IN VITRO AND IN VIVO IMAGING OF PEPTIDE-ENCAPSULATED POLYMER NANOPARTICLES FOR CANCER BIOMARKER ACTIVATED DRUG DELIVERY

BY

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THESIS

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ABSTRACT

Cancer creates one of the most significant public health problems not just in the United States, but worldwide. While one of the most effective treatment protocols for cancer is chemotherapy, the conventional agents used in chemotherapy affect normal tissue as well as cancerous tissue. This thesis reports the development of a new cancer drug delivery system based on nanoparticles, which is designed to target tumor sites better than previous practices.

In this study, nanoparticles coated with cathepsin D-specific peptides were developed as a vehicle for the targeted delivery of the cancer drug doxorubicin (DOX) to treat breast malignancy. Cathepsin D, a breast cancer cell secretion, triggers the release of DOX by digesting the protective peptide-coating layer of nanoparticles. Ultrasound imaging successfully detected fabricated nanoparticles in both in vitro conditions and in vivo mouse cancer models. Cell viability experiments were conducted to determine the efficacy of biomarker activation specific to breast cancer cell lines. These experimental results were compared with the outcome of a viability experiment run on non-cancerous cells. Viability decreased in human breast MCF7 cancer and mouse breast 4T1 cancer cells with no effect on fibroblast 3T3 non-cancerous cells. The next step was to obtain a real-time video of nanoparticle flow in mouse models using in-vivo ultrasound imaging. In vivo fluorescence imaging enabled the examination of cancerous mice injected with the drug-carrying nanoparticles. Results showed the

distribution of nanoparticles in subject mice bodies, with concentrations in bladder and tumor sites. This finding suggests that nanoparticles are able to specifically target tumor tissues. It also suggests nanoparticles are resistant to nonspecific disintegration of peptide coating and consequential system drug release. Thus, the results of this work can be of great value for the development of more effective cancer treatment methods.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

According to the International Agency for Research on Cancer (IARC), cancer caused 7.6 million deaths worldwide in 2008 and currently one in every four deaths in the United States is due to cancer [1]. Chemotherapy is widely used for cancer treatments despite the many side effects it imposes to a patient's health. High doses of chemotherapeutic agents are required to treat large tumors; however, their toxicity limits the dosage of chemotherapeutic drugs that can be given to a patient, resulting in suboptimal treatment of cancer.

1.1. Nanotechnology and Cancer Treatment

Several strategies have been proposed to target cancer cells, mostly based on biodegradable polymeric particles, which have attracted considerable attention due to their stability [2], versatility of surface modification [3], and potential for different drug release characteristics [4, 5, 6]. However, in these studies, although delivery efficiency increases with particle drug carrier, off-target drug release still exists due to the fast drug release of nanoparticles during circulation. One of the promising strategies, conjugating nanoparticles with antibodies that detect tumor-associated antigens, showed successful results for in vitro screening [7]. However, despite these encouraging results in vitro, the in vivo application might be restricted due to weak linkage stability and potential immunogenicity after

repeated injections [8]. Thus, the problem of finding an effective nanocarrier is still unresolved despite the aforementioned strategies.

In this project, we report using innovative nanoparticle drug capsules, through which the chemotherapy drug release can be triggered and tuned by nothing but the biomarker protease enzymes that are secreted in breast cancer cells and their extracellular matrix. That is to say, the cancer drug is only released in the vicinity of breast cancer tissue and the release dosage is inherently proportional to localized cancer status. Drug release from nanoparticle capsules in living breast cancer animal models can be monitored in real time through the use of high-resolution ultrasound imaging and fluorescence imaging to demonstrate the targeted release localized near the breast tumors. The main objective of the study described in this thesis is to develop an innovative design of drug delivery nanocapsules that are activated by cancer-specific biomarker enzymes for high-precision cancer chemotherapy. This thesis discusses work done towards this goal, particularly it discusses imaging results and evaluation of the effects of nanoparticles in in vivo and in vitro conditions.

1.2. Scope of the Research

1.2.1. Unique Aspects

Currently available strategies based on biodegradable nanoparticles impose early polymer degradation and risk of off-target drug delivery. In this

study, a unique design of nanocarriers prevents polymer degradation before the nanocarriers reach the tumor sites, largely due to a conjugated peptide layer on the nanocarrier surface that prevents the drug from being released early. Additionally, although there have been studies that showed encouraging results in terms of off-target drug release, nanoparticles still impose toxicity due to the silicon nature of the nanocarriers used in the studies [9, 10, 11]. This thesis, however, discusses the work done with nanoparticles made of biodegradable and non-toxic material. Finally, the time-lapse drug delivery and release dynamics information acquired from ultrasound and fluorescence imaging is extremely valuable for breast cancer chemotherapy studies. Suitability of proposed nanoparticles for ultrasound detection makes them a perfect combination of contrast agent and cancer drug carrier in one.

1.2.2. Impact of Study

This study directly addresses the most challenging problem in standard chemotherapy, off-target drug release, and advances a new potential cancer treatment. Proof of the concept opens the door to a more precise and effective method of cancer treatment that does not impose all the side effects traditional chemotherapy carries. Technologies originated from this concept could be applied to available chemotherapy drugs on the market and can make use of the drugs that are much safer and more efficient than those that are currently used in breast cancer treatment. By varying the substrate peptide sequences used for

nanoparticle coating, different cancer biomarkers can be targeted and the hybrid drug nanocapsule may be tailored for different subtypes of breast cancers for personalized medicine and therapy. Thus, this technology opens broad opportunities for a new generation of cancer treatments.

1.3. Overview

This work focused specifically on breast cancer malignancy. In order to achieve the final goal of the work - to show the effect of nanoparticles on breast cancer tumors in in vitro and in vivo conditions - the project comprised four main stages. Before the main study was conducted, in order to determine the best method for effective coating of nanoparticles, preliminary studies of conjugation techniques for small molecules were carried out. After the preliminary work came the main part of the study: the fabrication of peptide-coated and drug-loaded nanoparticles. The second stage of the study included evaluation of effects of nanoparticles on cancer cells in in vitro conditions. Finally, the concluding stage consisted of in vivo and in vitro imaging of biomarker activated nanoparticle capsules in animal models.

This thesis is broken up into several chapters that describe relevant stages of the study. Chapter 2 discusses the background information about existing problems in nanoparticle drug delivery and introduces the concept behind the

design of proposed nanoparticles. Chapter 3 discusses the methods used in the research completed for this thesis. It describes the parameters examined and outputs collected during the various stages of this work. Chapter 4 reviews the results and analysis obtained from the experiments described in Chapter 3. It reflects on and explains the outcomes of the research done during each stage of the study. Chapter 5 summarizes the main results and establishes their relevance to our study's primary objective. This chapter then continues with future recommendations and perspectives for this research project.

CHAPTER 2: THEORY AND CONCEPT OF THE WORK

2.1. Standard Chemotherapy and Its Limitations

Cancer chemotherapy was first successfully used in the 1950s when nitrogen mustard, previously used as a war gas, was found to be useful in inhibiting tumor growth [12]. However, due to its toxicity, chemotherapy with anticancer drugs took until the 1960s to be widely applied and it started to gain popularity in 1970s as a means to cure or inhibit the growth of certain types of cancers [12]. Currently there is an immense number of different anticancer agents available for chemotherapy; however, drugs that are more effective tend to be more toxic. As a result, off-target cancer drug delivery causes serious side effects and systemic damage to a human body going through chemotherapy. For example, doxorubicin (DOX), the most effective and widely used anticancer drug, is reported to cause adverse effects including nausea, vomiting, anorexia and heart damage (cardiotoxicity), which considerably limit its applicability [4, 13, 5]. Therefore, prevention of systemic drug release is crucial to improving current chemotherapy. With the emerging field of nanotechnology and huge progress in nanoparticle technology, development of nanoparticles of biodegradable polymers as an effective drug delivery vehicle for chemotherapy has become a new breakthrough among alternative methods of cancer treatment.

2.2. Nanoparticle Drug Delivery and Its Limitations

In the past few decades, research has emerged on the development of targeted drug delivery to cancer cells using nanoparticles. Nanoparticles, which have a small size suitable for intracapillary passage, provide an ideal solution for the mentioned challenge of current chemotherapy: toxicity of chemotherapeutics [14, 15, 16]. Additionally, nanoparticles can provide a controlled and targeted way to deliver the encapsulated cancer drugs and consequently provide high efficacy and minimized, or even completely mitigated negative side effects of off-target drug release [17, 18, 19]. However, despite the emerging and promising research done in in vivo therapy based on nanoparticles, there are still many difficulties to overcome in order to create an effective vehicle for targeted drug delivery. Some of the important challenges current nanoparticle-based drug delivery research encounters are the conjugation of developed nanoparticles with host molecules, multiple loading functionality, biodegradability, and toxicity [20, 21]. Additionally, 40% of anticancer drug candidates suffer from poor solubility due to crystal phase formations on nanocarriers [22]. Under intense study, porous silicon films and microparticles have been proposed for biomedical applications [23, 24, 25, 26, 27], showing high loading and releasing capacity, in vivo monitoring, and easy chemistry. However, these studies were carried out on micrometer-sized particles, while nanoparticles with diameters between 20 and 100 nm have been speculated to be ideal for cancer therapy [21, 28-30]. Off-target drug release of proposed particle carriers still exists due to the fast drug kinetics

during circulation. Additionally, for effective nanoparticle-based systems, it is essential to monitor drug delivery to targeted sites and verify the efficacy of the encapsulating peptide or antibody coating. However, it is difficult to monitor drug carrier transport due to lack of sufficient contrast of existing nanoparticles for current monitoring systems. The whole field of cancer treatment can be substantially improved by engineering such drug nanocarriers that can provide better contrast for imaging studies, prevent systemic drug release and achieve the correct specificity.

2.3. Concept of Novel Nanoparticles for Targeted Drug Delivery

In this study, we report a novel gelatin nanoparticle carrier for the targeted delivery of DOX to treat breast malignancies, which avoids problems of early nonspecific dissolution and off-target drug release and is suitable for high-resolution ultrasound and fluorescence imaging in animal models. The versatile nanotechnology here could be applied for treatment of different kinds of cancer with the change of biomarker specific peptides. A schematic diagram of the chemotherapeutic drug delivery vehicle is shown in Fig. 1.

The nanoparticle core was fabricated by the Electric Field Assisted Precision Particle Fabrication (E-PPF) method using acidic gelatin, loaded with DOX [31, 33]. The resulting nanospheres were coated with a high-density peptide

layer, the hydrolysis of which is catalyzed by cathepsin D, a specific biomarker protease hypersecreted by breast cancer cells. Thus, the core is protected from general proteolysis, wherein DOX is safely contained, until the digestion of the peptide shell is catalyzed by the secretory protease enzyme cathepsin D in the proximity of breast cancer cells. As the peptide shield is removed, gelatin is exposed to general proteases abundant in all cell secretions, triggering the release of DOX. As a result, the drug is released only in the vicinity of the target cancer cells and its release dosage is controlled by the localized secretory protease concentration. Because of the low presence of targeted protease in benign tissues, the peptides covering the nanocapsule surface remain intact and the drug inside the nanocapsule is well contained. By these means, a most effective chemotherapy may be achieved with minimal side effects.

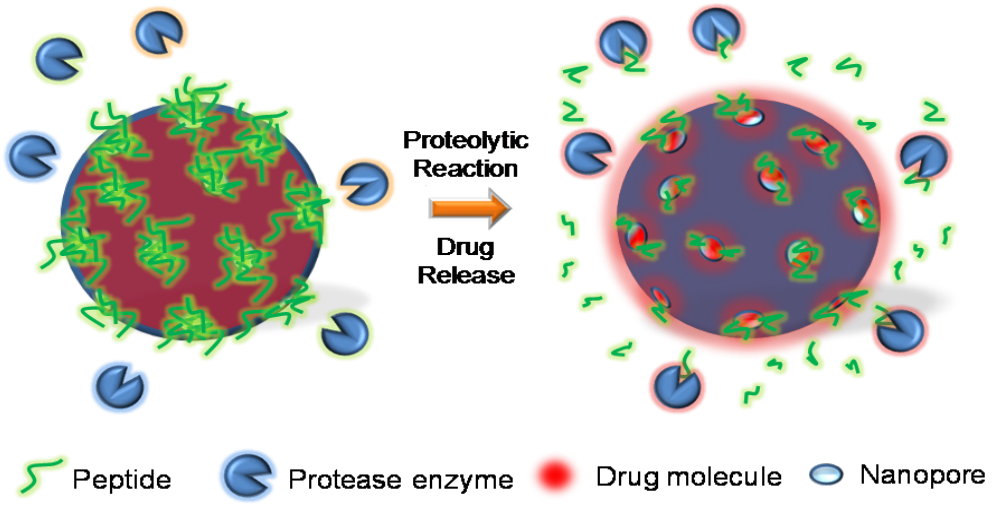

Fig. 1 Illustration of gelatin nanoparticle drug carrier protected by protease substrate peptides.

CHAPTER 3: METHODS

3.1. Preliminary Work: Conjugation Procedures

Chemical structure of the drug-loaded nanoparticles plays an important role in determining the adhesion of nanoparticles to and their interaction with cells. Conjugation of molecules to and their amount on the surface of nanoparticles have a significant effect on drug encapsulation efficiency, since the coating serves as a barrier to protect the leakage of the drug from the nanoparticles. Better conjugation of molecules to the surface prevents the early degradation of nanoparticles in the system, improving the targeted drug delivery efficacy. Therefore, prior to utilizing the peptide coating design, which is further discussed in the following subsections, various conjugation techniques and procedures were investigated as a preliminary stage of this project.

3.1.1. Antibody-Antigen Conjugations

The conjugation of small molecules (SM) to proteins is widely used in medical diagnostics and is valuable for nanoparticle drug delivery systems. Labeled antibodies and antigens are required in many diagnostic techniques. During this stage of the work, we experimented with enzyme-linked immunosorbent assay (ELISA) protocol conjugation. Polycarbonate surface was chosen as a substrate for antibody-antigen conjugation experiments for potential

usage of these results for another study [32]. Several strategies for activation of polycarbonate surface have been used.

Polylysine treated surface. In order to create a layer that is welcoming to conjugation of SMs, particularly Bovine Serum Albumin (BSA) (Sigma-Aldrich) molecules, a CD surface was treated with polylysine (Sigma-Aldrich). Figure 2 shows the schematics of ELISA performed on a compact disk. First, 0.5 ml of 2 mg/mL polylysine was applied to the CD surface and incubated in humid conditions for 24 hrs. After washing the surface, 0.5 ml of 1% glutaraldehyde (Sigma-Aldrich) was applied to the surface and incubated for 2 hrs. Glutaraldehyde (GA) is a cross-linker that has a carboxyl group on both ends. The carboxyl group of GA bonded with amine groups of previously applied polylysine. The other end of the GA was conjugated with an amine bond of primary antibody, Anti-Bovine Serum Albumin antibody (Abcam), that was applied as the next step in the experiment, and the layer was incubated for 2 hrs. This incubation step ensures the immobilization of antibodies to the surface so that antibody-specific molecules could be used for binding. Washing with PBS accompanied each incubation step. For the conjugation experiment, immobilized antibody specific antigen BSA and its secondary antibody FITC-IgG (Sigma-Aldrich) were used. Secondary antibody FITC-IgG that is specific to BSA molecules was applied for 2 hrs, after the BSA molecules were conjugated with the primary antibody. FITC-IgG has a fluorescence tag that emits fluorescence when the

conjugation between antibody and antigen occurs. Due to emitted fluorescence the whole conjugation could be confirmed via fluorescence microscopy.

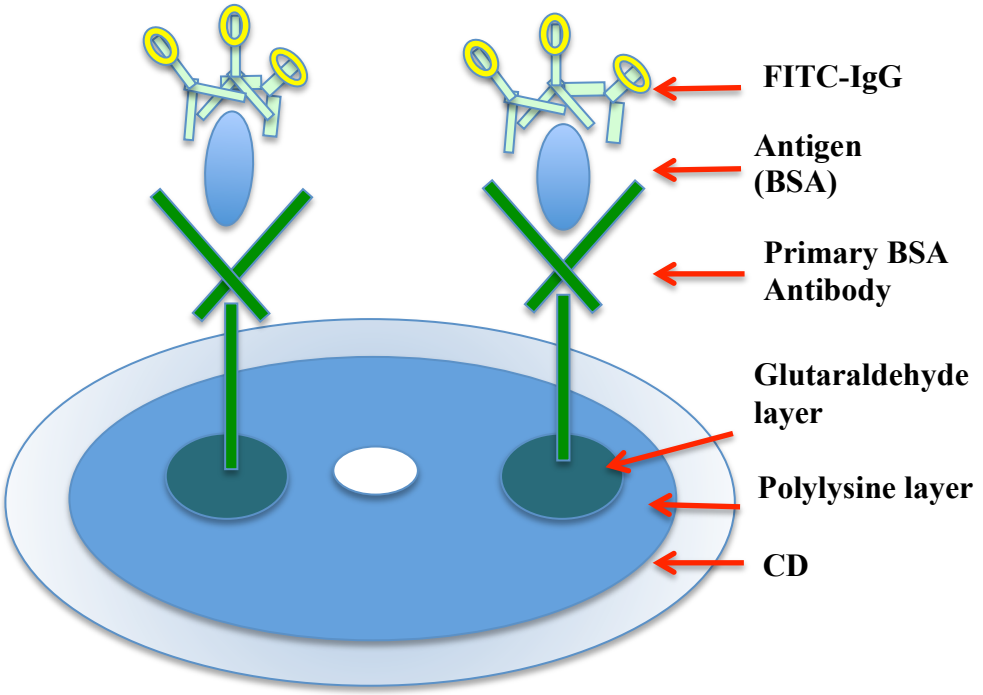

Fig. 2 Schematics for antibody-antigen conjugation to polycarbonate surface of a compact disk

Oxygen plasma treated surface. Another way of immobilizing the proteins on the polycarbonate surface could be creating an abundance of –OH bonds and having antibodies bind to the polycarbonate surface directly without the use of the GA cross-linker. For this purpose, our substrate was treated with oxygen plasma,

and primary antibody-antigen-secondary antibody conjugation was performed following the previously described procedure for the ELISA experiment. An advantage of this method is that it allows skipping the glutaraldehyde step, since proteins could bind to the oxygen-treated surface directly. A limitation that we observed in this experiment was that oxygen plasma applied to the surface of the CD was unstable and conjugation had to be done in short periods of time. Since the incubation steps in ELISA were lengthy, treating with oxygen plasma was a problematic solution to creating bonds on the polycarbonate surface.

Reactive Ion Etching activated surface. Previous studies have reported the usage of reactive ion etching (RIE) for preparing substrate materials for protein arraying applications that involve fluorescence-based detection [34]. In RIE, plasma etches the surface of a substrate using both chemically reactive species and ion bombardment. The resulting volatile byproducts get removed in vacuo during the process. RIE process enhances the roughness and porosity of the polycarbonate surface, thereby facilitating the protein adsorption on the surface of RIE-etched polymers [34]. Additionally, there is evidence, such as the enhanced surface hydrophilicity, of chemical effects that may have improved the retention of adsorbed protein on the etched surface [34]. However, in order to minimize the physical damage to the substrate that is typically caused by ion bombardment, in this study all parameters had to be carefully chosen for effective RIE-activation of the polycarbonate surface. In order to RIE-activate the CD surface, the following

parameters were used: 30% RF power, 80% oxygen, 20% CF4, 100 mTorr throttle pressure. Etching time varied from 20 to 40 minutes for this set of experiments. After RIE-activation, standard procedure of sandwich ELISA could be performed. Additionally, a simple antibody-antigen procedure could be applied; however, in the choice of antibodies, it is important to use fluorescently labeled antibodies for conjugation detection purposes.

Glutaraldehyde conjugated blue microparticles. Results of this conjugation experiment were published elsewhere as an application for microparticle counting using a standard CD drive [32]. Figure 3 shows the schematics behind the immobilizing BSA molecules on the polycarbonate surface. First, the surface of a standard CD is treated with polylysine, and glutaraldehyde modified microparticles that are coated with BSA molecules are incubated on top of the polylysine layer. For glutaraldehyde conjugation of microparticles, 10 μm blue polystyrene microparticles (Polysciences, Inc.) were conjugated with 8% glutaraldehyde. The conjugation protocol is described elsewhere [32]. One ml of glutaraldehyde modified blue microparticles was incubated with BSA antibody solution for 10 hrs. This solution was then incubated on the polylysine-activated CD surface for 3 hrs and was followed by washing.

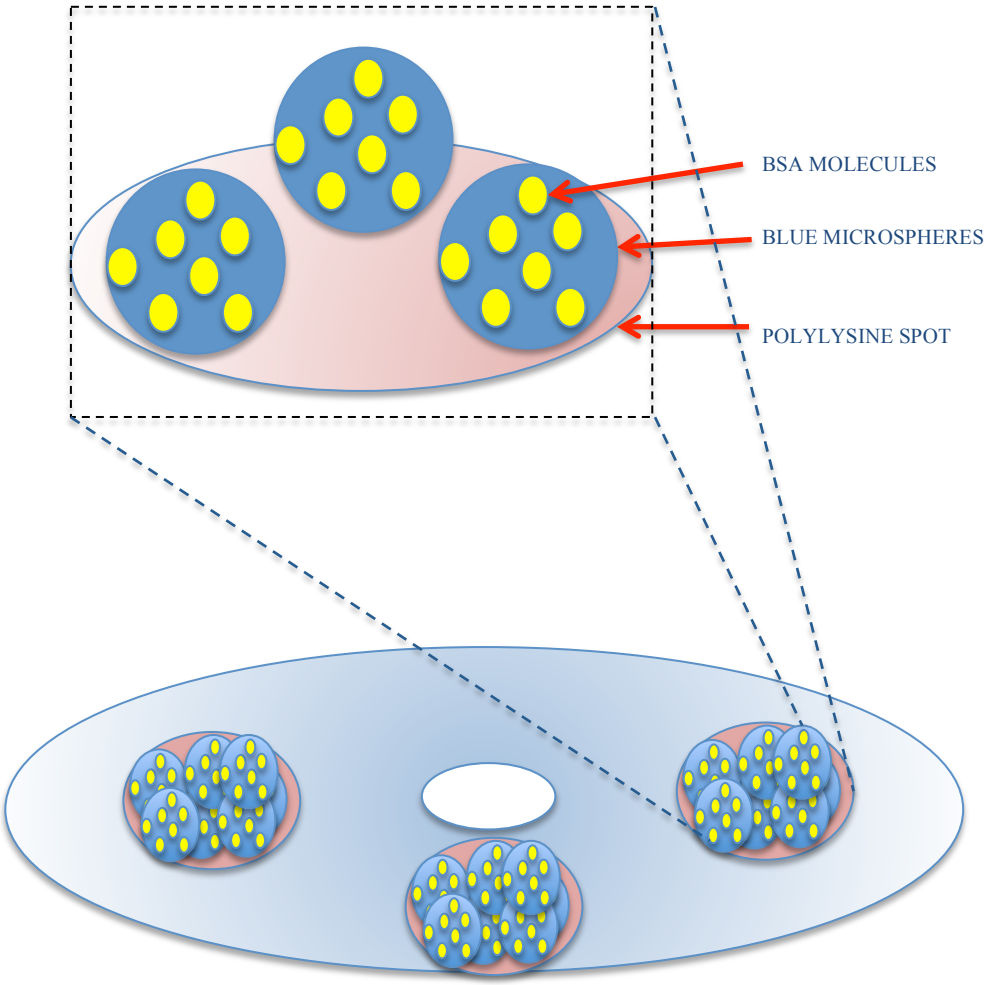

Fig. 3 Schematics of immobilizing the antibody conjugated polystyrene microparticles to CD surface.

3.2. Fabrication and Characterization of Drug Nanocarriers

Following the preliminary experiments for conjugation techniques of proteins applied to different surfaces, which are described in the previous chapter, fabrication of nanoparticles was established as the first main stage in this study.

3.2.1. Fabrication, Cross-linking and Drug loading

Gelatin (225 g bloom, BioReagent) polymer nanoparticles were prepared by the E-PPF [33]. In order to create cross-linking moieties on the surface of nanoparticles for consequent conjugations with peptide (which will be described in the next subsection), fabricated gelatin nanoparticles were cross-linked using GA that forms carboxyl groups on the surface of the nanoparticles. Fabricated nanoparticles were imaged with high-resolution ultrasound to show that they can provide sufficient contrast to monitor the drug transport in the consequent in vivo experiments.

To prepare nanocapsule carriers from the fabricated nanoparticles, DOX molecules (Sigma-Aldrich) were loaded into the polymer matrix through diffusions in excess drug solutions. Upon inward diffusion, the drug molecules were ionically impregnated to the polymer matrix so that unless attacked by an enzyme, they may not be released.

A standard parameter used for characterizing nanoparticles in drug delivery studies is swelling ratio, which could be characterized by using equation (1). Swelling ratio was calculated for fabricated nanoparticles using the dimensions of dry and wet particles.

$$Swelling\ \% = 100(W_{wet} - W_{dry})/W_{dry} \quad (1)$$

where W_{wet} and W_{dry} are the weight of the wet and dry sample, respectively. Wet nanoparticle samples were prepared by immersing dry nanoparticles in a phosphate buffer saline (PBS) (Sigma-Aldrich) solution at room temperature for 24 hrs.

3.2.2. Coating the Nanoparticle Surface

In order to keep the drug inside the capsule, the peptide strands were synthesized and a cross-linker moiety group was grafted at the terminal. Peptides were attached through covalent bonding between the cross-linking amine moiety groups at the peptide terminal and the carboxyl groups on the surface of the gelatin nanoparticles, which were previously formed via glutaraldehyde. In order to catalyze the conjugation of peptides to the nanoparticle surface, catalyst EDC-NHS (Sigma-Aldrich) was successfully employed. The specificity of the designed peptide substrate to the cathepsin D enzyme secreted by breast cancer cells was examined via the fluorescence emission from the terminal methoxycoumarin

(MCA) fluorophore molecule. The fluorescence was quenched by the dinitrophenyl (Dnp) molecule before the proteolytic reaction due to near field fluorescence energy transfer between the MCA fluorophore and Dnp quencher molecule.

3.3. In Vitro Chemotherapy

3.3.1. Cell Culture

To evaluate target cell specificity, nanoparticle mediated chemotherapy on three different types of cells was carried out. MCF7 human mammary adenocarcinoma, 4T1 mouse mammary carcinoma, and 3T3 mouse fibroblast were chosen (all from ATCC, Manassas, VA) for the experiment. ATCC-formulated Eagle's Minimum Essential Medium with 0.01 mg/ml bovine insulin, 10% final bovine serum (FBS) was used as a culture medium for MCF7, while 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 breast cancer cells were cultured using RPMI-1640 media mixed with 10% FBS. All media were filtered using a 0.22 μm vacuum filter for sterilization. The cells were added to the cultured media and then kept in 75 sq. cm flasks for culturing in incubator with 5% carbon dioxide at 37.0 °C.

3.3.2. Effect of Drug Nanocarriers on Cell Viability

Prepared cell cultures were incubated with $2x10^6$ drug loaded nanoparticles coated with peptide strands mixed in phosphate buffer solution (PBS) for 7 hrs. Optical images were taken every 2 to 3 hr and cell viability was measured at different time points.

Additionally, a MTT cell proliferation assay (ATCC) was carried out for cultured human breast MCF7 and mouse breast 4T1 cancer cells. This assay provides a reliable and quantifiable means of measuring the absorbance values of cell populations through spectrophotometry. Each 30,000 cells/mL solution of 4T1 cells and 38,000 cells/mL solution of MCF7 cells were placed in 2 and 4 wells of 96-well plates in equal amounts for each cell line, respectively. While half of the wells for each cell line were treated with drug carrying nanoparticles, the remaining half was incubated without nanoparticles for control measurements. Cell viabilities of treated MCF7 and 4T1 cells were assessed after incubation time using the MTT proliferation assay procedures. The results were compared to viabilities of the cells that were incubated under the same conditions, but without any addition of drug loaded and peptide coated nanoparticles.

3.4. In Vivo Chemotherapy

In order to test fabricated nanoparticles in in vivo conditions, we conducted extended animal studies. For this stage of the study, the experimental protocol was 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 a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (Rockville, MD) and provided food and water *ad libitum.*

3.4.1. Animal Models

Female 5-week-old athymic nude mice (19-23 grams on arrival) were ordered from Harlan Laboratories and individually housed in separate cages. For tumor inducement, mice were anesthetized under isofluorane (2% isofluorane, 2% oxygen flow rate) and subcutaneously injected with 100 μL of media containing $1x10^5$ 4T1 cells in the lower abdominal mammary gland. Following injection, mice were monitored every 1-3 days. Mice that did not display tumor growth after 6 weeks were reinjected under the lower right abdominal glands. Tumors were allowed to grow up to a maximum size of 10 mm before exposure. In vivo whole-body imaging studies, which are described in the consequent sub-chapter, followed similar anesthesia.

3.4.2. In Vivo Ultrasound and Fluorescence Imaging

For in vivo ultrasound detection, nude mice (Harlan, Indianapolis, IN) weighing 19-21 g were injected via the lateral tail vein with 100 μL of saline solution containing $2x10^9$ nanoparticles per mL that ranged between 200 and 900 nm in diameter. Real-time video of nanoparticle flow in the superior vena cava was captured starting from the injection time using a VisualSonics Vevo 2100 High Frequency Ultrasound Imaging System, indicating the potential application of the drug-carrying nanoparticles in serving as ultrasound imaging contrast reagents.

Along with ultrasound imaging, due to a fluorescent nature of DOX, mouse models injected with fabricated nanoparticles were imaged with fluorescence microscopy. Following the method described in the previous subsection for tumor inducement into nude mice, animals were tumor induced before fluorescence imaging. These 4T1 tumor mice were then injected with 100 μL of saline solution containing $2x10^9$ peptide coated drug nanocarriers. Due to the naturally fluorescing nature of DOX, fluorescence images of the injected and control mice were obtained for accessing the distribution of the drug in the mouse body after injection. Based on the DOX fluorescent profile, an excitation of 470 nm and emission of 590 nm were used.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1. Gelatin Nanoparticles

The scanning electron microscope (SEM) image in Fig. 4(A) and optical image of particles in saline solution (Fig. 4(B)) show that the particles fabricated by the E-PPF method are spherical and uniform in size.

The swelling ratio, defined as the ratio of diameters of wet (1.62 μm) and dry (0.9 μm) particles, was 1.8. Figures 4(C) and 4(D) show optical images of dry and DOX-loaded nanoparticles after centrifugation and washing.

The specificity of the designed peptide to cathepsin D was examined via fluorescence emission from the terminal MCA fluorophore molecule on the peptide strand. Figure 5 schematically illustrates gelatin particles conjugated with peptides containing a Leu-Phe-Phe-Arg-Leu sequence, which can be recognized by cathepsin D, an aspartic protease enzyme prominent in breast malignancies [18]. Once hydrolysis of the peptide is catalyzed by cathepsin D, the peptide substrate fluoresces as an indicator of the proteolytic activity of the peptide coating layer on the nanoparticle surface. When the peptide-coated particle loaded with DOX is incubated, respectively, with purified cathepsin D and with the MCF7 breast cancer cell media, the fluorescence intensity, as shown in Fig. 6, increases.

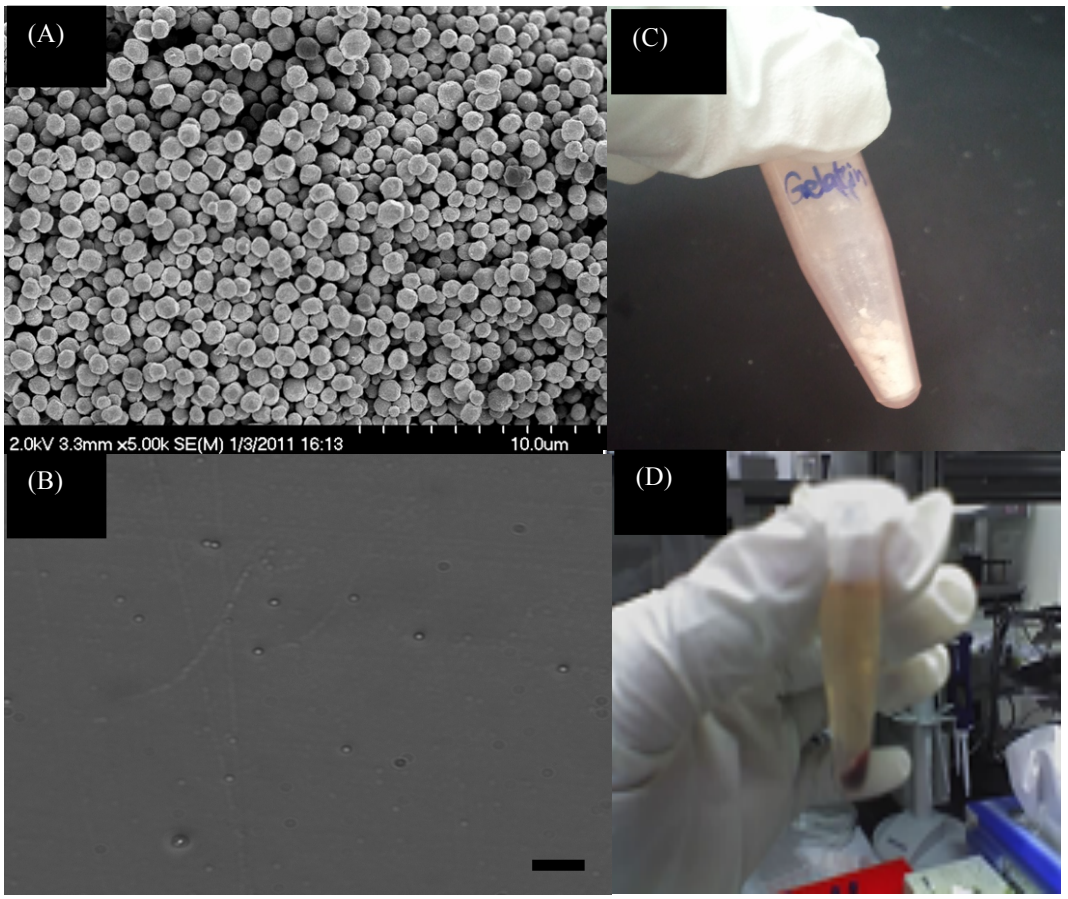

Fig. 4 (A) Scanning electron microscope images of gelatin submicron particles; (B) Optical image of gelatin particles after swelling in saline solution (200 nm – 5 μm); (C) Optical image of dry nanoparticles; (D) Nanoparticles loaded with cancer drug (DOX) after centrifugation. The scale bar represents 10 μm.

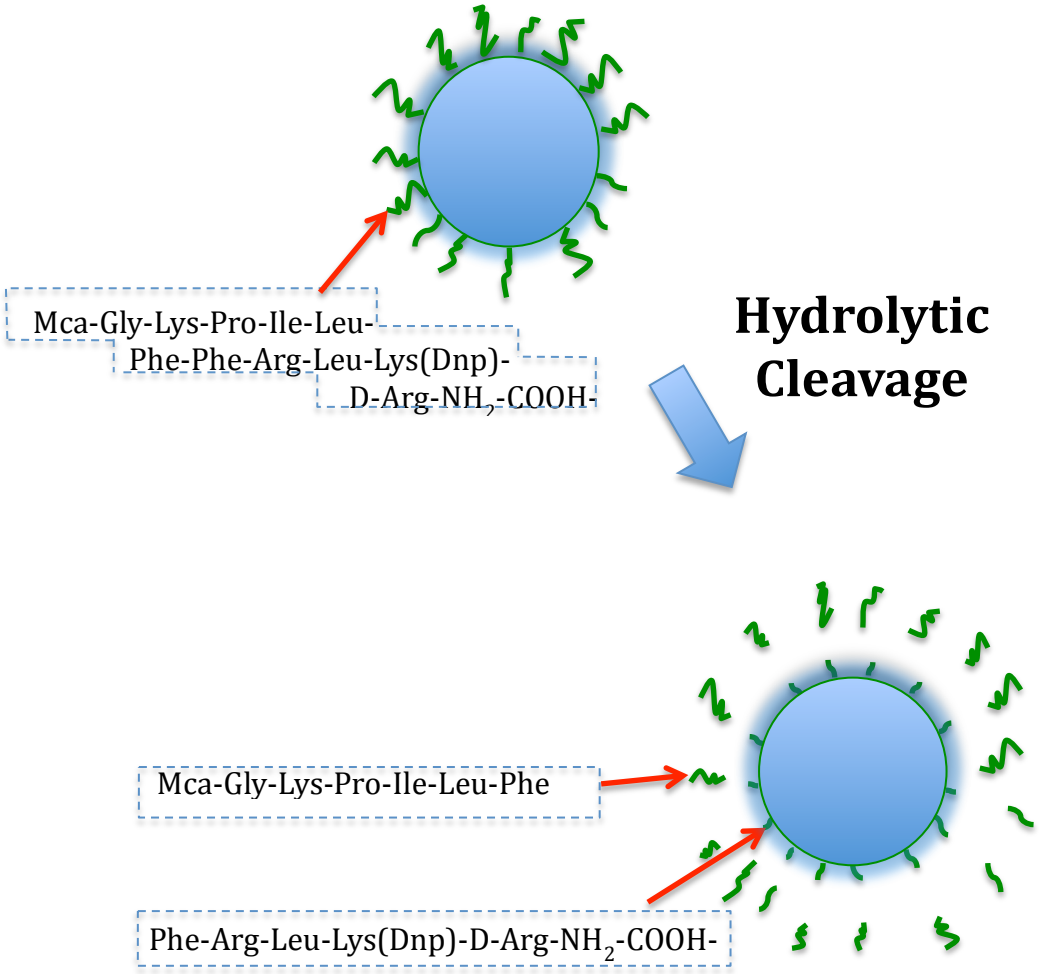

Fig. 5 Schematic illustration of peptide-conjugated nanoparticles. The peptide sequence is shown. The peptide can be hydrolyzed at the Phe-Phe bond by cathepsin D enzymes and then shows the fluorescence.

This observation indicates active proteolytic reactions on the particle surface. On the other hand, the blue fluorescence intensity remains unchanged when the particles are incubated with nontargeted protease enzymes, e.g., collagenase 1A and nontargeted human cell lines (e.g. Hela cells), which strongly indicates the specificity of the peptide layer to the targeted cancer biomarker, in this case cathepsin D. For 3T3 mouse fibroblast cell secretions, possibly due to the nonspecific proteolytic reactions of the peptides, the peptide fluorescence intensity is also elevated but the elevation level and sustainability are significantly lower than those for MCF7 mammary adenocarcinoma cells. Better design of peptides with higher specificity also will help to minimize the nonspecific proteolytic reactions.

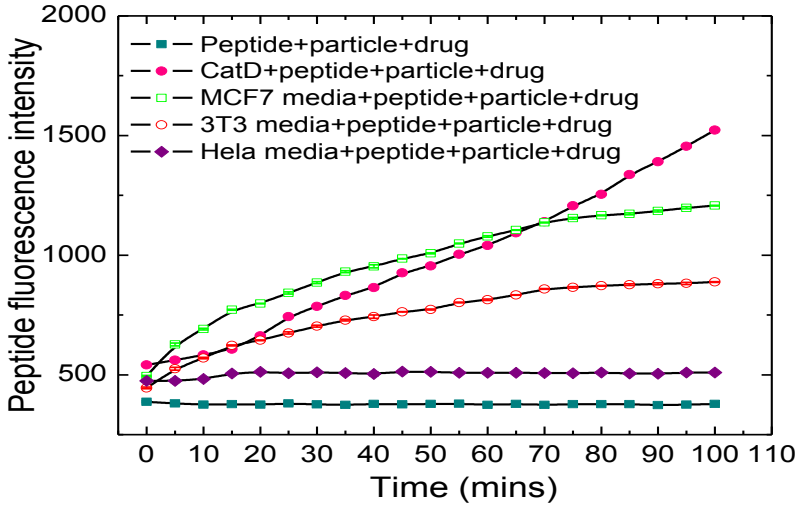

Fig. 6 Peptide fluorescence intensity under different incubated conditions.

4.2. Targeted Chemotherapy on Breast Cancer Cells

The morphology of peptide-coated nanoparticles cultured with three different cell lines was examined along with the number of resulting viable cells. MCF7 mammary adenocarcinoma cells and 4T1 mouse mammary carcinoma cells were specifically chosen to prove the peptide specificity to cathepsin D secreted by these cells, while 3T3 mouse fibroblasts were used as controls. Nanoparticles incubated with 4T1 cells significantly reduced the cell growth in the span of 7 hr (Fig. 7), but did not affect the growth rate of 3T3 cells (Fig.7). The number of 3T3 fibroblasts increased due to cell proliferation, which indicates negligible cytotoxicity to these nontargeted cells. In contrast, the number of 4T1 cells decreased dramatically by more than 80% after 5 hr and kept this downward trend temporally (Fig. 7).

The number of MCF7 cells, although with some oscillation temporally, eventually decreased to 50% of its initial concentration after 7 hr. These results demonstrate that the peptide coating enables the specificity of particle drug delivery system to only target cancer biomarkers and associated tumor cells. Results of these experiments using mice and human breast cancer cells are shown in Fig. 8 and 9. Cell concentrations of treated and untreated MCF7 and 4T1 cells were assessed after a 2-hr incubation time for each cell line.

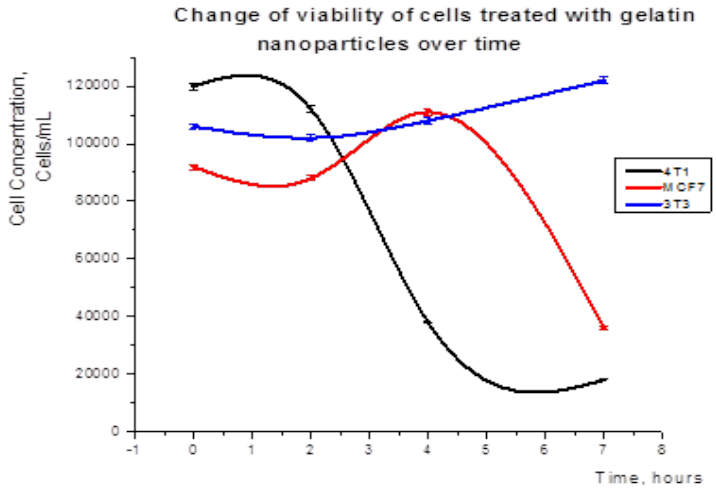

Fig. 7 Cell counting for 3T3 (control), 4T1 and MCF7 cells in 7 hr duration. Viability of 3T3 cells treated with nanoparticles increases, unlike mouse cancer 4T1 and human breast cancer MCF7 cells viabilities that significantly decrease after incubation with nanoparticles.

Figures show that cell viability was significantly reduced for cells incubated with nanoparticles, while control cells that were untreated with nanoparticles continued their growth, reinforcing the same result obtained in the discussed Fig. 7. While the concentration of 4T1 cells decreased from 30,000 to 26,000 cells/mL by the end of incubation time, the concentration of control 4T1 cells remained unaffected, increasing to 34,000 cells/mL Analogous trends for MCF7 cell growth were obtained. Thus, results demonstrate significant effects of drug-loaded nanoparticles conjugated with peptides on breast cancer cell viability. Figures 8 and 9 show that viability of nanoparticle treated human and animal breast cancer cells decreases, unlike untreated control cells.

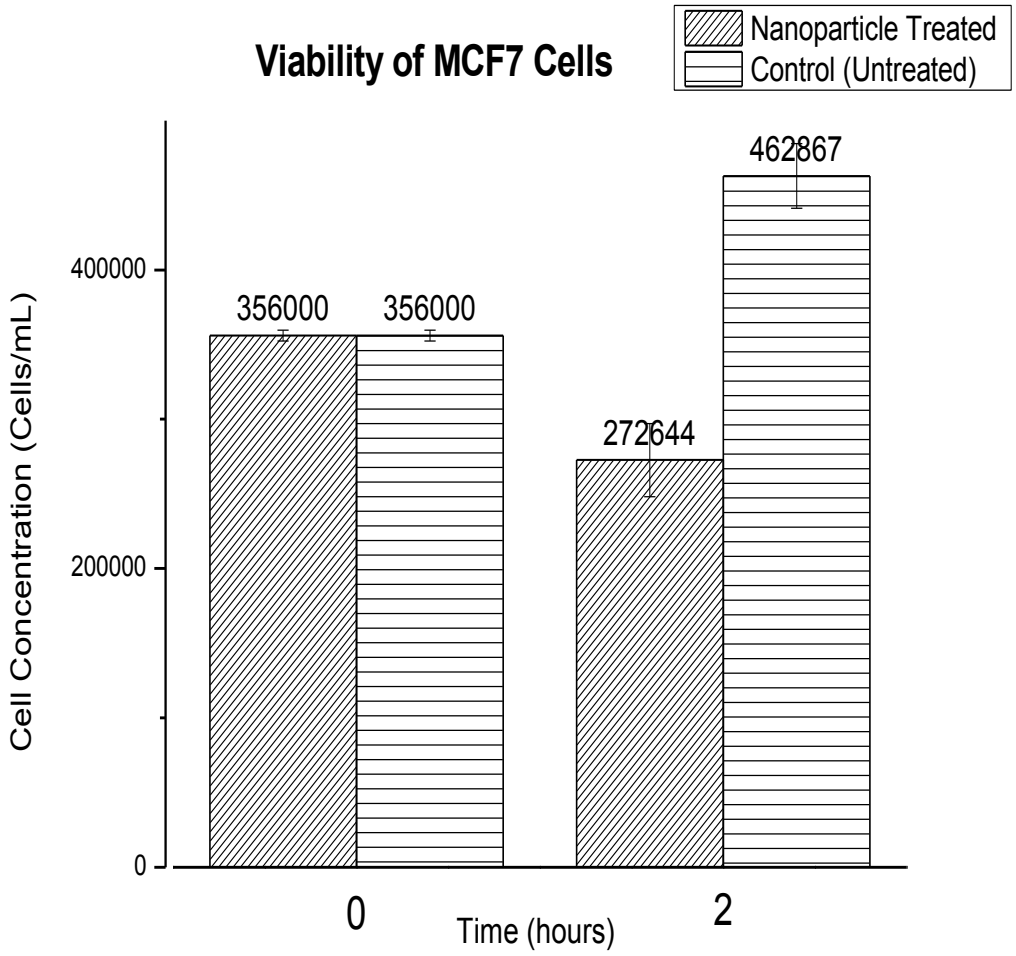

Fig. 8 Comparison of number of viable cells for MCF7 cells treated and untreated with nanoparticles.

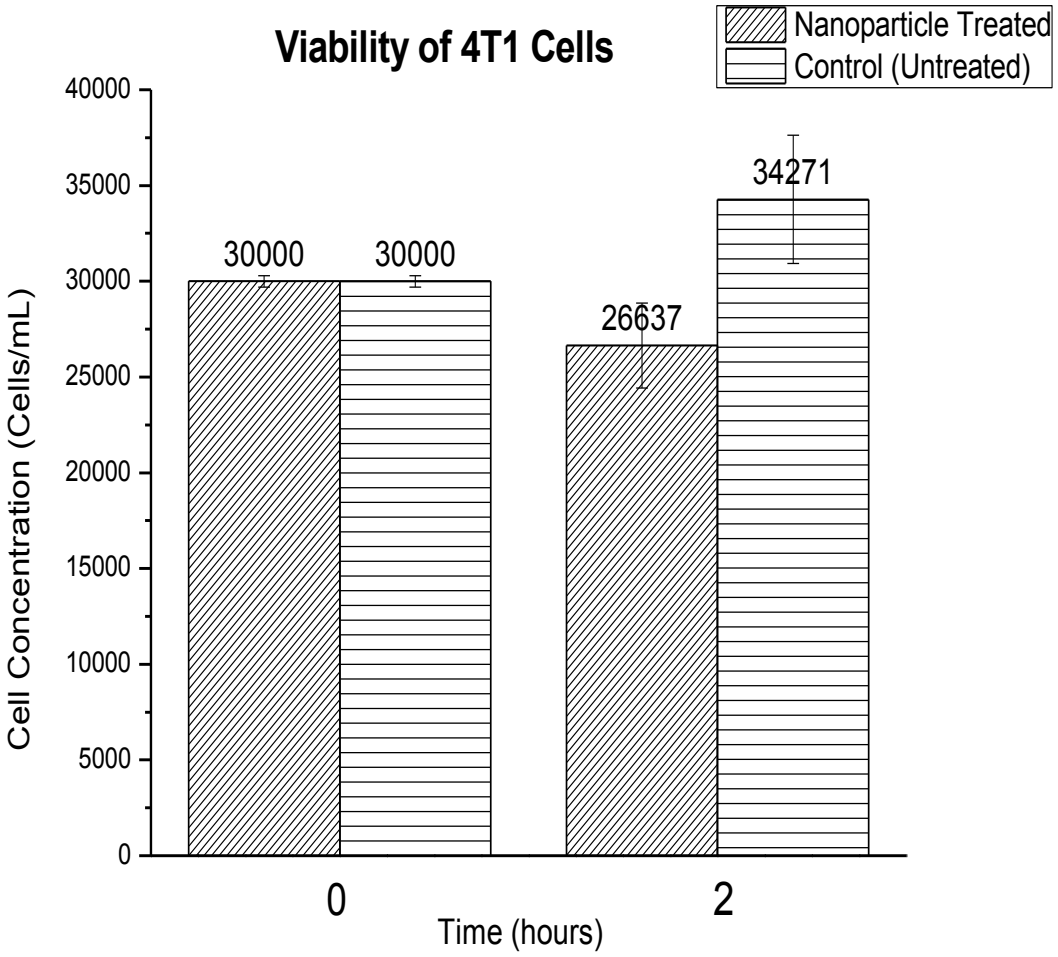

Fig. 9 Comparison of number of viable cells for 4T1 cells treated and untreated with fabricated nanoparticles.

4.3. In Vivo Ultrasound Imaging of Nanoparticles

Gelatin nanoparticles were injected into control mice via the lateral tail vein and real-time video of the superior vena cava was taken immediately after the injection. Snapshots of the particles passing through the vein located near the mouse heart are shown in Fig. 10(A) and 10(B). Figure 10(A) shows the vena cava before introducing the particles into the body, while Fig. 10(B) shows gelatin particles passing through the vein. Results suggest that the proposed nanoparticles can act as imaging contrast agents, facilitating in vivo high-resolution ultrasound imaging. This observation could be due to the swelling characteristic of the nanoparticles, which causes the formation of air gaps and free pores, giving them distinctive acoustic impedance. As a result, the particles can act as reflective mediums for ultrasound waves, allowing in vivo ultrasound detection, tracking of particle flow, and distribution in real time.

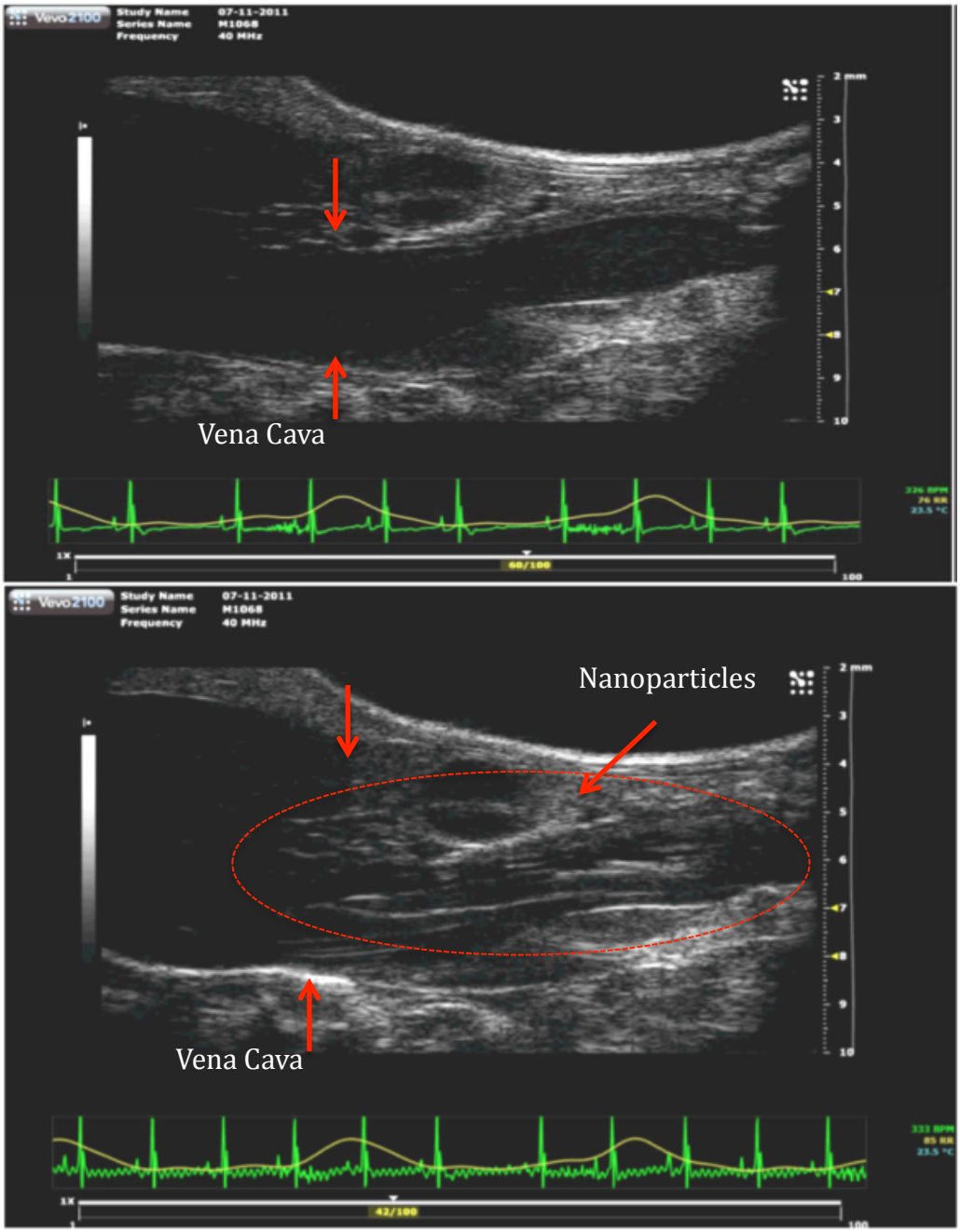

Fig. 10 High-frequency ultrasound images of the blood vessel in the heart of a nude mouse during the injection of nanocapsules. Ultrasound imaging of vena cava vessel (A) before and (B) several seconds after the injection of particles via the tail vein into the mouse body. The nanocapsules in flow can be clearly identified and equivalently act as imaging contrast agents.

4.4. Targeted Chemotherapy on Breast Cancer Mouse Models

The fluorescence nature of DOX used for preparation of the proposed nanoparticles allowed us to use fluorescence imaging techniques for monitoring the distribution of nanoparticles in vivo. Fluorescence imaging was performed to demonstrate peptide coating stability and nanoparticle drug release specificity to biomarkers secreted by tumor sites under in vivo conditions. As previously described both mice with and without cancerous growths were injected with 100 μL of fabricated particles mixed in saline solution and underwent whole body fluorescence imaging using a small animal fluorescence imaging system. The

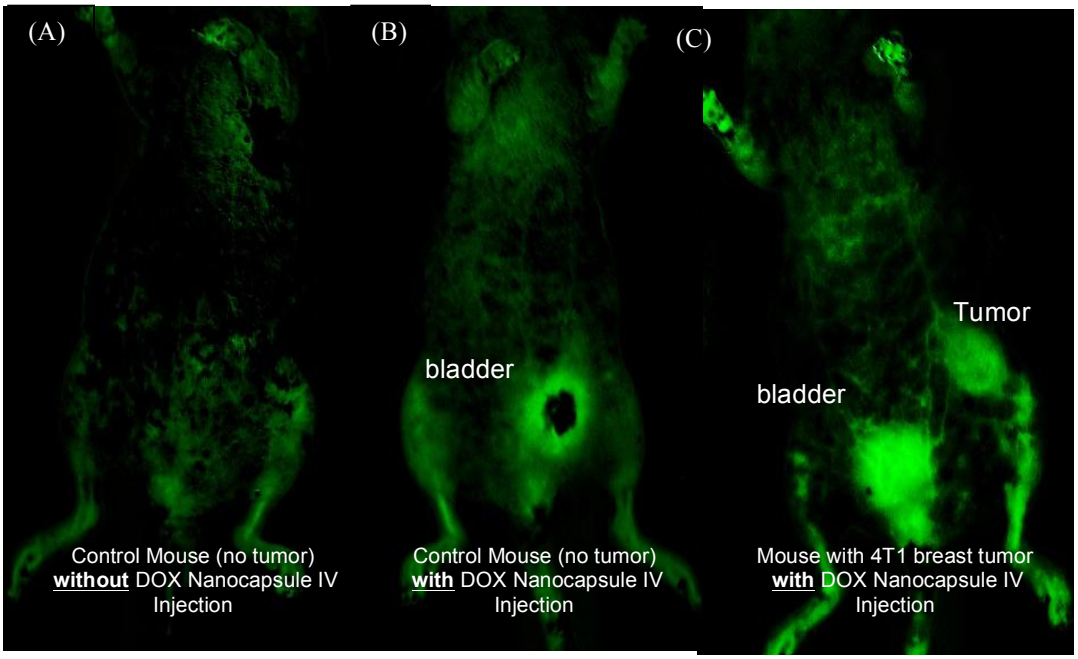

Fig. 11 (A) Fluorescence image of control cancer-free mouse not injected with DOX nanocapsules; (B) Fluorescence image of control cancer-free mouse model after the injection of drug loaded nanoparticles coated with peptide layer; (C) Fluorescence image of a cancerous mouse model with 4T1 breast tumor after injection of nanoparticles.

fluorescence images are overlaid on the bright field images to identify the locations of nanocapsules present in the mice (Fig. 11). The middle image in Fig. 11 shows that most of the particles are filtered out and end up in the bladder, while the image of the 4T1-tumor mouse displays that proposed nanoparticles concentrate not only in the bladder, but also reach the tumor site located at the right side of the mouse body. Evidently, DOX fluorescence is strong in the tumor and at the locations near the tumor, suggesting the targeted delivery through the nanocapsules. Thus, the concentrated distribution of drug carrying nanoparticles in the 4T1 tumor supports the hypothesis that introduced particles are biomarker activated through peptide encapsulation.

CHAPTER 5: CONCLUSION AND FUTURE STUDIES

5.1. Conclusion

In this study, nanoporous gelatin nanoparticles were fabricated as a vehicle for high-specificity and targeted drug delivery to breast cancer cells. Chemotherapeutic DOX drug molecules were loaded in the gelatin nanocarrier and coated with protective peptide strands to prevent the early degradation and nonspecific drug release. Release of drug immobilized by cross-linked gelatin, the loading efficiency of which can be optimized by controlling the cross-linker concentration, is triggered only by the biomarker protease enzyme cathepsin D secreted by the breast cancer cells. In comparison with the chemotherapy with free-form drug or uncoated nanoparticles, our peptide-coated nanospheres can significantly improve the specificity of cancer chemotherapeutic drug delivery and mitigate the adverse side effects due to the off-target drug release. Varying the peptide sequences for surface coating can also target different cancer biomarkers, the nature of which depends on cancer type. Thus, hybrid drug carriers may be tailored for different subtypes of cancers for personalized medicine and therapy.

The nanoscale size of particles allowed us to extend the studies to in vivo drug delivery. We achieved high contrast for fabricated gelatin nanocarriers during in vivo ultrasound imaging to monitor the nanocarrier transport after its IV

injection. The targeted drug delivery scheme was successfully demonstrated in vitro and in vivo for breast cancer malignancy with fluorescence imaging techniques. We anticipate that with higher specificity and stability of our peptide conjugated drug nanocarriers, systemic drug release and off-target drug delivery problems can be addressed to remove adverse side-effects of current cancer malignancy treatments.

5.2. Recommendations for Future Studies

In Chapter 3 we presented methods for in vivo whole body fluorescence imaging experiment, results of which in Chapter 4 showed the specificity of nanoparticles to tumor sites. DOX that was loaded in the particles was released in the vicinity of the tumor site and degraded DOX was filtered out in the organs of an animal. In vivo fluorescence imaging can be improved by investigating in vivo chemotherapy efficiency. Running these experiments again, but this time with the purpose of monitoring the tumor shrinkage level, which is treated with nanoparticles, will be a good step to move this project forward.

Looking broadly at the overall future perspectives, the work reported in this thesis shows the pathway toward the development of smart nanoparticle drug carriers. The drug delivery dosage is fine-tuned by the localized biomarker concentration and the chemotherapy drug is released in the vicinity of the tumor

rather than upon the physical binding with the tumor. All these unique properties make the reported enzymatically activated nanoparticle drug carrier a better one than antibody based carriers by providing lower systemic release and high delivery efficiency. The targeted drug delivery in the coming decade will rely on nanoparticle drug carriers that actively search for potent cancer cells, diagnose the cell condition and decide drug delivery dosage and rate autonomously. After drug delivery, the nanoparticle drug carrier can dissolve itself and be cleared out of a body. Negative side effects can be reduced to nearly zero and the precision drug delivery can result in optimized, rapid and effective cancer chemotherapy.

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