ULTRASOUND AND FLUORESCENT EVALUATION OF BIOMARKER ACTIVATED NANOPARTICLES FOR CHEMOTHERAPEUTIC BREAST CANCER DELIVERY

BY

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INDEPENDENT REPORT

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in the schools of Integrative Biology and Molecular & Cellular Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

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Abstract

Chemotherapy is an area in need of medical advancement due to its wide use for cancer treatment and unavoidable side effects which affect overall patient outcome. Targeted nanoparticle drug delivery has shown the potential to address challenges presented by modern chemotherapeutic treatments. This field of study focuses nano-scaled vehicles composed of various biocompatible polymers to diagnose, monitor, and treat diseases at a molecular level. Because of their novel designs, nanoparticles have the potential to improve drug solubility, prolong systemic circulation, help localize delivery, and increase drug efficacy. This study reports a novel biomarker activated nanovehicle for the delivery of doxorubicin to treat breast malignancies. Cell culture models are used to investigate biomarker specificity and efficacy while animal models are studied for release dynamics. Results show localized drug release with the capability to detect these nanoparticles through ultrasonic and fluorescent imaging techniques.

I. Introduction

The US has the highest annual incidence rate of cancer. Statistics from the American Cancer Society estimate that in 2010, over 1.5 million new cases were diagnosed among the population [1]. These numbers may be declining each year due to new advances in medical technology but the financial and emotional burdens created by cancer continue to take its toll on the US. This reality is further perpetuated by the fact that there are no clear causes for the onset of this disease. In terms of fighting cancer, the best one can hope for is an effective treatment plan.

Once diagnosed, patient outlook is best when the cancer is detected and treated early with treatment options ranging from surgery, radiation, hormonal, and chemotherapy. Chemotherapy,

in particular, is one of the most effective methods for treating cancer as it has specificity for neoplastic cells and is a minimally invasive treatment option [2]. While this type of treatment offers positive results, the benefits are not without costs. Off-target chemotherapeutic uptake by benign tissues often causes unwanted side effects and systemic damage. Physically, moderate side effects include nausea, vomiting, and hair loss with ratings strong enough to affect compliance with treatments [3-4]. Severe side effects, which are also not uncommon, include cardiotoxicity, brain dysfunction, immunosuppression, and damage to other body parts potentially leading to organ failure. These physical manifestations present clear challenges to cancer therapy and are further complicated by the psychological aspects of treatment. Therefore, the prevention of systemic drug release is crucial to the improvement of chemotherapy.

To overcome these challenges, strategies have been developed around the use of nanovehicles in hopes of localizing drug delivery to target sites while mitigating off-target release. An ideal nanoparticle must demonstrate *in vivo* efficacy, minimal toxicity, and traceable drug release [5]. The concept behind this study aims to investigate the therapeutic potential of novel nanoparticle vehicles in localizing chemotherapy delivery in breast cancer animal models. The use of biomarker technology is used to trigger drug release in target tissues while minimizing systemic release. Recent advances in cancer research have begun to shed light on different micro factors that are unique to tumors [6]. In parallel to the uncontrolled growth of tumors, the overexpression of specific proteins has been considered to be good tumor marker candidates in screening tests. With respect to breast cancers, cathepsin D has been identified as a key protease enzyme involved in extracellular matrix degredation for tumor development and metastases with hyperexpression profiles linked to cancer progression [7]. To avoid general proteolysis within the body, the nanoparticles are coated with a high density peptide layer of

cathepsin D substrate. This protective coating limits particle degredation in non target tissues while concentrating drug release in the cathepsin D rich tumor environments. A schematic diagram of the nanoparticle vehicle is shown below in Fig. 1.

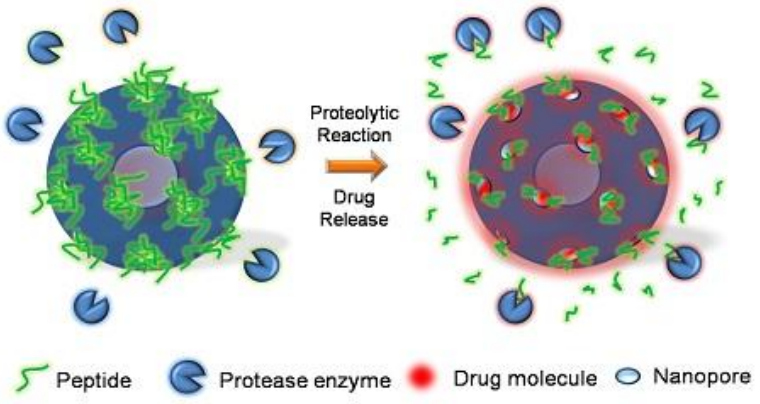

Fig 1. Illustration of gelatin nanoparticle drug release via proteolytic peptide cleavage.

4T1, MCF7, and 3T3 cell culture models are used to test cathepsin D peptide specificity and drug release *in vitro*. The rationale behind the use of these cell lines comes from their respective association with breast tumors. 4T1 cells are a common syngeneic model for studying tumor growth and metastasis in mice while the MCF7 line is ideal for breast cancer studies due to their retention of several ideal characteristics particular to mammary epithelium [8-9]. On the other hand, the 3T3 mouse embryonic fibroblast line is not commonly associated with breast cancer models and can be used to investigate the nonspecific effects of nanoparticle-cell interaction.

Athymic nude mice were chosen for *in vivo* drug studies due to their susceptibility to tumor induction and light skin phenotype. The nanoparticles were loaded with doxorubicin hydrochloride (DOX), a commonly used and naturally fluorescent chemotherapeutic agent. The

combination of the particles' distinct acoustic impedance and DOX's fluorescent nature allowed the use of both ultrasonic and fluorescent imaging modalities to capture particle flow and whole body drug localization *in vivo.*

II. Material & Methods

Particle Drug Loading and Peptide Application

Following fabrication, gelatin particles were received in a lyophilized state. As seen in Figure 2, the nanoparticles were uniform and spherical in size.

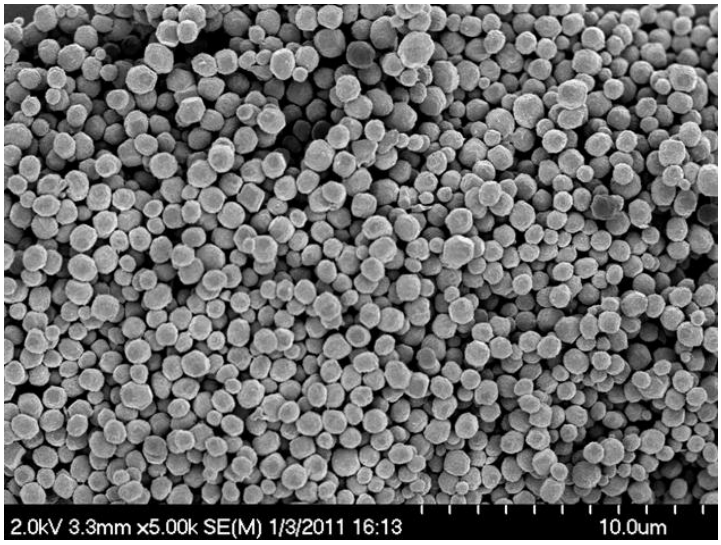

Fig 2. Scanning electron microscope image of gelatin nanoparticles 200-900nm in diameter.

To reconstitute them to a viable condition, 2mg was weighed out and submerged in 2mL of DOX. The particle concentration per mg was calculated as a function of weight, where d represented the diameter of a single gelatin sphere (μm) and D denoted the density of gelatin ($1.2 g/cm^3$).

$$W = \frac{4}{3}\pi\left(\frac{d}{2}\right)^2 * D$$

Immediate after submersion, the solution was sonicated using a Model 500 Ultrasonic Dismembrator (Fisher Scientific, Waltham, MA) at a 20% power level for 5 cycles. Each cycle consisted of a 30 second on pulse and 10 second rest period and was performed with the centrifugation tubes placed in an ice bath to avoid overheating of the sample. The particles were then left to sit for at least 5 hours to allow DOX impregnation. The solution was then spun down in a centrifuge, excess DOX solution removed, and particles rinsed with PBS.

To prevent degradation in non specific tissues, particles were ionically complexated with Cathepsin D substrate specific peptides. 500μL of 0.1 M PBS solution, 100μL of 0.33 M 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Sigma-Aldrich), and 100μL of 0.5 mM N-hydroxysuccinimide (NHS) (Sigma-Aldrich) solution was added to form a PBS-EDC-NHS solution as a catalyst for the conjugation process. To this mixture, 200μL of 100 nM drug loaded nanoparticles were added and kept overnight. The resulting mixture was centrifuged and washed with dimethylsufoxide prior to collection.

In Vitro Ultrasound Detection

To characterize the acoustic responses of a novel nanoparticle, solid gelatin spheres 200-900nm in diameter were exposed to ultrasound pulses using a double passive cavitation detection method [10]. This manually constructed setup involved the use of two 15 MHz receiving transducers and a 2.8 MHz center frequency transmit transducer, all confocally aligned within an enclosed degassed water tank. Particles were impregnated with saline and loaded into a syringe at a concentration of $2x10^9$ particles/mL. Single droplets were gradually introduced into the detection field until signals appeared on the oscilloscope. Over 1500 signals were collected as a

function of peak rarefractional pressure (1.1 to 6.5 MPa) and categorized into seven postexcitation schemes using both automatic and manual screening criteria.

In Vivo Ultrasound Detection

Saline loaded nanoparticles were prepared at a $1x10^9$ particles/mL concentration and loaded into syringes in 100uL volumes. BALB/c mice were anesthetized with 5% isofluorane 2% oxygen for inhalational induction, and moved to a 2% isofluorane facemask setup for anesthesia maintenance. The skin over the tumor was shaved and remaining hair removed with a depilating agent in order to maximize sound transmission and imaging. Mice were then placed in dorsal recumbency and a Vevo 2100 40-MHz linear array was used to monitor blood flow within the superior vena cava. Particles were introduced via a bolus injection into the lateral tail veins and real time video was captured.

Cell Culture

MCF7 human mammary adenocarcinoma, 4T1 mouse mammary carcinoma, and 3T3 mouse fibroblasts (all from ATCC, Manassas, VA**)** were cultured for this study. ATCC-formulated Eagle's Minimum Essential Medium with 0.01 mg/ml bovine insulin, 10% fetal bovine serum was used as culture medium for MCF7 cells. 3T3 Swiss mouse fibroblast cells (ATCC) were cultured using ATCC-formulated Dulbecco's Modified Eagle's Medium mixed with bovine calf serum to a final concentration of 10%. 4T1 mouse mammary cells were cultured using RPMI-1640 media mixed with 10% FBS. The cells were added to the media and then kept in 75 sq cm flasks (Corning Glass Works, Corning, NY) for culturing in CO_2 incubators at 5% carbon dioxide and 37.0 °C.

In Vitro Chemotherapy

To evaluate the specificity of the biomarker peptide coating on cell cultures, nanoparticle mediated chemotherapy was carried out on three different cell types. MCF7 human breast cancer cells were plated on a treated 96 well plate at a concentration of 90,000 cells/mL. 3T3 (100,000 cells/mL) and 4T1 (120,000 cells/mL) were also individually plated on a 96 well plate. All cell lines were allowed to adhere for 24 hours before experimental use. The cell cultures were then incubated with $2x10^6$ DOX loaded nanoparticles that were previously coated with peptide strands and mixed in PBS solution for 7 hours. Optical images were taken every 2-3 hours and cell viability was measured at different time points.

Additional proliferation measurements were taken using the 4T1 and MCF7 cell lines. For these experiments, an MTT proliferation assay (ATCC) was used to confirm previous viability test results. A 4T1 solution with a 30,000 cells/mL concentration was placed into 4 wells of a 48 well plate in equal amounts. Half of the wells served as experimental groups and received DOX loaded nanoparticles coated with cathepsin D substrate peptides. The remaining wells served as controls and were not exposed to the nanoparticles. Absorbance values were measured at various time points using a microplate reader (BioTek, Winooski, VT) and correlated to cell concentrations using a previously determined optical curve for the 4T1 cell line. Similar measurements were also taken with MCF7 cells, however a 2 hour time point was assessed only for this group. Graphs for nanoparticle exposed 4T1 and MCF7 cells were plotted to visualize the proliferation rate and for comparison to control groups.

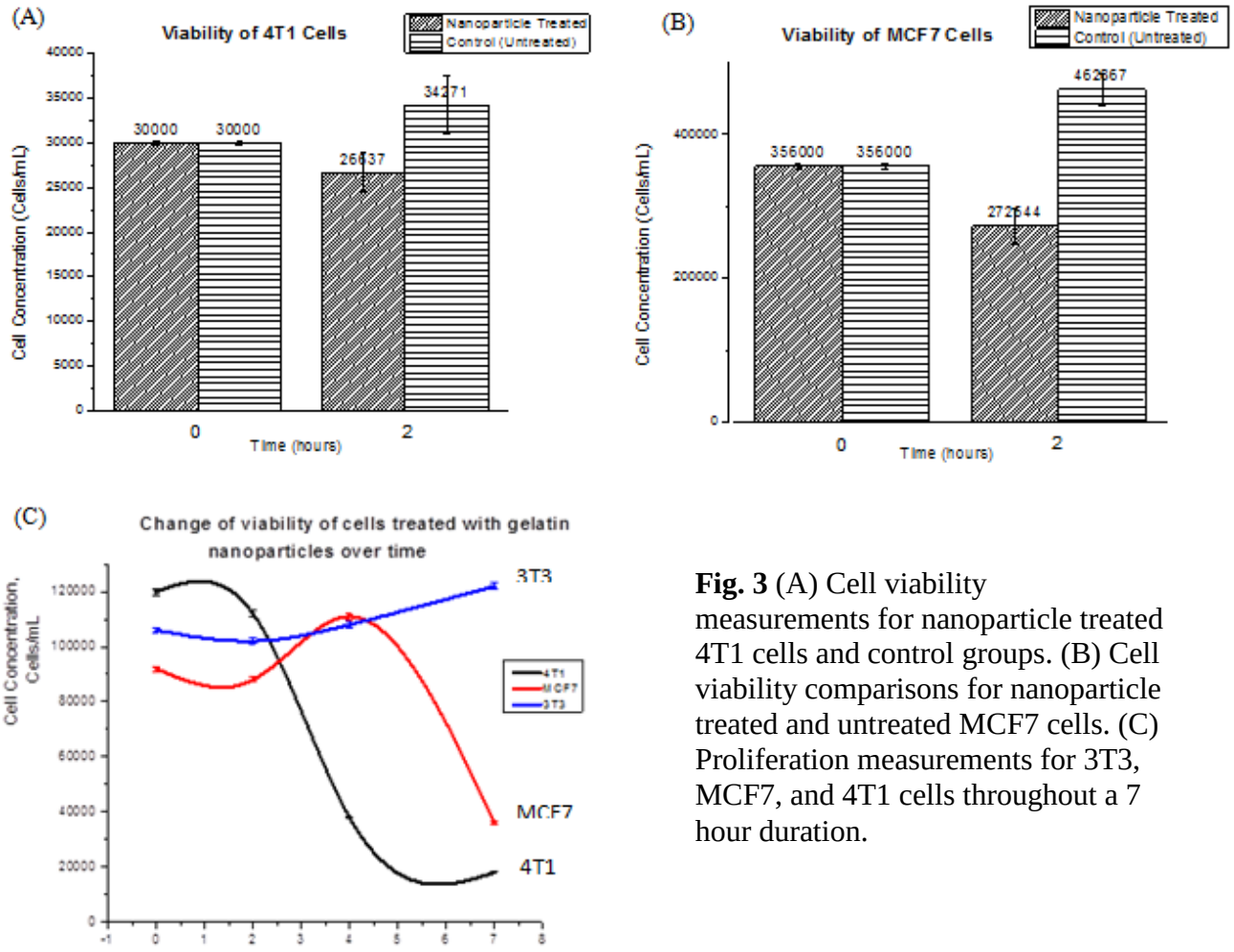

Fig. 3 (A) Cell viability measurements for nanoparticle treated 4T1 cells and control groups. (B) Cell viability comparisons for nanoparticle treated and untreated MCF7 cells. (C) Proliferation measurements for 3T3, MCF7, and 4T1 cells throughout a 7 hour duration.

Tumor Induction

5 week old female nude mice (Harlan, Indianapolis, IN) were individually housed with free access to a sterile water supply and irradiated food chow. For tumor inducement, mice were anesthetized under isofluorane (2% isofluorane, 2% oxygen flow rate) and subcutaneously injected with 100 uL of media containing a concentration of $1x10^3$ cells/mL into the lower left abdominal mammary gland. Following injection, mice were monitored for every 1-3 days. Those that did not display tumor growths after 6 weeks were re-injected under the lower right

abdominal glands at a higher concentration of $1x10^5$ cells/mL. Tumors were allowed to grow up to a maximum diameter of 10mm before exposures.

In Vivo Evaluation

100uL of saline containing $1x10^8$ DOX loaded and peptide coated nanoparticles were loaded into 1mL syringes and injected in the lateral tail vein of anesthetized nude mice bearing 4T1 tumors. During this time, ultrasound detection was performed using a Vevo 2100 High Frequency Ultrasound imaging system (VisualSonics, Ontario, Canada). A 40-MHz linear array was focused on the tumor growth to enhance particle extravasation into the tumor tissue and to monitor vasculature circulation. Within a 20 minute timeframe post injection, whole body imaging was performed with a Maestro Imaging System (Cri, Inc., Woburn, MA) within DOX's fluorescent profile (470nm excitation, 580nm emission) [11]. Injected mice were kept under gas anesthesia alongside a control mouse and fluorescent images were generated using Maestro imaging software. Skin autofluorescence from the control mouse was analyzed and subtracted from the injected group, resulting in images representative of nanoparticle distribution within the body.

All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of the University of Illinois and satisfied all campus and National Institutes of Health rules for the humane use of laboratory animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (Rockville, MD)-approved animal facility and provided food and water *ad libitum.*

III. Results & Discussion

In *Vitro/Vivo* Ultrasound Evaluation

Compared with DPCD responses from commercial ultrasound contrast agents, the gelatin nanoparticles exhibited a unique signal as shown in Figure 4.

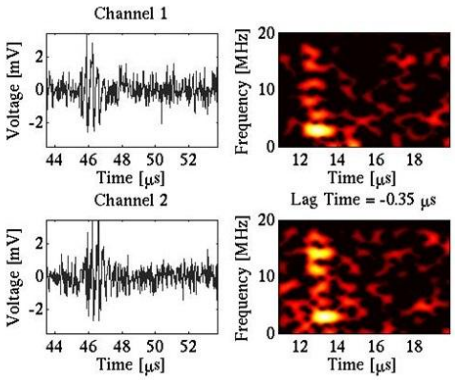

Fig. 4 Dual aligned acoustic echo response of gelatin nanoparticles at 5.5 MPa.

At higher pressure exposures, it was observed in frequency-time plots that the particles displayed a strong principal response at 3 MHz with weaker harmonics at higher frequencies. This observation was also noted by the absence of secondary broadband spikes in the corresponding voltage-time channels. These signals indicate that the gelatin spheres are relatively robust when exposed to ultrasound pulses while still retaining echogenic properties that allow them to be detected.

In vivo ultrasound detection of saline impregnated nanoparticles further revealed potential imaging capabilities. As seen in Figure 5, the particles were successfully detected in the superior vena cava after vasculature injection via the lateral tail veins. Mice were also allowed to recover from anesthesia post injection and monitored for immediate signs of distress of trauma. No signs of discomfort were observed. Together these results display the nanopaticles' biocompatibility and distinctive acoustic impedance which allow them to safely be imaged within mice.

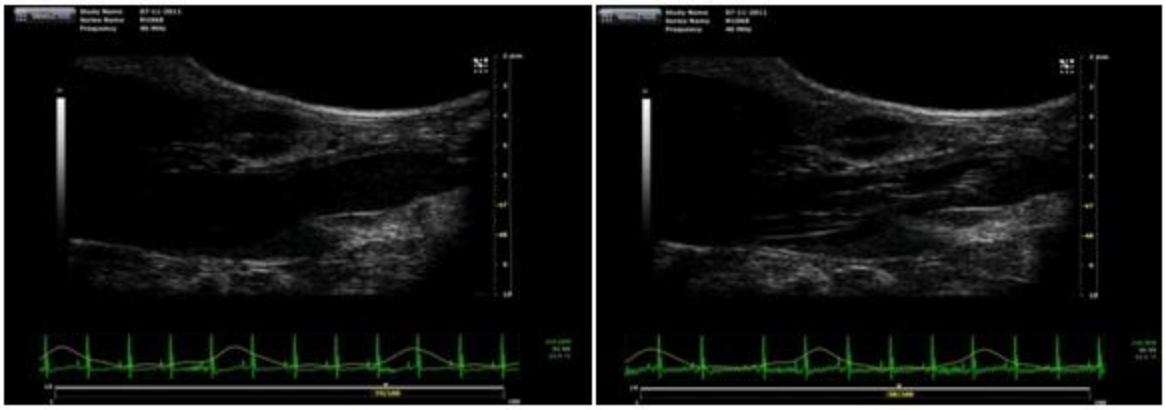

Fig. 5 High-resolution imaging of nanoparticle circulation in mice vasculature before and after injection.

Nanopaticle Mediated Chemotherapy on Breast Cancer Cells

The morphology of the peptide coated nanoparticles was used an indicator of drug release and confirmed through cell viability measurements. MCF7 human mammary adenocarcinoma and 4T1 mouse mammary carcinomas were chosen to test the peptide specificity to cathepsin D, a hypersecreted protease found within breast tumors, while 3T3 mouse fibroblasts were used as controls. Throughout a 7 hour time span of nanoparticle incubation, reduced growth trends were observed for both MCF7 and 4T1 cells. In contrast, there was no observable affect on the 3T3 growth rate.

The inhibited growth rates of the MCF7 and 4T1 cells were validated through an MTT cell proliferation assay at 2 hour time points. Each cell line was divided into an experimental group that received nanoparticles, and a control group that did not receive treatment. 4T1 concentrations decreased from 30,000 cells/mL to 26,000 cells/mL at the end of the incubation time while MCF7 cells also displayed reduced cell growth. Both control groups remained unaffected. The data is consistent with previous observations that the introduction of nanoparticles mediates drug release and decreases in cell proliferation. Overall, these experimental results demonstrate the peptide coating’s ability to prevent nanoparticle

degredation and drug release within non specific cell environments. The particles are specifically degraded in breast cancer cells that express the desired biomarker, in this case cathepsin D.

In Vivo Drug Localization in Breast Cancer Animal Models

The naturally fluorescent profile of DOX was used to study the biodistribution of nanoparticles in tumor bearing mice models. To evaluate this, DOX loaded nanoparticles were peptide coated and injected into the cardiovascular circulation system of nude mice models. Figure 6 shows the representative images from *in vivo* whole-body imaging.

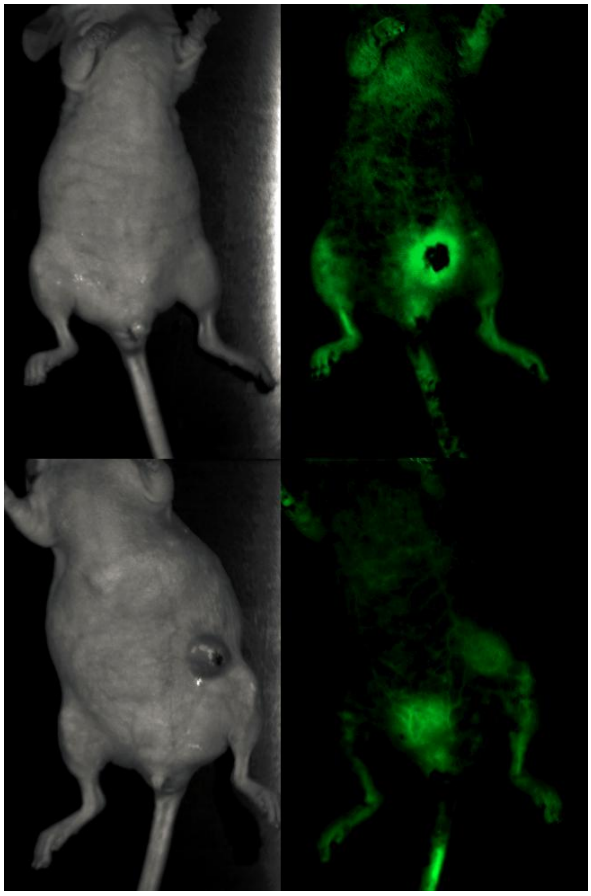

Fig. 6 Top Row - Brightfield and fluorescent imaging of control mice displaying nanoparticle biodistribution within the bladder.

Bottom Row – Brightfield and fluorescent imaging of 4T1 tumor bearing mice. Particles can been traced to the tail vein injection site, the bladder, and the tumor growth.

In control mice, the fluorescence signal can be traced to clearance organs such as the bladder and less noticeably the liver. Injected mice also display strong fluorescence localization within the

bladder, tail vein injection path, and most notably the tumor site. This demonstrates that the nanoparticles had distributed throughout the circulation system and accumulated in the bladder and tumor tissue within a 30 minute timeframe.

IV. Conclusion

The value of assessing the biodistribution of drug delivery systems holds tremendous potential in improving chemotherapy treatment and mitigating negative side-effects due to off-target drug release. In this study, biomarker activated nanoparticles with narrow particle size distributions were developed for breast cancer treatment in nude mice models. Passive acoustic cavitation measurements were used to assess the nanoparticles' experimental response and determined to lack signals indicative of transient collapse or fragmentation. Cell models were used to validate biomarker activated specificity for cathepsin D and measured directly through decreases in cell proliferation rates. Additional *in vivo* ultrasound and fluorescent imaging both revealed sufficient contrast within mice vasculature to monitor the circulation path as well as drug localization within tumor sites. Furthermore, there were no observable signs of complications due to the introduction of nanoparticles in mice vasculature.

Future studies for this design should be planned to examine the longitudinal potential for tumor regression as well at the clearance mechanisms for drug and nanoparticle removal within the body. It has been reported elsewhere that silica based nanoparticles can be detectable for up to 3 months [12]. Given that the nanoparticles used in this study are composed of biocompatible gelatin polymers, it would be interesting to look at the difference in clearance times. Additionally, this use of nanoparticle vehicles and biomarker technology may potentially be applied with other candidate chemotherapy drugs. By varying the substrate peptide for surface coating, other cancer biomarkers can be targeted to treat different subtypes of cancers.

V. Acknowledgements

This work was supported in part by the *University of Illinois* Center for Nanoscale Science and Technology (CNST), the *National Cancer Institute*-funded *Siteman Center for Cancer Nanotechnology Excellence* at Illinois, *Mesothelioma Applied Research Foundation*, the *Naional Research Foundation of Korea*, *U.S. ARMY Medical Research Acquisition Activity* under the contract # W81XWh-10-1-0859 and NIH R37EB002641. The author would like to express sincere appreciation to Dr. William O'Brien Jr. for his assistance in the preparation of this report and mentorship throughout this research effort. In vivo whole body imaging studies were carried out in cooperation with Stephen Boppart, M.D. Ph.D, and Eric Chaney from the Biophotonics Imaging Laboratory at the same university. The author would also like to thank Rami Abuhabsah, Emily Hartman, RDMS, and Rita J. Miller, D.V.M, for their respective assistance in tumor inducement preparation, in vivo mouse ultrasound imaging, and supervision in all animal work.

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