3-4]. The resulting structure was an 8 x 2 mm cylindrical dehydrated, macroporous, biphasic ferrogel. After rinsing the gels for three days, they were kept in -20°C and -80°C freezer (on previously frozen 24-well plates at -20°C and -80°C temperatures). Structural analyses of freeze-dried ferrogels were performed using Scanning Electron Microscopy (SEM) with a Zeiss SIGMA VP Field Emission-Scanning Electron Microscope (FE-SEM) equipped with an energy dispersive X-ray spectroscopy EDS detector for elemental mapping. Backscattered electron imaging was done using identical conditions at an accelerator voltage of 20 keV and a chamber pressure of 5×10^{-6} torr. 17.2 nm thickness of gold sputter coating was applied to the ferrogels for

imaging. Pore size was measured using ImageJ software, and pore interconnectivity was measure by water wicking test.

S.5.2 Ferrogel mechanical characterizations and cyclic compressions

Fully swollen ferrogels were subjected to compression tests using Instron 3345 (Norwood, MA) single column apparatus with a strain rate of 2 mm/min until reaching 90% strain to avoid the risk of damaging the instrument. Engineering stress and strain were recorded from *Instron Bluehill 3* software package. Young's modulus was calculated from the linear portion of the stress-strain curve according to Hooke's law: $\sigma = E\varepsilon$. Toughness was calculated from the area under the stress-strain curve. Ferrogels were compressed cyclically using LFS-200N-A BiSS (Bangalore, India) material testing machine. Gels were subjected to 10 min compression cycles at 0.01 Hz to 3 Hz at 0.04 mm/mm maximum strain in a closed chamber of 20 mL PBS buffer solution. Relative force and displacement vs. time data were collected from the BiSS MTL software package. Relative force and displacement data were used to calculate the strain, stress, phase lag, storage moduli, loss moduli and, damping vs. frequency.

S.5.3 Therapeutic molecule release studies

Lyophilized ferrogels were loaded with 65 μ L solution of 125 μ g/gel dextran or anticancer agents or 65 ng/gel proteins and left in a closed cap scintillation vial overnight. To remove unincorporated molecules, ferrogels were then soaked in 5 mL of PBS for 1 hour. Immediately the following rinse, gels were placed in 1 mL of PBS, and the release study was initiated. Ferrogel-containing scintillation vials were placed on top of a custom magnet stimulation system described previously [6] and exposed to 0 to 5.6 kGauss graded magnetic stimulation signals (or no signal for control

experiments) at frequencies between 0.1 to 14 Hz). Samples were taken after 10 min stimulation by fully removing the 1 mL of PBS, reserving it for later analysis. The concentration of FITC-dextran release was quantified using fluorescence ex/em at 495 nm and 525 nm in a BioTek Cytation3 microplate reader against a standard curve. The concentrations of mitoxantrone, 5-fluorouracil and irinotecan release samples were quantified by measuring optical absorbance at 610 nm, 350 nm, and 370 nm, respectively, on the plate reader against a standard curve. ELISA was used to measure the concentration of signaling factor release (VEGF and PDGF).

S.5.4 Data representation and statistical analysis

All quantitative data presented here are represented by means $\pm$ standard deviation, unless otherwise specified. For all statistical analyses, student t. test was used and p-values of less than 0.05 being our benchmark for statistical significance. *, **, ***, and **** indicates statistical significance of $p < 0.05$, 0.01, 0.001, and 0.0001 respectively. n.s. indicates no statistical significance. ($p > 0.05$).

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S.7. References

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