Nanopreparations for Cancer Imaging and Theranostics

Doctoral Thesis

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Director of the Graduate School

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<td>ABTS</td>
<td>2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid</td>
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<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
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<tr>
<td>CA-125</td>
<td>Cancer antigen–125</td>
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<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
</tr>
<tr>
<td>CdSe</td>
<td>Cadmium selenide</td>
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<tr>
<td>CEA</td>
<td>Carcinoembryogenic antigen</td>
</tr>
<tr>
<td>CGM</td>
<td>Carcinoembryogenic antigen -Gene Family Member</td>
</tr>
<tr>
<td>CHN</td>
<td>Center for High-rate Nanomanufacturing</td>
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<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
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<tr>
<td>CMT</td>
<td>Critical micelle temperature</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DOXO</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DSP-PEG5K</td>
<td>1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]</td>
</tr>
<tr>
<td>EBL</td>
<td>Electron-beam lithography</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>$E_g$</td>
<td>Energy gap</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factors</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assays</td>
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<tr>
<td>EPR</td>
<td>Enhanced permeability retention</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FPA</td>
<td>Focal plain array</td>
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<tr>
<td>GPCRs</td>
<td>G-protein coupling receptors</td>
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<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
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<tr>
<td>ICAM-11</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICG</td>
<td>Indocyanine green</td>
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<tr>
<td>LCTF</td>
<td>Liquid crystal tunable filter element</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Mce6</td>
<td>Mesochlorin e6</td>
</tr>
<tr>
<td>MION</td>
<td>Micelle-encapsulated superparamagnetic iron oxide magnetic nanoparticles</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
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<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
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<tr>
<td>NIR</td>
<td>Near infrared</td>
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<tr>
<td>NIRS</td>
<td>Near infrared spectroscopy</td>
</tr>
<tr>
<td>NS</td>
<td>Nucleosomes</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly (acrylic acid)</td>
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<tr>
<td>PAI</td>
<td>Photoacoustic imaging</td>
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<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
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<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
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<td>PEG-PE</td>
<td>Poly (ethylene glycol)-phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG-PLA</td>
<td>Poly (ethylene glycol)-poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly (L-glutamic acid)</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly (methyl methacrylate)</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>Rh-PE</td>
<td>Rhodamine – phosphatidylethanolamine</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SBR</td>
<td>Signal-to-background ratio</td>
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<tr>
<td>SLN</td>
<td>Sentinel lymph node</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
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<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
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<tr>
<td>SPION</td>
<td>Superparamagnetic iron oxide nanoparticles</td>
</tr>
<tr>
<td>SQUID</td>
<td>Superconducting quantum interference device</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>US</td>
<td>Ultrasound</td>
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<tr>
<td>US-FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>ZnSe</td>
<td>Zinc selenide</td>
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ABSTRACT

Nanopreparations, such as micelles, liposomes and polymeric microcapsules are used to incorporate diagnostic and therapeutic agents in order to improve their performance. Lipid-core nanopreparations composed of polymeric micelles were employed to incorporate quantum dots for near infrared imaging of tumors. Tumors were imaged rapidly at half the dose of a commercially available quantum dot formulation. Additionally, these quantum dots containing polymeric micelles were targeted with a cancer-specific monoclonal antibody (2C5) that selectively accumulated in the tumor. This system produced a higher signal compared to untargeted quantum dot micelles.

Cancer was also detected indirectly by making use of circulating biomarkers, such as nucleosome and carcinoembryogenic antigen. A microchip-based diagnostic tool was fabricated that detected low levels of these biomarkers. The device was sensitive enough to capture picogram concentrations of antigens in biological fluids. Tumor development could be tracked by following the cancer biomarker levels. This system proved to be more sensitive than commercially available ELISA kit. Thus, it may aid in the detection of small changes in biomarker levels associated with an early cancer stage.

In a combined diagnostic and therapeutic approach, a micellar nanoparticle that incorporated both superparamagnetic iron oxide nanoparticles and paclitaxel for application as a theranostic agent was developed and characterized in vitro and in vivo.
STATEMENT OF HYPOTHESES

1) Quantum dot-micelles will produce an increased signal-to-noise ratio compared to commercial PEGylated quantum dot and will more rapidly detect tumors in vivo using NIR imaging.

2) Quantum dot-immunomicelles will demonstrate an enhanced accumulation and increased signal-to-noise ratio compared to quantum dot-micelles and will detect tumor metastasis in vivo using near infrared imaging.

3) Monoclonal antibody-coated polystyrene nanobeads assembled on a micron-size biochip will detect nucleosome and carcinoembryogenic antigen cancer biomarkers at picogram concentrations.

4) Poly (ethylene glycol)-phosphatidylethanolamine theranostic micelles containing superparamagnetic iron oxide nanoparticles and paclitaxel will produce strong T2-weighted magnetic resonance image and will be cytotoxic to cancer cells in vitro.

5) Poly (ethylene glycol)-phosphatidylethanolamine theranostic micelles will generate T2-weighted contrast using magnetic resonance imaging and show tumor apoptotic activity in vivo.
OBJECTIVE AND SPECIFIC AIMS

The long-term objective of my dissertation was to design, characterize and test different nano-preparations for imaging of tumors, detection of biomarkers and simultaneous therapy and detection of cancer.

The following are my specific aims:

1) To rapidly image and detect tumors using quantum dot loaded in phospholipid micelles.

2) To achieve enhanced tumor accumulation and to detect tumor metastasis using quantum dot-loaded immunomicelles.

3) To prepare and characterize antibody-coated polystyrene nanobeads assembled on a micron-size biochip for detection of the nucleosomes and carcinoembryogenic antigen cancer biomarkers.

4) To develop and characterize a poly (ethylene glycol)-phosphatidylethanolamine-based micellar theranostic agent for magnetic resonance imaging and evaluate its cytotoxicity on cancer cells in vitro.

5) To evaluate the T2-weighted contrast properties and tumor apoptotic effect of this lipid-based theranostic agent in vivo using 4T1 and B16F10 tumor mouse models.
CHAPTER 1. INTRODUCTION: NEED FOR DEVELOPMENT OF NEW NANOMATERIALS

1.1 Statement of the problem

The NCI defines cancer as “a term used for diseases in which abnormal cells divide without control and are able to invade other tissues”, these abnormal cells can be characterized by certain changes in the cellular physiology that command their uncontrolled malignant growth [1]. Currently, a variety of chemotherapeutic agents are clinically available to treat cancer. However, these chemotherapeutic agents pose potential disadvantages such as toxicity or side-effects to normal cells, premature drug degradation resulting in short in vivo half-life, poor bioavailability and thus, repeated administrations [2].

Addressing a different issue, the ACS established 3 major goals to be met by 2015: 1) to reduce cancer mortality by 50%, 2) to reduce cancer incidence by 25%, and 3) to improve the quality of life of cancer patients [3]. In attempts to achieve this goal, cancer detection has received great importance and it is critical to rapidly detect cancer for appropriate treatment.

Additionally, cancer can also be detected by the circulating biomarkers in the body fluids. Current diagnostic systems such as 96-well plate ELISA kits can be used to detect biomarkers. However, they suffer from lack of sensitivity making them less reliable, thus calling forth new technologies that can overcome this challenge and improve sensitivity.
Upon detection of cancer, current treatment regimens such as radiation, surgery and chemotherapy can be used. Although over 90 chemotherapeutic drugs have been approved by the FDA for clinical purposes, their use has been hindered by dose-limiting toxicity and patient morbidity [4]. However, not all patients respond with the same intensity to the same therapy which, by the way, is the current trend of “one-size-fits-all” based chemotherapy. In the past three decades, the pharmaceutical industries have sustained this blockbuster model of drug development. As a consequence, many drug molecules have failed to make it out of phase 1 or phase 2 clinical trials and the need for clinical efficacy and cost-effectiveness of new molecules has resulted in increased cost and decreased productivity of research and development.

1.2 Rational to overcome current chemotherapeutic challenges

To overcome this challenge, a major stratagem is to detect cancer early on. Rapid detection of cancer or identification of cancer biomarkers remains a critical goal for diagnostic imaging. The key is to detect and/or image cancer at the smallest possible number of tumor cells, ideally before the angiogenic switch from diffusion-limited nutrition to neovascularization [5]. Mapping these differences at various stages provides potential targets for treatment and further diagnosis. Identification of cancer and cancer biomarkers will give us the opportunity to arrest the development of cancer at its earliest, thereby preventing subsequent cost and cumbersome treatment modalities. Prevention of mortality
and disability through detecting cancer early remains the best possible strategy. Hence, appropriate visualization techniques or imaging modalities must be employed to assist in detecting cancer and offer as guide to monitor the response to therapy.

Also, it is important to increase the amount of a therapeutically active entity at the site of action or pathological area and to reduce the toxicity by embedding drugs into non-toxic biodegradable polymers [6]. At the same time it is important to have sufficient signal from the pathological area in order to distinguish it from the normal tissues. To achieve this goal, a variety of delivery systems such as synthetic polymers, microcapsule, lipoproteins, liposome, micelles and various other nanoparticles are currently applied or under development [7] offering a wide range of advantages in delivery of chemotherapeutic agents. Nanomaterials have also been employed in the development of contrast agents and radiopharmaceuticals intended for applications in the field of imaging [8].

1.3 Imaging modalities for cancer detection

Imaging is an indispensable tool in cancer research that enables clinicians to visualize expression of cells and biological processes that influence tumor behavior after therapy. Over the past three decades there has been an increase in the number of imaging technologies that have enabled clinicians and researchers to locate the tumor, visualize the expression and activity of cells that
manifest different behavior of tumors and their responsiveness to therapeutic drugs [9]. Presently, various imaging modalities (CT, US, MRI, SPECT, PET and optical imaging) are used in the clinic to diagnose and help treat cancer patients. Out of which CT, MRI, SPECT, and PET imaging techniques are capable of three dimensional detection of cancer in the human body [10].

Amongst various imaging modalities, non-invasive techniques such as MRI, optical bioluminescence, fluorescence, NIR, US, SPECT, and PET are used to visualize, characterize, and measure biologic processes at the cellular and molecular levels in living organisms. From the variety of imaging techniques available, NIR and MRI imaging are extensively studies here in. More detailed discussion on the principle and working of NIR is described in chapter 2. The application of NIR imaging technology in rapid tumor detection is described in chapter 3 and chapter 4.

1.4 Delivery systems for cancer therapy and diagnosis.

Lately, premature drug degradation, undesirable side-effects and low bioavailability due to the poor solubility of many anti-cancer agents are challenges that have begun to be overcome using nanotechnology. In addition to chemotherapy, nanotechnology has been applied for delivery of contrast agents that accumulate more substantially in areas of interest to form a highly localized signal and differentiation from surrounding tissues [11]. An assortment of nanoparticles, including micelles, liposomes, niosomes, polymeric and metal
nanoparticles, solid lipid nanoparticles, quantum dots, microcapsules, dendrimers, cells, cell ghosts, lipoproteins and various other nanoassemblies are now available [12]. Polymeric micelles made up of amphiphilic conjugates such as PEG-PE are promising nano-systems used in our study.

Micelles are self-assembling colloidal particles that consist of a hydrophobic core and hydrophilic corona [7]. These polymeric micelles belong to a group of amphiphilic molecules, which form spontaneously under certain conditions of concentration and temperature. At low concentrations in the aqueous medium, these amphiphilic molecules exist as monomers. As the concentration is increased, these molecules begin to self-assemble into micelles at a concentration known as the CMC. The temperature below which amphiphilic molecules exists as unimers and above which aggregates, is called the CMT [13]. The major driving force behind self-association of amphiphilic copolymers is the decrease in the free energy of the system due to removal of hydrophobic fragments from the aqueous surroundings with the formation of a micelle core stabilized by the hydrophilic corona exposed to water [14]. In addition, van der Waals interaction amongst the hydrophobic blocks forming the core of the micelles significantly contributes to the stability of these polymeric micelles [15]. The characteristic size of polymeric micelles ranges from 5 and 50 or 100 nm [16], which makes them ideal sized candidates for delivery of therapeutic and contrast agents to the tumor tissue. Because of their small size, they are able to
extravasate into the interstitium of body compartments with leaky vasculature (tumors and infarcts) by the EPR effect [17].

For example, it has been shown in tumors [18] that the blood vessel walls are leaky and the permeability of vascular endothelium increased to a size range within 0.4 to 0.6 microns [19]. This gives polymeric micelles with small ‘cut-off’ sizes [20] an advantage to be used as pharmaceutical nanocarriers. One more contributing factor that aids accumulation of polymeric micelles to the tumor area is its longevity in the blood. PEG chains, that are known to be highly water soluble and highly hydrated, provide better steric protection against opsonins in the biological environment [21] as a result, make polymeric micelles more stable and long–circulating for delivery of their load into tumors [22]. Several studies have used PEG-PE micelles to incorporate chemotherapeutic and contrast agents, qualifying them as good nanopreparations [23; 24; 25; 26; 27; 28] for treatment and detection of cancer.

1.5 Cancer detection using biomarkers

Cancer can also be detected indirectly by identifying biomarkers. They are cancer cell-associated protein products, which when present in abundance, help to distinguish between normal and cancer cells [29]. They are good indicators of the presence and status of cancer and can help in management of treatment. Many biomarkers such as PSA, CEA, CA-125 are available for clinical use [30].
Additionally, NS can also serve as a potential biomarker for detection of cancer [31]. A more detailed explanation and background is given in chapter 5.

Currently, there is an urgent need for improved systems to detect cancer biomarkers in blood. Since conventional ELISA systems have poor sensitivity, new technologies such as DNA micro-array, nanocantilevers, nanoparticle-based bio-barcodes are possible solutions to current biomarker detection systems. However, they add to the cost of health care and need expensive readout devices. More promising are antibody arrays that have a capacity to detect biomarker at concentrations below the current detection limit of 96-well plate kits without modifying the simplicity of the ELISA technique itself. We have fabricated one such device and its application is discussed in chapter 6.

1.6 Theranostics: new paradigm for cancer therapy and diagnosis

Once cancer is detected, its treatment and management are of prime importance. Most of the current chemotherapeutics follow the one-size-fits-all trend, and not all patients respond to the therapy in the same way. Theranostics has the potential to positively impact the challenges that would change the trend of chemotherapy to a more personalized form. Theranostics is a system that integrates diagnostic and therapeutic agents within a common platform. It offers a tool to treat and simultaneously image the tumor allowing clinicians to better understand if the therapy is effective. This real-time treatment and imaging is an essential component of theranostic nanomedicine. The advantage of such agents
is that they will provide us with early feedback of the chemotherapeutic agents’ associated risks and toxic effects.

The diagnostic agent used in our theranostic preparation is SPION. Amongst the various MRI contrast agents such as chelated paramagnetic metal ions (gadolinium, manganese, dysprosium), SPION are used as diagnostic agent in conjunction with various chemotherapeutic agents [32], and their lack of toxicity has been extensively studied and reported [33; 34]. Detailed discussion on SPION and its MRI application is given in chapter 7.

We used PTX as a chemotherapeutic agent within the theranostic micelles. PTX (Taxol®) belongs to the family of taxanes, which is an important anti-cancer drug first isolated in extremely low yield from the bark of the western yew, Taxus brevifolia in 1963 and used against a broad range of cancers that are considered to be refractory to conventional therapy [35]. Wani and coworkers, were the first to publish its structure in May 1971 [36]. PTX received attention after P. Schiff with Susan Horwitz and J. Fant found its unique mechanism of action in 1979. They discovered that PTX promoted polymerization of tubulin and at sub-nanomolar concentrations, it inhibited the disassembly of microtubules, thereby causing cell death by disrupting the normal microtubule dynamics required for cell division and vital interphase processes [37]. Striking clinical results with ovarian cancer were reported in 1989, and Taxol was approved by the US-FDA for refractory ovarian cancer in December, 1992 and for refractory breast cancer in April, 1994 [38].
However, its poor water solubility and high lipophilicity led to development of nanoparticle systems that incorporate PTX in polymeric micelles to improve its solubility and bioavailability. PTX has since been studied extensively in the field of nanomedicine for the treatment of a variety of cancers. Application of PTX in a theranostic agent is discussed in chapter 8 and chapter 9.
References:


CHAPTER 2. NEAR-INFRARED IMAGING USING QUANTUM DOTS

2.1 Introduction

As summarized in the previous chapter, several diagnostic imaging technologies developed over the past three decades have had a profound impact on clinical medicine. However, only few technologies have been introduced as laboratory tools for imaging small animals at high resolutions. Although, clinical imaging systems are primarily used for displaying anatomical, physiological, and metabolic parameters, experimental animal systems are additionally being developed to be imaged at the cellular and molecular level in vivo [1]. Current imaging technologies differ in five main aspects: resolution, depth of penetration, energy expended for image generation (ionizing versus non-ionizing), availability of injectable, biocompatible molecular probes, and the detection threshold of probes for a given technology [1].

Imaging plays a key role in helping oncologists meet several goals such as detection of solid tumors; detection of recurrence and evaluation of the success of a treatment regimen. Thus, compared with the physical exam, imaging technology has represented a quantum leap in how disease can be detected, quantitated, and characterized [2].

Given this intertwining of oncology and imaging, it is imperative that those involved with cancer care have an understanding of what imaging can achieve
today. The impressive technological advances currently evaluated in mouse models are on the horizon for significantly improving the use of imaging in clinical practice [2]. Herein, the focus is on a NRI technology composed of imaging hardware for visualization of near-infrared light coupled with QD probes that allow detection and their application in vivo.

2.2 NIR imaging for in vivo applications

NIR imaging is a particularly promising method of imaging since it is non-invasive, requires relatively simple and easy to use equipment, and can take place in real time [3]. Historically, it was discovered by Herchel in 1800’s who separated the electromagnetic spectrum with a prism and found out that the temperature increased markedly towards and beyond red, i.e. in the NIR region [4]. However, it was only in the mid-1950’s when Kaye and Beckman instruments published and introduced NIRS into practice [5]. Due the low absorbance of tissue chromophores such as oxy- and deoxy-hemoglobin, melanin and fat, NIR light of 700–900 nm can propagate by multiple scattering through several centimeters of tissue, enabling imaging of tissues using non-ionizing radiation [1; 6]. In other words, in this region of the spectrum, light absorption and scattering from the tissues is minimal, thus light is able to penetrate further through the body giving the greatest tissue penetration depth and optical signal, which is one of the major shortcomings of fluorescence imaging. Because of this property,
imaging at the NIR region is non-invasive [7], allowing for better observation and imaging using suitable NIR emitting fluorophore [8].

There are two key fundamental modalities of NIR imaging namely, reflectance and tomography. In reflectance imaging, there is a continuous incident excitation beam of light on the object to be image. The light reaches the tissue and emitted light is reflected back to the surface where it is measured. There is an exponential decay of light intensity with tissue penetration making this technique limited to several millimeters at best. On the other hand, in tomographic imaging, the signal intensity falls more linearly with target depth in tissue, permitting measurements to be made up to 30-40 cm into tissue [9]. However, in such cases, the images acquired must be reconstructed using various equations and mathematical calculations.

NIR imaging has been used in the field of cancer diagnostics with in vivo applications including visualization of molecular aspects of tumors such as VEGF receptors, molecular marker for angiogenesis [10], EGFR in breast tumors [11], SLN mapping before surgery [12] and cell trafficking studies, hence allowing study of various phenomena within our body. Additional applications of NIR in cancer research include [13]:

1. detection of tumors through antibody-mediated targeting,
2. detection of apoptosis through detection of the caspase activation,
3. detection of viral probes for gene transfection or cancer therapy,
4. imaging of the tumor response to the anticancer treatment,
5. dual imaging of luminescence and near infrared probes in tumor and
6. engineering bio luminescent cells as the neural precursor for gene therapy of brain cancer together with the NIR imaging of the tumor cells.

In the field of nanotechnology, NIR probes can be used for the detection of the accumulation of nanoparticles and determination of their stability in vivo [14; 15]. This trend in the use of nanosized imaging agents is particularly valuable for the imaging of intracellular organelles; especially mitochondria (their fluorescent imaging can offer information about the Ca\(^{2+}\) levels inside them and in the sarcoplasmic/endoplasmic reticulum, the redox state of the organelle, the activation of apoptosis proteins) and nuclei, where the transcription of genes can be observed [16].

2.2.1 NIR imaging: principle, instrumentation and working

In the electromagnetic spectrum, the infrared refers to that part between the visible and microwave regions (Figure 1). Electromagnetic spectrum refers to the seemingly diverse collection of radiant energy, from cosmic rays to X-rays to visible light to microwaves, each of which can be considered as a wave or particle traveling at the speed of light which differ from each other only in their wavelength and frequency [17].
The combination of NIR with digital image processing is termed NIR imaging. The basic components of a NIR system are a source of illumination, imaging-optics a wavelength encoder and a FPA as described in Figure 2.
During imaging, the NIR light is incident on the sample. After reaching the target organ, the diffuse reflected light is captured by the image optics that can be configured based on the sample size or sample type. In case of microscopic or macroscopic imaging, usually, a focusing lens is used. Following the collection of the data, a series of images are recorded on the NIR FPA at a particular wavelength by spectral encoder such as a LCTF or an interferometer. This generates a 3D data set with \( x \) and \( y \) axis representing spatial information and the \( z \) axis representing the spectral information. The data can be quantified and analyzed by programmed software for detailed spatial analysis and location of the target organ.

**Figure 2:** Basic components of NIR imaging [4].
With technological advancements, a variety of NIR instruments have become available that are capable of acquiring accurate images and quantifying data based on an in-built mathematical equation. Regarding instrumentation, there are two fundamental approaches to imaging [4];

1) Push-broom method; wherein there is movement between the camera and the sample. In this case, the spectral information is recorded line-wise providing information for each pixel along the line. Such encoding is obtained by linear variable filters, a digital micro-mirror array in combination with a grating, or dispersive optics. After the image is acquired, the software combines the images, derives the axis and reconstructs the image.

2) Wavelength scanning method; wherein the camera and the sample are stationary and for each wavelength, single images are acquired. The spectral information obtained is by a number of discreet filters, tunable filters or combination with an imaging Fourier transform spectrometer. Images acquired for different wavelengths are combined by the software and quantified. This method is also known as the staring imager method and is used in research wherein the acquisition time is 2 mins or less. However, the acquisition time varies between different instruments. An example of one such instrument used in our research is discussed in the following section.
2.2.2 KODAK in vivo FX system: instrumentation and working

The Kodak Image Station *in vivo* FX system (in Dr. Amiji’s Lab) is used for NIR whole body *in vivo* imaging. A schematic representation of the instrument is depicted in Figure 3.

![Scheme of NIR imaging using Kodak in vivo FX system (modified from [18])](image)

**Figure 3:** Scheme of NIR imaging using Kodak *in vivo* FX system (modified from [18]).

The animal is stationary and positioned in an animal chamber, which is directly above the imaging chamber. In the case of NIR imaging, the light is incident directly from a high intensity lamp. Depending on the wavelength, the light is directed through a series of excitation filters and reflected onto the animal. The light from the imaging agent inside the animal is then emitted and separated from the excitation light as it passes through the patented Kodak Wide Angle emission filter [18], following which, the fluorescence enters in the 10x zoom lens.
and is focused onto a 4 million pixel cooled CCD following which the image is
digitized. The read–out is efficiently interfaced with a software. Broadly the
components and working can be classified into a) Optics, b) Software and c)
Samples [18].

a) Optics: The chief characteristic of the Kodak image station is a very
sensitive camera that collects the photons from the sample. The light source has
a number of excitation filters that control the wavelength of the light that irradiates
the sample. The light is reflected from a two way mirror. The fluorescence from
the sample is collected in the camera as the photons pass through the two way
mirror and through an emission filter. The instrument is also equipped with a
sliding panel that permits the visualization of x-rays or autoradiography
simultaneously with the fluorescence imaging.

b) Software: In some cases, the system software permits the
transformation of the arbitrary units in mJoules/cm$^2$. The photograph is colored to
depict the light intensity emitted from the sample. ROI can be chosen to quantify
the light emitted from a particular area of the photograph. This system is more
useful in electrophoresis, where standards can be automatically inserted in the
system, and the quantification is a simple one-step process. However, this
procedure cannot be used in the case of small animals. Alternate settings are
required for imaging of small animals.

c) Samples: The instrument allows the visualization of both small animals
and gels. In the case of animals, it is best to use near infrared probes, as the
absorption from the animal tissues is minimum (~680nm). However, the scattering remains intense and this creates quantification problems. Removal of the skin decreases the scattering and increases clarity of the images.

Apart from the current emphasis on the technology, it is essential to have a highly contrasted image in order to visualize the sample and the target tissues. Due to the fact that most of the endogenesis lack the unique optical properties within the NIR range, the extrinsic NIR fluorescent exogenous contrast agents make the in vivo study possible [19]. Over the past decade, many organic fluorophores have been used as contrast agents. These include polymethines, heptamethine cyanines, benzoazole, benzothiazole, indolyl, 2-quinoline and 4-quinoline. ICG is an FDA approved fluorophore used in humans for NIR imaging at excitation and emission values of 760-800 nm and 790-830 nm, respectively [9]. However, conventional organic dyes suffer from limitations such as small excitation/emission bands, biodegradation and photo-bleaching that restrict their application in vivo. Additionally it is a challenge to control their excitation and emission wavelengths since this parameter depends on their chemistry. To overcome these limitations, QDs have emerged as an alternative to conventional organic dyes.

2.3 Quantum dot as NIR contrast enhancing agents

QD represent a new class of fluorescent probes for cellular and biomolecular imaging. They are colloidal inorganic semiconductor nanocrystals
that are made of CdSe or ZnSe. They possess unique optical properties such as size tunable light emission that allow simultaneous excitation of multiple fluorescence at different wavelengths [20]. Change in particle size of the QD, leads to a significant change in the photoluminescence emission which gives size-tunable properties to QD [21]. Coincidentally, these properties are of utmost importance for improved sensitivity during molecular and cellular imaging, since QD remain bright and stable under complex in vivo conditions allowing them to be used in two spectral windows for imaging (one at 700-900 nm and another at 1,200-1,600 nm) [22]. Their main advantage compared to other fluorescent probes is the resistance to photo-bleaching, prolonged fluorescence and tunable wavelength resulting in better quality of images [23].

2.3.1 Quantum dots: basics; preparation and working

The fluorescence of QD is more intense and lasts much longer than conventional fluorophores (this is particularly useful as it can increase the signal-to-noise ratio for the fluorescence imaging). Their excitation spectrum is broad, permitting the simultaneous excitation of different QD with one beam. The emission spectrum of QD depends solely on their size: the larger they are, the more their emission shifts towards the red part of the spectrum. Their size is smaller than the Bohr excitation radius [24]. This property is also known as the quantum confinement effect [25] i.e. increase in the band gap of the semiconductor material leads to a large Stokes shift within the QD. Most of the
QD consists of two compartments, the outmost known as the shell and the innermost known as the core (Figure 4). They are made up of different chemical combinations to give different types of QD.

![Shell and Core Diagram](image)

**Figure 4**: Single QD nanocrystal.

For example, in a CdSe semiconductor QD, the outer shell is made up of ZnS and the inner core is made up of CdSe. QD with a core have only low quantum yield and limited resistance to photobleaching and their fluorescence emission intensity is easily altered because of the charges and/or free radicals present in their environment [26]. However, QD with both core and shell tend to resist photobleaching. Thus the shell is responsible for optical and chemical stability of the inner core. It is known that QDs have size tunable optical properties meaning that they can emit in the UV, fluorescent or infrared spectrum by changing their size. This can be explained by a simple illustration below (Figure 5).
Figure 5: Quantum properties of (a) CdSe bulk semiconductor and (b) CdSe nanocrystals [27].

A semiconductor bulk material has a continuous conduction between the valance band and conduction band that are separated by a fixed $E_g$ and the color of the light emitted is determined by the distance between the gap. The $E_g$ is also the distance between the excited and the ground states. In case of QD nanocrystals, this $E_g$ is determined by the size (radius) and for description sake, they can be represented as energy states in atomic-like energy levels (1S, 1P, 1D) [27].

<table>
<thead>
<tr>
<th>Core(core) materials</th>
<th>Core diameter size range (nm)</th>
<th>Typical emission range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnS</td>
<td>0.7-2.1</td>
<td>300-400</td>
</tr>
<tr>
<td>ZnSe</td>
<td>2.0-5.0</td>
<td>325-450</td>
</tr>
<tr>
<td>CdS</td>
<td>2.8-5.3</td>
<td>375-475</td>
</tr>
<tr>
<td>CdSe(CdS)</td>
<td>2.0-8.0</td>
<td>480-650</td>
</tr>
<tr>
<td>CdTe</td>
<td>3.2-9.0</td>
<td>540-750</td>
</tr>
<tr>
<td>CdTe(CdSe)</td>
<td>4.0-9.4</td>
<td>640-860</td>
</tr>
<tr>
<td>InP</td>
<td>2.6-4.5</td>
<td>625-720</td>
</tr>
<tr>
<td>InAs</td>
<td>3.4-6.0</td>
<td>860-1250</td>
</tr>
</tbody>
</table>

Figure 6: Size dependent optical properties of QD [27; 28].
Additionally, this $E_g$ can be altered in QD to produce range of energies between valance band and conduction band. Hence, as the radius changes, so does the energy gap and the color of the emitted light (Figure 6). QD have very large molar extinction coefficients on the order of $0.5-5 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ which are 10-50 times higher than conventional organic dyes and suggests that the absorption rates will be faster at the same excitation [29], making them brighter fluorophores under photon-limited conditions (in vivo).

QD are synthesized by a variety of different methods such as microfluidic synthesis, electrostatic squeezing, EBL, laser beam, electron grid and chemical synthesis. The mostly widely used method is by organometallic chemical synthesis [30]. High quality colloidal semiconductor nanocrystals are prepared using simple chemical synthesis at high temperatures resulting in nucleation and subsequent formation of QD crystals [20].

Most of the time, QD obtained by chemical reactions are hydrophobic and hence their surfaces must be modified or replaced with amphiphilic molecules. For in vivo applications various techniques such as simple ligand exchange with thiol containing groups or peptides or dendrons are used [25]. Additional techniques include encapsulation using triblock co-polymers phospholipids, polymer beads or diblock co-polymers. Some techniques use both ligand modification and encapsulation to ensure stability. A core/shell QD can be surface-modified with additional ligands for application in biological systems.
2.3.2 Applications of quantum dots

QDs in general are valuable tools for in vivo imaging as they are photostable, bright and can be engineered to observe at depths up to 1 cm in a living organism, despite the scattering and the auto fluorescence of the tissue. Using multiphoton microscopy it is possible to visualize capillaries at a depth of several hundred micrometers.

Figure 7: Various applications of QD in biomedical imaging [25].

In addition, conjugation of QD with biomolecules including peptides, DNA and antibodies etc. allows its use in a variety of different applications like cell
labeling, cell trafficking, lymphatic imaging, tumor detection, brain imaging, detection of apoptosis, biopsy and surgery (Figure 7) [31].

In the field of cancer detection, NIR QD has been used in identification of SLN, a common procedure in breast cancer. SLN is the lymph node closest to affected tissue that is monitored for presence of disseminated cancer cells [32]. Kim et al. showed that QD allowed for precise imaging of SNL after intradermal injection of QD in live mice and pigs [12]. These QD had a broad emission at 850 nm. Following accumulation in the SNL, they were surgically removed. This indicates that QD can be used real-time in situ to remove lesions and metastatic tumors. Similar studies were reported using QD that identified SNL from 1 to 5 mins in various tissues [33; 34; 35; 36].

QD can also be conjugated to macromolecules exactly as other fluorocromes, with the difference that, due to its large size, it can be attached to a number of proteins at the same time, acting as a multifunctional nanoparticle-biological hybrid. Akerman and coworkers reported the use of QD modified with peptide conjugates to target tumor vasculature [37]. Similar multifunctional QD was constructed by Gao et al. using amphiphilic triblock copolymer and PSMA for active targeting of tumor and imaging in live animals [22]. On the other hand, Zhou et al. biofunctionalized a peptide ligand targeted to GPCRs for whole cell and single molecule imaging [38]. Similar cell membrane targeting and imaging of living cells were carried out using Fe$_2$O$_3$–CdSe magnetic QD [39]. QD were also conjugated with biotin-streptavidin for quantitative analysis of viral binding
such as the human T cell leukemia Type I virus [40]. In a different study, imaging of live cells were carried out by conjugating QD with folic acid [41] or with anti-Pgp [42].

### 2.3.3 Quantum dot toxicity

Although QD offers a plethora of advantages and applications, there is some concern regarding its toxicity in biological applications. Naked CdSe QD were shown to be toxic towards primary rat hepatocytes when exposed to UV irradiation [43]. This is because UV radiation dissolves the semiconductor particles by photolysis thereby releasing free Cd$^{2+}$ ions into the medium. These heavy metals can also cross the blood-brain barrier, can accumulate in adipose tissues and can be toxic to the liver and kidneys [44]. In addition, release of this ion can have toxic effects \textit{in vivo} to the lungs [45]. However, in the absence of UV there was no effect on ATP production or cell division (D. Stuart, X. Gao and S. Nie, unpublished data). These results were confirmed when Ballou \textit{et al.} coated QD with amphiphilic PAA polymer and tested \textit{in vivo} [46]. By changing the surface chemistry of QD with the view to improve their toxicological profile, Hoet \textit{et al.} showed that PEGlyated quantum dots with sizes greater than 10 nm were passively excreted by the kidney [47].

These studies suggest that by attaching molecules such as PEG to QD, the toxic burden to the animal could be reduced. Their application for \textit{in vivo} imaging makes it essential to avoid any long-term toxicity [48; 49]. For example,
amphiphilic polymer conjugation makes QD more water-soluble and resistant to chemical or enzymatic degradation. Also, great attention has been focused to increase the circulation time of the QD for better imaging. However, clearance from the body is important for any contrast agent for its clinical application and the dose used should be below the toxic levels for Cd [12].

2.4 Conclusion

Whole body in vivo imaging of nanoparticles, macromolecules and living cells can be carried out in the NIR region of the spectrum and offers results with high sensitivity and precision.

NIR imaging has many advantages to offer, since deep tissue imaging requires the use of far-red or NIR light. This provides a clear window for in vivo applications since it separates the major absorption peaks of blood and water resulting in minimum interference. Various parameters have to be taken into consideration during NIR imaging to optimize the image quality and minimize any SBR. Image contrast can be enhanced using contrast agents such as QD.

QD possess unique physiochemical properties which determine its potential application in NIR imaging since different spectral windows are available for in vivo imaging.

In this chapter the NRI imaging technique, principle and instrumentation are summarized. This technology allows rapid detection of cancer in vivo with the use of contrast agents such as QD.
References:


CHAPTER 3. RAPID IMAGING AND IDENTIFICATION OF TUMORS USING QD LOADED PHOSPHOLIPID MICELLES IN VIVO

3.1 Introduction

As mentioned in chapter 2, NIR technique for whole body imaging is a powerful non-invasive method for the visualization of complex biological phenomena such as tumors, tumor vasculature, VEGF and EGF receptors in tumors and whole body studies. In this chapter, the application of NIR imaging technique using a novel NIR contrast agent QD encapsulated into PEG-PE-micelles (QD-M) is described.

QD-M accumulated maximally in the tumor area within one hour compared to four hours for the commercially available PEG-modified QD (QD-PEG) and allowed for the visualization of both tumor and internal organs.

QD-M exhibited a high signal-to-noise ratio that allowed the quantification of the micelle biodistribution using image analysis. The signal obtained with the QD-M was higher than the commercial formulation at half the QD dose. Overall, the QD-M appear to be a powerful and fast-working nanosized imaging agent that allows for effective visualization of tumors using NIR imaging.
3.2 Materials and Methods

3.2.1 Materials

Cadmium selenide (CdSe) QD (QD-800) and PEGylated QD (PEG-QD) (QDtracker® 800) were purchased from Invitrogen (Carlsbad, CA, USA). 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol) 2000] (PEG–PE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Isopropanol, methanol and chloroform and all other chemicals were purchased from Sigma Aldrich Chem., Inc. (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) and Penicillin/Streptomycin stock solutions were purchased from CellGro (Kansas City, MO, USA). Phosphate buffer saline (PBS) pH 7.4 was prepared in deionized (DI) water. Tissue culture grade fetal bovine serum (FBS) and trypsin were obtained from ICN Biomedicals (Costa Nesa, CA, USA).

3.2.2 Preparation of QD-micelles

Commercially available QD-800 in decane were mixed in a fourfold volume of 1:3 isopropanol-methanol solutions and centrifuged at 3500 rpm for 5 minutes. The supernatant was removed and the QD pellet was re-suspended in chloroform. QD-M were prepared using lipid film hydration method [1] by mixing 2.7 µmol of PEG-PE in chloroform with 20 pmol of QD in chloroform. The organic solvent was removed by rotary evaporator to get a lipid film. Traces of solvent
residue were removed by freeze-drying overnight under vacuum. The dry film was then hydrated with PBS pH 7.4 and vortex to get QD-M.

3.2.3 Particle size distribution of QD-micelles

The micelle size was measured by dynamic light scattering using Coulter® N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). The micelle suspension was diluted with DI water until the concentration providing a light scattering intensity of $5 \times 10^4$ to $1 \times 10^6$ counts per second was achieved. The particle size distribution was measured in triplicate.

3.2.4 Cell culture

Murine mammary carcinoma (4T1) cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM cell culture medium at $37^\circ$C, 5% CO$_2$. DMEM media was supplemented with 10% FBS, 1mM Na-pyruvate, 50 U/mL penicillin, and 50 µg/ml streptomycin.

3.2.5 Tumor inoculation in Balb/c mice

All experiments were performed in 6 – 8 weeks old female Balb/c mice, purchased from Charles River Laboratory (Wilmington, MA, USA), following a protocol approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health’s Principles of Laboratory Animal Care (publication 85-23, revised 1996). These animals were allowed free access to food and water.
For biodistribution and whole body *in vivo* imaging of QD-M, mice were administered with 150,000 4T1 cells suspended in 150 µl PBS pH 7.4 subcutaneously in the right flank. Tumors developed within a period of 14 days after injection.

**3.2.6 In vivo NRI imaging**

For biodistribution experiments, commercially available PEG-QD and QD-M were injected intravenously at a dose of 40 and 20 pmoles per mouse respectively, via tail vein into tumor bearing mice. The injection volume was 200 µl. Following administration, mice were anesthetized (ketamine/xylazine) intraperitoneally and depilated using hair removal cream (Nair, USA) and imaged with a Kodak Image Station *In vivo* FX (Carestream Health, Rochester, NY, USA) system using excitation at 710 nm and emission at 790 nm. At the end of experiments, animals were sacrificed using CO$_2$ or anesthesia followed by cervical dislocation, their skin removed and re-imaged to precisely locate the internal organs.

All images were analyzed using Kodak image analysis (Carestream Health) or the Image J (NIH, Bethesda, MD, USA) software. The ROI were determined using threshold analysis by comparing the whole-body images with images without the skin and exposed organs. The mean pixel intensity, the background signal and the auto-fluorescence of the animal was determined at each time point. The mean pixel intensity at the ROI was expressed as an absolute number by comparison with the scattered light (background noise from the internal
organs near the ROI), and all images were normalized using a ROI over the hip of the mouse to compensate for differences between the NIR images of the different animals and time points that result from differences in the overall image luminosity.

3.3 Results

3.3.1 QD-micelle particle size and signal-to-noise ratio

![Figure 8: Particle size distribution of QD-M (a) immediately after preparation and (b) after 3 months storage at 4°C, (n=3) [2].](image)

The average size of QD-M was 17.6 ± 1.2 nm and remained unchanged over a period of 3 months (Figure 8).
Since our main aim was to clearly identify the tumor area from the rest of the body, it was important that the formulation produced sufficient signal compared to the background. The signal-to-noise ratio was estimated over the tumor region in vivo. From Figure 9 the signal-to-noise ratio for QD-M was higher at one and two hours after the administration (t-test, P < 0.05) at half the dose, compared to commercial formulation. This suggests that QD-M accumulates faster in the tumor area. The signal-to-noise ratio for the QD-M was at near maximum at one hour, whereas for commercial PEG-QD this maximum occurs only after three hours. Also, the standard deviation for the QD-M was consistently low (< 5%), whereas for the commercial PEG-QD, it was approximately equal to the mean value at three and four hours. Additionally, over the four hour imaging period, the signal-to-noise ratio changed proportionally for the commercial PEG-
QD preparation, whereas the signal-to-noise ratio remained unchanged in case of QD-M. This information was critical, since it helped us in studying the effects of QD-M during whole body imaging.

### 3.3.2 Whole-body NIR in vivo imaging

Whole body *in vivo* imaging was carried out using the Kodak *in vivo* FX camera in Dr. Amiji’s Lab. NIR images of animals injected with QD-M were highly contrasted at half the dose compared to those injected with commercial preparation. As seen in Figure 10, the QD-M rapidly accumulated in tumor and internal organs; both were easily visible leading to sharp images. The liver was clearly visible, especially in the ventral view. At one hour, we could observe fluorescence from all organs. Organs such as liver, kidney, and gastrointestinal (GI) tract were clearly visible. Also at two hours, we could clearly identify the spleen mainly because the background fluorescence from the liver had decreased.
The decrease in the fluorescence from the GI tract allowed clear identification of the kidneys. Some fluorescence from the thoracic cavity was detected, but the intensity was not high. Because of the biodistribution of the QD-M, the background of the scattered light was not constant and it changed with time. Two hours post-injection of the QD-M the overall intensity increased and all the organs were clearly detectable, as suggested by the color-coded mapping of the images. The tumor site was also clearly detectable at one and two hours with
high fluorescence (dorsal view). By changing the angle, the biodistribution (internal organs) and the imaging or detection of tumor was possible.

Figure 11: Whole body color-coded images of mouse injected with commercial PEG-QD. Internal organs were best viewed on ventral side while tumor was clearly visible on the dorsal side [2].

In comparison with QD-M, when commercial PEG-QD were injected (Figure 11), the fluorescence from the formulation was diffused over the entire body of the animal resulting in a poor contrast image. Although the signal decreased with time, no internal organs were seen. Very high signal was detected compared to QD-M, but it was observed from all the parts of the animal.
suggesting us diffused distribution of the formulation, perhaps in the circulation. The images acquired at one, two hours were similar using the commercial preparation rendering image analysis very difficult. Additionally, the tumor could not be identified at one or two hours post injection since the fluorescence from the tumor was not higher than from the overall background.

3.3.3 Composite images to study tumor localization

In order to pinpoint the tumor, the white-field image was superimposed over the color-coded image. As a result, the pixels values with the highest intensities were observed eliminating the low fluorescence intensity regions from the color-coded image. This revealed the position of the tumor with great precision. Images obtained were from two mice injected with respective formulations.
Figure 12: Composite images of two mice injected with commercial PEG-QD (top) with the cumulative histograms for the tumor region and the entire mouse body (below). Fluorescence intensity values are calculated by selecting the ROI [2].

In Figure 12, (Mouse 1 and 2), large parts of the body emitted fluorescence, and the tumor area could not be identified. In fact, the highest fluorescence was emitted from the area around the tumor and not from the tumor itself and from tissues around the tumor area, where blood vessels leading to the tumor were located. However, this could be explained only by the presence of long-circulating commercial PEG-QD in the blood. The histograms represent the cumulative distribution of the pixels for mouse 1 and 2 in the tumor region and mouse body, indicating a more diffuse pattern in the mouse body compared to that of tumor area.
Figure 13: Composite images of two mice injected with commercial QD-M (top) with the cumulative histograms for the tumor region and the entire mouse body (below) [2].

On the contrary, in Figure 13, (Mouse 1 and 2), the tumor area in the image contained pixels with the highest value. The pixels values at the tumor area were in the upper limit of the values obtained from the whole-mouse image. The signal is visible only from the tumor area, indicated by the arrow which is also evident in the histogram analysis. The signals are more sharp and distinct compared to that obtained from commercial PEG-QD. A more diffuse fluorescence was observed from mice injected with PEG-QD compared to QD-M suggesting that tumors were best identified using our formulation.
3.3.4 Biodistribution of QD-micelles and ex vivo imaging

Further biodistribution of commercially available PEG-QD and QD-M was carried out using NIR imaging.

Figure 14: Biodistribution of the preparation in mice injected with commercial PEG-QD (A) and QD-M (B), (Mean ± S.D., n=5) [2].

Figure 14 shows the biodistribution of NRI signal from various tissues at different time points following administration of formulation. The signal obtained in different tissues was normalized relative to the signal over the hip to allow
quantification using the same signal-to-noise ratio as maximum to compensate for the difference in the overall luminescence at various time points. After administration of commercial PEG-QD, high variability was observed in the biodistribution pattern affecting our quantification due to the variable background noise. The commercial PEG-QD fluorescence accumulated in kidney and spleen over time. Signal from the tumor was observed at the end of three hours and showed a similar pattern at the end of four hours. Following administration of QD-M, a stable pattern was observed within one hour. Signal was detected from various organs and the biodistribution at one hour was similar to that after four hours post administration. Additionally, the ratio of the signal amongst the various organs remained the same at different time points of imaging. This can be explained by the scattering of the NIR fluorescence.

After comparing the two treatments, high tumor signal was achieved at one – two hours using QD-M compared to commercial PEG-QD (P < 0.05). Kidney, lung and heart also showed a remarkable increase in the accumulation of QD-M (P < 0.05), however compared to commercial PEG-QD the signal reached its peak at the end of one hour and remained higher during the course of the study. On the contrary, the fluorescence intensity for commercial PEG-QD increased over the time of imaging and reached its maximum value at the end of four hours (twice that of one hour). Significantly higher signal was observed from the QD-M than from commercial PEG-QD in the organs such as the liver and
heart (P < 0.005) which allowed us to clearly image these organs and also led to better quantification.

![Image](image.png)

**Figure 15:** *Ex vivo* color coded map images of organs (tumor $Tu$, lung $Lu$, heart $He$, spleen $Sp$, liver $Li$, kidney $Ki$), excised from mice injected with QD-M and PEG-QD at the end of the imaging experiment [2].

Additionally, *ex-vivo* imaging was carried out at the end of the study (**Figure 15**), showing us that animals injected with QD-M showed limited fluorescence (except liver) while all the organs from mice injected with commercial PEG-QD showed high signal intensity. This data corresponded to the whole-body imaging.
3.4 Discussion

The detection of tumors and distribution within the body using QD-M and NIR imaging was studied. Both, the commercial PEG-QD and the QD-M possessed long polymer chains of PEG, that allowed prolonged circulation. As shown previously, they tended to accumulate in tumors and organs rapidly, resulting in high fluorescence [3]. Due to a high signal-to-noise ratio from the tumor region, it was possible to quantify the signal in a way similar to dual wavelength NIR whole body imaging [4]. For NIR image weighting, multiple light scattering was used as our external reference. This was done by calculating the signal intensity from the ROI, taking into consideration the intensity of the scattered light and the background noise. Previous studies of live animal imaging used a similar technique of image weighting that allowed definition of the ROI with great precision and pinpoint tumor and other organs [5]. However, the most challenging part of the imaging studies was the presence of background noise which altered the quality of the image as the light scattering took place [6].

To overcome this, background noise was eliminated mathematically. However, use of state of the art instruments and detection of signal at a fixed wavelength can sufficiently eliminate background noise [7]. To quantify the data, it is important to normalize the intensity in planar imaging. For example, in the planar fluorescent imaging, both dark-field and epiluminescent images were used, and their difference in the intensity values were used to obtain a composite image [8]. The noise in NIR whole body imaging is due to the scattered light from
the organs, so the ROI values were expressed as a ratio over the background fluorescence.

It is well-known that the background in fluorescent tomography can be considered homogeneous, and hence it can be eliminated using mathematical methods or tomography [9]. However, if the images of the samples are well-contrasted and the contrast agent used is bright enough to produce a high signal, the background need not be eliminated. The signal can be directly detected over the noise. Previous tomographic studies suggested that the ratio of signal in the excitation and the emission wavelengths were used to distinguish the emitted light from the scattered light for quantification purposes [6].

In this study, the QD-M was used to achieve all of the above by enhancing the signal and reducing the time of imaging. A reproducible biodistribution pattern of QD-M was obtained that allowed us a better quantification at half the dose, compared to commercially available PEG-QD. Additionally, using the QD-M, we obtained images within one hour of injection compared to other ‘smart’ NIR contrast agents that produce images only after 24 hours [10]. However, these planar imaging studies cannot be compared to other tomographic studies using more elegant techniques. Compared to commercial PEG-QD, QD-M are advantageous for tumor detection and estimation of the effectiveness of chemotherapy [11].

The QD-M accumulated faster in the tumor area allowing rapid imaging to take place with less noise or background (Figure 9). However, the PEG-QD took
much longer where the tumors were visible only after several hours. In other observations, it was noted that, the commercial PEG-QD preparation circulated for a long time emitting fluorescence from vessels close to the skin surface and thereby reducing the image contrast (Figure 11, 12).

The difference in the image contrast between the QD-M and PEG-QD can be attributed to the various physiochemical properties of micelles and its architecture that allow a different biodistribution profile (Figure 14). These results are in agreement with previously reported work using QD [12]. In this study, the images showed signal from the vasculature close to the tumor area and the vessels close to the skin. However, the image of the tumor itself was not clear. Also, the accumulation of the QD was probably due to the leaky vasculature in the tumor area, and its rate was likely not constant.

The high signal intensity and sharp contrast of organs compared to the surrounding tissues with QD-M led to good quality images (Figure 10, 13). QD-M is a highly effective contrast agent due to its increased accumulation and rapid blood clearance. This rapid clearance from the circulation made the organs more visible. Some background noise was observed due to the scattering of the near infrared fluorescence from QD. However, this did not compromise the clarity of the images.

In general, it is challenging to obtain a clear NIR image. Thus far using QD to image tumors has only been able to identify the target after hours [13] or days post-administration or by using advanced microscopic techniques such as two
photon microscopy [14]. Additionally, several authors have superimposed NIR images over white field to appropriately locate the desired organ or tissue [15]. However, advances have been made that increase contrast, for example, using bioluminescent cells, MRI multifunctional nanoparticles and plasma mass spectrometry [16], or self-illuminating quantum dots[17].

3.5 Conclusion

The QD-M proved to be an improved agent both for tumor imaging and quantification. They offer higher contrasted images at a high signal-to-noise ratio compared to the PEG-QD at half the dose of PEG-QD in significantly less time. QD-M can be manufactured easily and reproducibly. Their potential as contrast agents for tumor imaging may be further improved with actively targeted QD-M using tumor-specific antibodies. The applicability of QD-M was further improved by making them multifunctional as described in the following chapter.
References:


CHAPTER 4. DETECTION OF TUMOR METASTASIS AND ENHANCED TUMOR ACCUMULATION USING QUANTUM DOT-IMMUNOMICELLES IN VIVO

4.1 Introduction

Here, a tumor-targeted NIR imaging agent composed of cancer-specific anti-NS mAb 2C5 coupled to QD-containing PEG-PE-based polymeric micelles is described.

mAb 2C5 has been previously shown to be reactive against a variety of tumor cells of murine and human origin and does not recognize the surface of normal cells. Many studies have reported the NS-restricted activity of mAb 2C5.

To improve the imaging potential of QD-M, they were decorated with mAb 2C5 to make QD-immunomicelles (QD-IM) by technique that has been previously developed.

QD-IM imaging potential is great since the fluorescence intensity in the tumor was twofold that of non-targeted QD-M after one hour post-injection. In addition, the QD-IM detected small cancer cell clusters in the lungs and so, may potentially serve as an early detection system for metastases.
4.2 Materials and Methods

4.2.1 Materials

Cadmium selenide (CdSe) QD (Qdots 800) was purchased from Invitrogen (Carlsbad, CA, USA). 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol) 2000] (PEG–PE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). p-Nitrophenylcarbonyl-polyethyleneglycol-3400-phosphatidylethanolamine (pNP–PEG–PE) was synthesized in our lab according to the procedure described earlier (Torchilin et al. 2001). Monoclonal antibody (mAb) 2C5 was produced and purified by Harlan Bioproducts (Indianapolis, IL, USA) using 2C5 hybridoma cell line from our laboratory previously described (Iakoubov et al. 1997). Isopropanol, methanol and chloroform and all other chemicals were purchased from Sigma Aldrich Chem., Inc. (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) and Penicillin/Streptomycin stock solutions were purchased from CellGro (Kansas City, MO, USA). Phosphate buffer saline (PBS) pH 7.4 and citrate buffer saline (CBS) pH 5.0 were prepared in deionized (DI) water. Tissue culture grade fetal bovine serum (FBS) and trypsin were obtained from ICN Biomedicals (Costa Mesa, CA, USA).

4.2.2 Preparation of QD-immunomicelles using para-nitrophenol chemistry

Commercially available QD (Qdots 800) in decane were mixed in a fourfold volume of 1:3 isopropanol-methanol solutions and centrifuged at 3500
rpm for 5 minutes. The supernatant was removed and the QD pellet re-suspended in chloroform.

For preparation of QD-IM; a lipid film was prepared by removing chloroform from the mixed solution of 2.7 µmol of PEG-PE, 5 mol % of pNP–PEG–PE and 20 pmol of QD. The film was hydrated with 200 µl of 5mM CBS pH 5.0 and vortexed to form micelles. When required, 339 ml of a 2.84 mg/ml solution of mAb 2C5 was added to three-fold excess borate buffer, pH 9.3, and incubated with the micelle solution with constant stirring overnight at 4°C to allow the attachment of antibody to the micelles. The formulation was then dialyzed against PBS, pH 7.4, using cellulose ester membranes with a cut-off size of 250,000 Da (Spectrum Medical industries) overnight at 4°C.

4.2.3 Particle size distribution of QD-immunomicelles

The micelle size was measured by dynamic light scattering using a Coulter® N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). The micelle suspension was diluted with DI water until the concentration providing a light scattering intensity of 5 x 10^4 to 1 x 10^6 counts per second was achieved. The particle size distribution was measured in triplicate.

4.2.4 Cell culture

Murine mammary carcinoma (4T1) and Murine melanoma (B16-F10) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM cell culture medium at 37°C, 5% CO₂, DMEM
media was supplemented with 10% FBS, 1mM Na-pyruvate, 50 U/mL penicillin, and 50 µg/ml streptomycin.

4.2.5 Tumor inoculation in Balb/c mice and pseudo-metastatic mouse model.

All experiments were performed in 6 – 8 week old female Balb/c mice, purchased from Charles River Laboratory (Wilmington, MA, USA), following a protocol approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health’s Principles of Laboratory Animal Care (publication 85-23, revised 1996). These animals were allowed free access to food and water.

For biodistribution and whole body in vivo imaging of QD-IM, mice were administered 150,000 4T1 cells suspended in 150 µl PBS pH 7.4 subcutaneously in the right flank. Tumors developed within a period of 14 days after injection.

Ex vivo imaging of lungs was carried out using a pseudo-metastatic melanoma mouse model. Mice were injected intravenously via tail vein, with 80,000 B16-F10 cells suspended in PBS pH 7.4. After two weeks, cancer nodes developed in the lungs.
4.2.6 In vivo NRI imaging and detection of cancer nodes in the pseudo-metastatic mouse model

For biodistribution experiments, QD-M and QD-IM were injected intravenously via tail vein into tumor bearing mice, at a dose of 20 pmoles per mouse. The injection volume was 200 µl. Following administration, mice were anesthetized (ketamine/xylazine) intraperitoneally and depilated using hair removal cream (Nair, USA) and imaged with a Kodak Image Station In vivo FX (Carestream Health, Rochester, NY, USA) system using excitation at 710 nm and emission at 790 nm. At the end of experiments, animals were sacrificed using CO₂ or anesthesia followed by cervical dislocation.

For the pseudo-metastatic mouse model, QD-IM was injected into the animals at 1/10th of the i.v. dose for PEG-QD (40 pmoles per mouse). At one hour and twenty-four hours post-injection, animals were sacrificed using CO₂ or anesthesia followed by cervical dislocation. Their lungs were removed for ex vivo imaging using Kodak Image Station In vivo FX (Carestream Health, Rochester, NY, USA) system.

All images were analyzed using Kodak image analysis (Carestream Health) or the Imaje J (NIH, Bethesda, MD, USA) software. The ROI were determined using threshold analysis by comparing the whole-body images with images without the skin and exposed organs. The mean pixel intensity, the background signal and the auto-fluorescence of the animal was determined at each time point. The mean pixel intensity at the ROI was expressed as an absolute number.
by comparison with the scattered light (background noise from the internal organs near the ROI), and all images were normalized using a ROI over the hip of the mouse to compensate for differences between the NIR images of the different animals and time points that result from differences in the overall image luminosity.

4.3 Results

4.3.1 QD-immunomicelle particle size and signal-to-noise ratio

QD-IM were prepared by a protocol previously established in our laboratory. The size of the micelles was 21.0 ± 6.7 nm and were stable at 4°C for at least fifteen days (Figure 16).

Figure 16: Particle size distribution of QD-IM, (n=3).
Similar to the previous study in chapter 3, signal-to-noise measurements were carried out at the tumor area. From Figure 17, it is observed that the signal-to-noise ratio was high within the first hour and remained practically unchanged for the duration of the imaging. One hour after injection, the signal-to-noise was $47.1 \pm 3.1$, while at the end of imaging it was $49.8 \pm 2.7$. This signal was double that of the non-targeted QD-M and almost four times higher than that of the commercially available formulation of PEG grafted QD administered at twice the dose, as reported in chapter 3. As mentioned earlier, the signal-to-noise ratio in the non-targeted QD-M is maximum at one hour after administration and for the commercially available long-circulating PEG-QD, this maximum is reached after four hours post-administration.
4.3.2 Whole-body NIR in vivo imaging

Figure 18 shows composite NIR images of two mice injected with QD-IM one hour after the injection, superimposed over a white field image. The signal is visible only from the tumor area, indicated by the arrow. Some signal was detected from hairs that were not completely removed. Pixel values for the tumor area had the highest values compared to the rest of the animal body. For instance, the mean value in the mouse body ROI is $42.7 \pm 23.4$, while in the tumor area is $62.2 \pm 16.1$. It is of particular interest that the pixel distribution is much narrower in the tumor ROI, while this is not the case for the more heterogeneous mouse body image. The high slope of the pixel value distribution allowed the tumor to be identified clearly.
4.3.3 Biodistribution of QD-immunomicelles

Figure 19: Biodistribution of the preparation in mice injected with QD-IM, (Mean ± S.D., n=5).

Further the biodistribution of QD-IM was investigated in various tissues (Figure 19). The methodology involves the generation of weighted signal-to-noise data for the duration of the imaging experiment. The signal-to-noise ratio is comparable among the different time points, as it represents normalized values with reference to the intrinsic fluorescence of the animal. These data allow us to express and quantify the imaging effect of the contrast agent in vivo. The highest signal was detected in the tumor area (47.2 ± 3.1 at one hour after the injection) and the kidney (51.4 ± 25.2). The rest of the organs exhibited a much lower signal, the lowest of which was from the liver with the signal intensity 4.97 ± 0.26. This pattern remained unchanged throughout the imaging period, so that four hours after the administration of the contrast agent, the signal in the tumor area was 49.8 ± 2.7. The liver again exhibited the lowest signal of 5.64 ± 0.82. The actively targeted QD-IM signal was much higher in the tumor area than in the
liver, but not the spleen. Thus, with the exception of the kidney, the actively targeted QD-IM accumulated preferentially in the tumor area with lower affinity for other organs.

4.3.4 Composite ex vivo images of lungs in pseudo-metastatic mouse model

![Composite ex-vivo images of lungs from mice bearing metastatic lung melanoma (B16F10) tumor.](image)

**Figure 20:** Composite ex-vivo images of lungs from mice bearing metastatic lung melanoma (B16F10) tumor.

Taking advantage of the fact that NS are present on the surface of uncontrolled proliferative cancer cells, we attempted to identify cancer metastasis using QD-IM in a pseudo-metastatic lung model. In **Figure 20**, B16F10
melanoma cells appeared black, while normal cells were white, since Balb/C mice are albino. The whole body images from control mice injected with B16F10 melanoma cells, but not the QD-IM, that represents the intrinsic fluorescence of the organ, showed a uniform low level of NIR fluorescence. The cancer cell clusters in the lungs of the mice were clearly identifiable by their black color. The background fluorescence was uniformly distributed over the lungs. A large number of cancer cell nodes could be seen in the white field photograph. One hour after the injection of the QD-IM, the NIR fluorescence was diffuse over the entire lungs with a pattern similar to the background fluorescence but with a slightly higher intensity. At 24 hours after injection, NIR fluorescence was much less uniform. The signal was concentrated mainly at areas near the small clusters of cancer cells and in the periphery of the lungs, pinpointing the smaller cancer cells clusters. This suggests that QD-IM can attach (be targeted) to cancer cells even when the tumor is small and may serve as a vehicle for early detection and treatment of cancer micrometastasis.
4.4 Discussion

The need for efficient contrast agents surfaced with the development of practical and easy-to-use instruments for NIR whole body imaging. Lipid-based coatings for QD have been earlier proposed by us and by other labs [1; 2; 3; 4]. Our approach described here aims to alter the biodistribution profile of QD to allow for effective and relatively rapid imaging of the tumor site. With this in mind, a contrast agent was developed to actively target tumor sites resulting in a high signal-to-noise ratio.

This formulation represents a significant improvement both over the commercially available PEG-QD such as the Qtracker®, and passively targeted QD-M as described earlier [5], since it allows for rapid imaging using a low dose of QD (half that of the Qtracker®) and with the two-fold enhancement of image intensity compared to non-targeted QD-M (at the same QD dose). The encapsulation of QD in PEG-PE envelopes offers great versatility and allows for the easy attachment of antibodies and other ligands without using complex surface chemistry [1; 3]. The attachment of antibodies was performed via the micelle-incorporated reactive pNP-PEG-PE component, which is simple and straight-forward compared to the use of the avidin-biotin linker that exposes the complex to opsonization by blood proteins and may decrease its circulation time [6].

In the previous chapter, encapsulation of QD into the PEG-PE envelope was shown to increases the signal in the tumor area compared to unmodified “native”
PEG-QD. It decreased the imaging time from four hours to one hour, and allowed for the decrease of the equivalent dose of the imaging agent by one half (Figure 17). The attachment of the anti-NS 2C5 antibody further doubled the signal by bringing more contrast into the tumor (Figure 18), similar to earlier demonstrated enhanced imaging by 2C5-targeted contrast agents with other imaging modalities [7]. This allowed us to image the tumor site with a signal much superior to that of the liver (Figure 19).

The use of lipid-based nanoparticles for delivery of QD has recently gained a lot of attention. A hybrid QD/cationic liposome system was recently proposed [8]. This system significantly enhanced the delivery of QD in tridimensional cell culture systems, mainly due to the positive charge of the nanoparticles. Another system used conventional immunoliposomes [9] with QD attached via PEG spacers at the surface of the nanoparticles. Although the latter system might offer bright images due to the presence of a large number of fluorophores per nanoparticle, the simplicity of this QD-IM may provide an advantage. QD-IM also provides improvement over QD directly conjugated to an antibody, where the QD/antibody complex lacks the steric protection provided by PEG coating [10].

The higher signal within a short time may be of great benefit for cancer imaging. Recent studies have shown that QD toxicity may be low enough for systemic administration [11]. There are a small number of studies on the use of QD for the detection of metastases [11; 12; 13] however, this could be attributed to the low sensitivity of the optical imaging and the intense scattering of the light
that makes pinpointing the source and estimation of the size of small tumor difficult. The clear benefit of our formulation is its high affinity towards cancer cells introduced by the 2C5 antibody [14; 15]. The period of 24 hours for the detection of metastases is longer than that of the imaging of a larger tumor (one hour). This is due to the need to allow the contrast agent enough time to circulate and attach in sufficient quantity to the cancer cells in loci containing small numbers of such cells (Figure 20). This observation also implies that both passive targeting and antibody-mediated attachment to the tumor are involved in the accumulation of the contrast agent at the target site. The lungs are saturated with QD-IM following the attachment to cancer loci. The insufficient staining of bigger cancer masses can be explained by the fact that QD-IM does not penetrate inside the tumor to produce high contrast.

The use of NIR optical imaging for the detection of metastases has gained a lot of attention recently and is a useful modality for the intra-operative detection of lymph node metastases. By administering QD before the operation the lymph nodes can be visualized during the operation for the removal of the breast cancer, greatly reducing the probability for the development of metastasis. The optical methodology in this particular case has obvious advantages, due to the simplicity of the instruments involved, as no nuclear or magnetic imaging modality can be used in the operating room [16; 17; 18; 19].

However, as a modality for the detection of metastasis, it has limits, because of the low sensitivity of the method and the inability to detect cancer clusters in
whole body imaging. Recent developments involving the quantification of the 
signal and the detection of fluorescence deep inside the body could, however, 
make this type of imaging possible and useful [20]. Although the time necessary 
for imaging may be longer with QD-IM than with some radio-imaging contrasts,
the benefits of using non-radiation emitting agents remain important. As evident 
from our data, there is a sufficient difference with the background signal of the 
healthy part of the lungs to permit an effective determination of cancer cells 
within healthy organs. Our system may be useful in the detection of the signal 
from remote sources throughout the body and visualization of small clusters of 
cancer cells before they develop into apparent tumors [21], especially when 
coupled with an advanced imaging modality that permits in vivo images in real 
time [22; 23].

4.5 Conclusion

In summary, QD-IM allow imaging as rapidly as the non-targeted micelles 
and produced twice the signal at the same dose. Their production is easy and 
requires no special equipment. With the development of advanced imaging 
instruments that allow signal detection deep within the body, their potential 
usefulness can be expected to be significantly enhanced.

The studies carried out so far have allowed rapid detection of cancer and 
cancer metastasis using NIR. However, in some unfortunate circumstances 
following metastasis, the cancer cells could remain undetectable. In such cases,
identifying a cancer biomarker would be an ideal alternative for detection of cancer.

References:


CHAPTER 5. BIOMARKERS: EMERGING TOOL FOR CANCER DIAGNOSIS AND TREATMENT

5.1 Introduction

Current cancer detection relies on various imaging modalities. Tremendous advances have been made in the imaging technology to improve sensitivity and allow detection of small lesions. However, these advances have also led to an increase in false positive findings and invasive procedures to make a definitive diagnosis [1; 2]. Identification and detection of cancer is currently possible only on macroscopic examination of the tumor tissue long after disease onset. Although these techniques are important for prediction of tumor behavior and prognosis, additional methods are necessary for cancer detection [3]. Hence there is a need for a non-invasive technique to detect cancer.

Biomarkers have emerged as potentially important diagnostic tools for cancer and many other diseases and their usefulness lies in their ability to provide early indications of disease or the progression of the disease. Additionally, they can help in risk assessment, chemotherapeutic response, evaluation of treatment and recurrence of cancer. From the variety of different biomarkers, nucleosomes (NS) and carcinoembryogenic antigen (CEA) are convenient antigens for early cancer detection. Current assessments of their blood or serum level offers the most useful single marker testing for different cancer diagnoses. However, these techniques suffer from low sensitivity, high
cost and false-positives that indicate a demand for improved techniques for biomarker detection.

5.2 Circulating biomarkers for early detection of cancer

Cancer cell associated protein products also known as biomarkers [4; 5] can aid in cancer detection. Identification of biomarkers present in excess distinguishes cancer cells from normal cells and should help to define the cancer, its nature and behavior. Biomarkers can help to identify the responsiveness to chemotherapy of the particular cancer and help to better manage therapy. Biomarkers are found in biological fluids or tissues in higher-than-normal amounts that can include patterns of SNP’s, DNA methylation, or changes in mRNA, protein, or metabolite abundance, provided that these patterns can be shown to correlate with the characteristics of the disease produced only by a malignant tissue [6].

Figure 21: Comparison of differences between normal and cancer cell [7].
As outlined in Figure 21, biomarker levels are increased in production due to genetic changes in the DNA. For example, changes such as point mutation, deletions, translocations, amplifications, and methylations alter mRNA transcripts from these affected genes. As a result, the cellular protein products are altered and expressed intracellularly, on the cell surface, or secreted into the extracellular space at higher or lower levels compared to normal cells [7]. Biomarkers are cellular indicators of normal physiological functions and changes during the disease state.

Ideally, they should be easy to detect, measurable across populations, useful in one or more of the clinical settings such as, detection at an early stage or identification of high-risk individuals or early detection of recurrence or as intermediate endpoints in chemoprevention [8; 9]. Various reports show an increase in the research effort on biomarkers for pancreatic cancer, melanoma, esophageal and lung cancer. However, only some of these biomarkers have clinical significance in diagnostic assays.

The FDA has approved several standard tumor-associated antigen assays for diagnostic use, such as PSA for prostate cancer, CEA for colon cancer and AFP for testicular and liver cancer. In addition, the FDA has also approved a HPV test for cervical cancer screening and several assays for monitoring or estimating the prognosis in patients with breast, bladder and ovarian cancer [10]. These biomarkers will not only help in early cancer detection and development of
preventive approaches, but will also help identify new targets for therapeutic development [4; 5].

5.3 Biomarkers types

According to the NCI, biomarker is a molecule found in blood, another body fluid or in a tissue that is a sign of a normal or abnormal process [11]. Currently, there are a large number of biomarkers that are been tested and under investigation, making non-invasive cancer diagnostics a highly feasible technology for early cancer detection. As discussed above, a variety of biomarkers have been discovered with state of the art methodology for quantitative analysis of circulating proteins, nucleic acids, metabolites and tumor cells which are products of cancer cells and the tumor microenvironment [3]. A number of biomarkers used in diagnosis and cancer therapies are summarized in Table 1.
Table 1: Cancer biomarkers and their applications [11].

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cancer type</th>
<th>Clinical use in diagnosis</th>
<th>Clinical use in therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>Colon</td>
<td>Monitoring</td>
<td>Passive and active therapy</td>
</tr>
<tr>
<td>Alpha-fetoprotein</td>
<td>Germ-cell hepatoma</td>
<td>Staging</td>
<td>not used</td>
</tr>
<tr>
<td>CA125</td>
<td>Ovarian</td>
<td>Monitoring</td>
<td>Passive therapy</td>
</tr>
<tr>
<td>EGFR</td>
<td>Colon</td>
<td>Prognosis</td>
<td>Passive therapy</td>
</tr>
<tr>
<td>KIT</td>
<td>Gastrointestinal</td>
<td>Diagnosis</td>
<td>Molecular therapy (Imatinib)</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>Thyroid</td>
<td>Monitoring</td>
<td>not used</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate</td>
<td>Screening and monitoring</td>
<td>Passive and active therapy</td>
</tr>
<tr>
<td>CA15-3</td>
<td>Breast</td>
<td>Monitoring</td>
<td>not used</td>
</tr>
<tr>
<td>CA27-29</td>
<td>Breast</td>
<td>Monitoring</td>
<td>not used</td>
</tr>
<tr>
<td>Cytokeratins</td>
<td>Breast</td>
<td>Prognosis</td>
<td>not used</td>
</tr>
<tr>
<td>Oestrogen and progesterone receptor</td>
<td>Breast</td>
<td>Prognosis</td>
<td>Hormonotherapy</td>
</tr>
<tr>
<td>HER2</td>
<td>Breast</td>
<td>Monitoring</td>
<td>Passive and active therapy</td>
</tr>
<tr>
<td>Fibrin/FDP</td>
<td>Bladder</td>
<td>Monitoring</td>
<td>not used</td>
</tr>
<tr>
<td>Mucin 1</td>
<td>Glandular epithelial origin</td>
<td>Diagnosis, monitoring</td>
<td>Passive and active therapy</td>
</tr>
<tr>
<td>CA19-3</td>
<td>Pancreatic</td>
<td>Monitoring</td>
<td>not used</td>
</tr>
</tbody>
</table>

Of the many different biomarkers available NS and CEA were chosen because they are released within the circulation in a variety of cancer types.

**5.3.1 Nucleosomes**

Cells die constantly as a response to diverse homeostatic stimuli and there are several studies that demonstrate the mode of cell death. For example, it could be the influence of inflammatory factors or a genetic background that manifests as augmented cell death [12]. As a consequence, NS are released into the surrounding area or blood circulation. NS represent the core of basic
chromatin units. It is a complex of DNA and histone core proteins that are released in the blood circulation because of cell death [13]. NS release is also a result of cell stress [14]. In mammalian cells, DNA is organized into compact nucleosomal subunits by wrapping around a histone octamer, containing H2A, H2B, H3 and H4 present in two copies of each. Histone protein H1 is located outside the NS on the internucleosomal DNA [15]. Nuclear chromatin occupies 99% of DNA and is organized in a systematic manner. In its secondary structure, chromatin is arranged as a chain of NS subunits.

Figure 22: Structure of Nucleosome [16].

Each subunit (Figure 22) is a disc shaped 206 kDa octamer. Wrapped around this complex are 147 base pairs of double stranded DNA or core DNA. In the core NS, the anionic DNA is tightly bound to the cationic H3-H4 histone tetramer [17]. The DNA linked between two NS subunits is called a linker DNA and runs about 10 to 100 base pairs. Histone protein H1 is positioned outside the NS and is responsible for stabilization of the NS in its tertiary structure as chromatin. This articulated arrangement plays an important role not only in
organization and stabilization of DNA, but also regulation of transcription of genetic information during DNA replication and repair [18].

Small amounts of NS are found in the plasma of healthy patients. To maintain homeostasis within the tissue, $10^{11}-10^{12}$ new cells are created by cell division every day. A comparable number of cells die during this time frame by well orchestrated apoptotic cell death. During this process, endonucleases are activated that cleave the chromatin into multiple oligo- and mononucleosomes [19] consisting of DNA in multiples of 180 to 200 base pairs wrapped around histones. High levels of NS are present in individuals suffering from diverse forms of cancer, particularly lung cancer, breast cancer, renal and prostate cancer and lymphomas as a consequence of elimination systems that are overloaded or impaired in such diseased state [14].

NS are cleared from circulation in a biphasic, saturable, and concentration-dependent manner with an initial half-life of 4 minutes. However, in blood of patients with cancer, there is an increased rate of cell death and NS appears in elevated levels [20]. Under normal physiological conditions, NS are packed into apoptotic bodies and engulfed by macrophages and surrounding cells [21]. However, in conditions such as cancer where high rates of apoptosis or necrosis occur, these phagocytosing mechanisms are saturated or compromised leading to increased levels of NS in circulating blood [22]. NS possess a broad range of clinical applications as a marker to predict the onset of
tumors at early stages of cancer and is used to predict tumor response to treatment of chemotherapy or radiation.

Several studies detected high NS levels in individuals with diverse forms of cancer, particularly in those with advanced stages, including colorectal and various other gastrointestinal kinds of cancer, lung cancer, breast cancer, ovarian and other gynecologic cancers, renal and prostate cancer, and lymphoma [13]. In some tumors, such as lung and breast cancer, high NS values were already observed in early stages.

The changes in the courses of circulating NS were associated with tumor response to therapy. While decreased levels were mainly found in patients achieving remission, constantly high or even increasing values were associated with progression in some solid and systemic tumors [13]. Kuroi et al. showed high levels of circulating NS in the plasma of 96 breast cancer patients as compared with 111 healthy controls [23]. Similar results were reported by Trejo-Becerril and coworkers in the plasma of 11 patients with cervical cancer when compared to healthy controls [24]. They also reported a direct correlation of changes in the NS levels to tumor load or tumor shrinkage. In a different study, Holdenrieder et al. reported elevated NS levels in 418 patients with various cancers including colorectal and other gastrointestinal cancers, lung cancer, ovarian cancer and other cancers [25]. Hence, circulating NS in plasma and serum are promising antigens that may be used to determine diagnosis and prognosis in many acute diseases [14].
To detect NS, mAb such as 2C5 is used. mAb are proteins which are produced by clones of a single cell, that bind to specific antigens for recognition and/or treatment of chronic infections and cancer [26]. The US-FDA has approved mAbs to treat cancer (Table 2), transplant rejection, and autoimmune diseases. At least 400 mAbs are in clinical trials worldwide.

Table 2: FDA approved monoclonal antibodies [11].

<table>
<thead>
<tr>
<th>Product</th>
<th>Type</th>
<th>Target</th>
<th>Indications</th>
<th>Date of approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trastuzumab (Herceptin)</td>
<td>Humanized</td>
<td>HER2</td>
<td>Metastatic breast cancer</td>
<td>1998 (US) 2000 (EU)</td>
</tr>
<tr>
<td>Gemtuzumab (Mylotarg)</td>
<td>Humanized, (coupled to calicheamycin)</td>
<td>CD33</td>
<td>Acute myeloid leukemia</td>
<td>2000 (US)</td>
</tr>
<tr>
<td>Alemtuzumab (Campath)</td>
<td>Humanized</td>
<td>CD52</td>
<td>Chronic lymphocytic leukemia</td>
<td>2001 (US) 2001 (EU)</td>
</tr>
<tr>
<td>Brentuximab (Ontuxan)</td>
<td>Chimeric; 131I radiolabelled</td>
<td>CD20</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>2002 (US) 2004 (EU)</td>
</tr>
<tr>
<td>Tositumomab (Bexxar)</td>
<td>Murine; 131I radiolabelled</td>
<td>CD20</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>2003 (US)</td>
</tr>
<tr>
<td>Cetuximab (Erbitux)</td>
<td>Chimeric</td>
<td>EGFR</td>
<td>Metastatic colorectal cancer</td>
<td>2004 (EU)</td>
</tr>
<tr>
<td>Bevacizumab (Avastin)</td>
<td>Humanized</td>
<td>VEGF-A</td>
<td>Metastatic colorectal cancer</td>
<td>2004 (US) 2005 (EU)</td>
</tr>
<tr>
<td>Panitumumab (Vectibix)</td>
<td>Human</td>
<td>EGFR</td>
<td>Metastatic colorectal cancer</td>
<td>2006 (US) 2007 (EU)</td>
</tr>
<tr>
<td>Cetuximab-monomer (Removab)</td>
<td>Hybrid rat and mouse (trifunctional bispecific)</td>
<td>EpCam</td>
<td>Malignant ascites</td>
<td>2009 (EU)</td>
</tr>
<tr>
<td>Ofatumumab (Arzerra)</td>
<td>Human</td>
<td>CD20</td>
<td>Chronic lymphocytic leukemia</td>
<td>2009 (US)</td>
</tr>
</tbody>
</table>

mAb have also been used as tools in biomedical research for the past three decades with their application in immuno-fluorescence, radioimmunoassay, ELISA and recently, in targeted delivery of imaging and therapeutic agents [27]. NS-specific mAb 2C5 as described previously [28], is reactive against various tumor cells of murine and human origin, but is unable to recognize the surface of
normal cells [29]. Many studies have been carried out in the field of nanomedicine, that have made use of mAb 2C5 as a targeting ligand towards various cancer cell lines [30; 31; 32; 33; 34; 35; 36] because of its cancer cell specific NS-restricted specificity [37]. Taking advantage of this property mAb 2C5 was used to fashion a diagnostic probe as a tool to detect circulating NS [38] in blood circulation that can be used for diagnosis and prognosis of cancer.

In addition to NS, CEA was used as a biomarker model for indirect cancer detection.

5.3.2 Carcinoembryogenic antigen

CEA was first described in 1965 by Gold and Freedman as the first tumor antigen to be identified [39]. In the late 1980’s extensive molecular cloning of CEA was carried out to study its structure. Subsequently, it was found that CEA belonged to family of CEA-related molecules termed CGM including CGM1, CGM2, CGM6 and CGM7. Later on, further analysis of the amino acid sequence revealed that CEA was a member of an immunoglobulin supergene family clustered on chromosome 19q composed of 29 genes [40] that encodes for ICAM-1. Additionally, the genes also encode for lymphocyte function-associated antigen 1 and major histocompatibility antigens [41].

In contrast to the above findings, when CEA was isolated from liver metastasis and analyzed, its structure revealed a glycoprotein that consists of 60% carbohydrate with a molecular weight of 180-200 kDa [40]. Mannose, galactose, N-acetylglucosamine, fucose, and sialic acid contributed to the
carbohydrate and variations in the side chains attributable to the heterogeneity of CEA [41].

CEA is bound to the cell membrane by a glycosyl phosphatidylinositol bond, and its soluble form is released by the action of phospholipase C or phospholipase D. Additionally, CEA’s structural similarity to ICAM-1 or ICAM-2 suggests that CEA acts as an adhesion molecule [42]. In cancer tissue, alterations in the cell adhesion involve cancer invasion and metastasis. It further suggested that CEA may be involved in these processes [43]. However, in normal tissue, CEA shows limited expression.

CEA is present in tissues such as columnar epithelial cells and goblet cells in the colon, in the mucous neck cells and the pyloric mucous cells in the stomach, in squamous epithelial cells of the tongue, esophagus and cervix, in secretory epithelia and duct cells of the sweat glands and in epithelial cells of the prostate [44].

CEA is indicative in many different forms of cancer. For example in breast cancer patients, CEA is predominantly found in the ductal region that suggests the initiation of tumor [45]. The main reason CEA is useful as a serum tumor marker for colorectal cancer and some other cancers is probably because CEA is a stable molecule and has a fairly restricted expression in normal adult tissues [44].
For example, in a normal healthy colon, CEA is released from the apical surface of mature columnar cells into the gut lumen and disappears with feces. As a result, low levels are seen in the blood (Figure 23) [44]. However, in cases of colon cancer, the cells are continuously proliferating and have a compromised basal lamina and as a result CEA is distributed around the cell surface. CEA has also been shown to be elevated in patients with pancreatic cancer [46] and colorectal cancer [47].

Normal levels of 2.5 µg/L are seen in healthy individuals and 5 µg/L in smokers [42]; and increase in patients suffering from various types of cancer. Additionally, the levels of CEA can be useful in the cancer prognosis and post-operative surveillance of colorectal cancer patients [48]. CEA is detected in the
serum of cancer patients by 96-well plate ELISA. Unfortunately this detection is limited by the high number of false positives due to its poor diagnostic sensitivity and specificity [49].

5.4 Imaging techniques for identification of biomarkers

To identify biomarkers with high precision, it is necessary to have a sensitive detection technique. Among the various technologies available, electrochemical and optical are the most widely used methodologies. With the application of nanotechnology many nanobiosensors are emerging with greater precision [50]. They still use optical imaging techniques, such as light absorption, light scattering, SPR and fluorescence to detect biomarkers.

Optical imaging enables real-time, noninvasive visualization of biomarkers, affording multiple and frequent measurements, thereby reducing or avoiding the need for costly, time-consuming and often painful biopsy and subsequent histological analysis. Optical imaging requires two components: a molecular-specific signal and an imaging system to detect this signal [51]. In optical imaging, when a photon of light interacts with the tissue of interest, it can be absorbed or scattered. Most optical imaging systems image the reflected excited light or fluorescence emission light from the surface. This principle is used in assays involving color changes that are produced using enzymes which bind to the biomarker of interest.

For example in one sandwich ELISA technique, gold and magnetic nanoparticles were coated with antibodies against CEA antigen. Upon capture of
CEA antigen, signal amplification was achieved using HRP TMB-H$_2$O$_2$ as substrate that produced a color change [52]. Additionally, CEA can be quantified by measuring the signal intensity at 450 nm. Zhou et al. and coworkers used DNAzymes as catalytic label moieties for detection of biomarkers such as AFP in substitution for HRP [53]. DNAzymes catalyze reduction of hydrogen peroxide in water in a way similar to HRP, producing chemiluminescence [54]. It has also been shown that DNAzymes react with ABTS reagent (present in some substrates) that produces a green color which can be measured at 410 nm [53].

Additionally, other imaging techniques such as fluorescence can be used for biomarker detection. Fluorescence refers to the inherent property of molecules to absorb light at a particular wavelength and emit light at a different wavelength. In fluorescence imaging, an external light of appropriate wavelength is used to excite a target fluorescent molecule, following which a longer wavelength, lower-energy light is emitted and used for imaging. Macroscopic imaging systems achieve a depth resolution of up to 800 micrometers and yield 3-D information from light emitted from different fluorescent objects that are detected through multiple channels [55]. Although confocal microscopy has been most widely used because it is more user-friendly and less expensive than macroscopic imaging techniques, it suffers the disadvantage of poor optical penetration [56].

Fluorescence microscopy has been widely used to study the interaction of nanoparticles with cells in vitro. For example, fluorescence microscopy was used
to observe the binding of Rh-PE-labeled PTX loaded PEG-PE micelles to several tumor cell lines and to measure cell viability of the MCF-7 (breast adenocarcinoma) cell line [30]. Fluorophores can be conjugated to target molecules specific against a biomarker to allow detection of captured antigen for a specific cancer type. For example, Jokerst et al. detected and quantified CEA, CA125 and HER2 using fluorophores [57]. They captured the antigen of interest with antibodies immobilized on an agarose support. A QD-conjugated detection antibody recognized the biomarker by emitting fluorescence.

Other electrochemical techniques have been widely studied for detection of cancer biomarkers in body fluids [58]. Using these techniques, biomarkers have been detected for screening, monitoring, diagnosis and prognosis of cancer. Techniques such as MS and MALDI-TOF are capable of detecting and identifying a large range of proteins. Additional assay platforms include a lateral flow immunoassay, microfluidic flow cells, an electrochemical cell, flow-through chips and microfluidic chips. However, the most widely used detection technique is ELISA. Since most of the biomarkers are present in low abundance in normal conditions, it’s important to detect small changes in levels of biomarkers at early stages of cancer development. Commonly, 96-well plate ELISA systems have been used widely for biomarker detection, but these devices are not suitable for sensitive detection and thereby appropriate diagnosis. Additionally, they lack sensitivity and specificity and provide only semiquantitative results. These shortcomings lead to high number of false-positives and false-negatives [59]. In
contrast, microchip technology allows detection of biomarker in very low blood sample volumes. This, in turn results in lower cost and avoids expensive kits. Additionally, because the microchips are very small in size, multiple detection assays can be run in parallel for biomarker detection. A variety of microchip technologies have been introduced recently (Table 4).

The use of these microchip devices may produce fewer false positives and false negatives. Additionally, a sandwich ELISA format can be performed on these devices where the primary antibody that captures antigens is immobilized and the secondary antibody generates a signal. This technique could lead to highly sensitive detection of biomarkers with reduced noise or background. Also the amount of blood serum, plasma or sample needed is reduced due to the miniature characteristic of the microchip.

Table 3: Microchip technologies in cancer diagnostics [60]
device. Studies on one such microchip device are presented in the following chapter.

5.5 Conclusion

Biomarkers such as NS are found in plasma of patients with tumors as they arise from apoptotic cells present in every in vivo developing tumor. CEA is produced during fetal development, but its production stops before birth at a basal level of 2.5 ng/ml. However, in some cancers CEA serum concentration increases. Thus, both NS and CEA should be considered as good candidates for detection of cancer, as monitors of the effectiveness of treatment and as predictors of recurrence of a variety of cancers. However, conventional diagnostic techniques limit detection of these biomarkers due to lack of sensitivity. Various new technologies have been developed to overcome this challenge, although they come with a very high cost. Using conventional diagnostic techniques, a detection system has been built to take advantage of these cancer biomarkers using fluorescence imaging as the imaging modality to aid in detection.
References:


CHAPTER 6. ANTIBODY-COATED POLYSTYRENE NANOBeads ASSEMBLED BIOCHIP BASED TOOL FOR DETECTION OF NS AND CEA

6.1 Introduction

Biomarker detection is a crucial tool for proper identification, early diagnosis and management of cancer. With the demand for a more sensitive detection of these biomarkers, mAb-coated polystyrene nanobeads have been prepared and assembled on chips to develop a biosensor for a highly sensitive antibody-based cancer fluorescent immunoassay. Chips were incubated with different dilutions of NS or CEA in different test media. The detection assay was developed similarly to a conventional ELISA assay on a plate format. These biochips demonstrated a very high sensitivity by detection of NS as low as 65 pg/ml and CEA at 15.6 pg/ml in serum using a fluorescence imaging technique. Thus, this system could serve as an effective tool for detection of small changes in biomarker levels and assist in the management of cancer therapy.
6.2 Materials and Methods

6.2.1 Materials

Carboxy-functionalized polystyrene (PSL) nanobeads (5% w/v) were purchased from Sperotech Inc. (Lake Forest, IL, USA). Nucleosome (NS) antigen (calf thymus) was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). mAb 2C5 was produced by Harlan Bioproducts (Indianapolis, IL, USA) using the hybridoma cell line from our laboratory [1]. Clarified mouse myeloma ascites IgG-2a (kappa) UPC10 antibody was purchased from MP Biochemicals (Solon, OH, USA). Poly-L-Lysine bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Chemical, Inc. (St. Louis, MO, USA). Goat anti-mouse IgG peroxidase-conjugate was obtained from ICN Biochemicals (Aurora, OH, USA). K-blue TMB peroxidase substrate was purchased from Neogen Corporation (Lexington, KY, USA). Oregon Green® 488 carboxylic acid succinidymyl ester-5-isomer was from Molecular Probes, Inc. (Eugene, OR, USA). The Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Mouse monoclonal anti-CEA antibody [1C7], CEA protein, and secondary fluorescein-labeled goat polyclonal antibody to mouse IgG-H&L were from Abcam, Inc. (Cambridge, MA, USA). Phosphate buffer saline (PBS) pH 7.4 was prepared in deionized (DI) water.

Female Balc/C mice, age 8-10 weeks, were purchased from The Charles River Laboratory (Wilmington, MA, USA).
**6.2.2 Immobilization of mAb 2C5 and mAb CEA on PSL Nanobeads**

PSL nanobeads were coated with IgG, mAb 2C5, or mAb CEA by adding 1.56 mg of an antibody to 10 mg of a 1% (w/v) suspension of the nanobeads in a total volume of 1 ml of PBS, pH 7.4. After an overnight incubation at room temperature with gentle shaking, the suspension was centrifuged at 1.5 x1000 rpm for 15 min. The supernatant was removed and the concentration of the free antibody in it was determined by using the BCA assay (according to the providers’ instructions). The pellet was re-suspended in DI water or PBS, pH 7.4, and stored at 4°C.

**6.2.3 Particle size and zeta potential**

Size and zeta potential of all preparations was determined with Dynamic Light Scattering and Zeta Phase Light Scattering, respectively, using a ZetaPlus particle size analyzer (Brookhaven Instrument, Holtsville, NY). The antibody-particle suspension was diluted with DI water for the size measurements and with 1 mM KCl for all zeta potential measurements. All analysis was repeated in triplicate.

**6.2.4 Activity of mAb 2C5-coated PSL nanobeads by indirect ELISA**

96-well plates were incubated with 50 µl of 40 μg/ml poly-L-lysine overnight at 4°C. Following incubation, the poly-L-lysine solution was discarded and the wells were blocked with 200 µl of Tris-buffered saline containing 0.05%w/v Tween 20 (TBST) with 2 mg/ml casein (TBST-casein) for one hour at
RT. After blocking, the wells were washed three times with TBST and incubated with 50 µl of 40 µg/ml of NS in TBST-casein for one hour at RT. The wells were washed three times with TBST and incubated with different concentrations of mAb 2C5 as a standard, clarified mouse myeloma ascites IgG-2a (kappa) UPC10 antibody as control, mAb 2C5-coated PSL nanobeads, IgG-2a (kappa)-coated PSL nanobeads and plain PSL nanobeads in TBST-casein for one hour at RT. After incubation, wells were washed three times with TBST and incubated with 50 µl/well of 1:5000 dilution of a goat anti-mouse IgG peroxidase conjugate in TBST-casein for one hour at RT. The wells were washed 3x again and incubated with 100 µl of enhanced K-blue TMB peroxidase substrate for 15 mins or until a blue color developed. The 96-well plate was read at a wavelength of 620 nm with the reference filter at 492 nm using a Labsystems Multiscan MCC/360 microplate reader installed with GENESISLITE Windows-based microplate software.

6.2.5 Labeling of nucleosomes with Oregon green 488®

The NS were labeled with Oregon Green® according to the Molecular Probes protocol. Briefly, 1ml of NS solution (5-10 mg/ml in PBS, pH 7.4) was added to 0.1 ml of 1M sodium bicarbonate solution in water to raise the pH of the solution to 8.3. Oregon Green® dye was dissolved in DMSO and added to the NS solution at a 16:1 M/M dye-to-protein ratio with stirring, and incubated for 1 h at RT. The mixture was then dialyzed against PBS, pH 7.4, using cellulose ester membranes with a cut-off size of 250,000 Da (Spectrum Medical Industries) overnight at 4°C. The concentration of NS was determined by the BCA assay.
The reactivity of mAb 2C5 towards the labeled NS was checked by indirect ELISA as described above.

6.2.6 PMMA biochip template fabrication

The nanochip template was fabricated by depositing layers of metals (6 nm Chromium/40 nm Gold) onto a 380-micron thick silicon wafer coated with 150 nm of thermal SiO₂. Then, a 300 nm thick PMMA film was spun on the gold template and baked at 100°C for 90 seconds. Trench array patterns were generated on the PMMA by EBL. After the exposure, the PMMA film was developed in a methyl iso-butyl ketone (MIBK): isopropyl alcohol (IPA) [1:3] mixture for 70 seconds followed by IPA rinse for 20 seconds and DI water rinse for 5 minutes.

6.2.7 Assembly of mAb 2C5- and mAb CEA-coated PSL nanobeads

For assembly into the trenches of the biochip, mAb-coated PSL nanobeads were diluted in DI water and NH₄OH was added to the solution in order to increase the pH and conductivity of the nanobead suspension. The nanobead concentration, pH, and ionic conductivity of the suspension were controlled. To perform electrophoretic assembly, patterned PMMA/gold chip (anode) and a gold substrate (cathode) were connected to a DC voltage source and inserted vertically into the nanobead suspension. For experimental consistency, a dip coater was used to control the speed of insertion and removal of the setup in the suspension. The pulling speed was maintained at 85 mm/min.
The electrostatic force acting on the negatively charged nanoparticles is directly proportional to the strength of the applied field and the charge on the nanoparticle. For a complete coverage a voltage of 2.5 V was applied for 1-2 minutes when the electrodes were spaced over a distance of 5mm. To keep the assembled nanobeads intact, the voltage was kept applied while removing the chips from the solution. The assembled chip was stored in DI water and used as required.

6.2.8 In vitro nucleosome detection by mAb 2C5-PSL assembled biochips

After confirming the activity of mAb 2C5-nanobeads towards NS, 2C5-biochips were incubated overnight in a 2% BSA solution at 4°C to block non-specific binding on the surface of the biochip. After washing with DI water, chips were incubated with different concentrations of fluorescently labeled-NS ranging from 500 pg/ml to 62.5 pg/ml in FBS at 37°C for 1 hour. Then, chips were washed again with DI water and stored in PBS, pH 7.4, until their analysis by fluorescence microscopy. Images acquired were analyzed by Image J software. Non-specific IgG-biochips were used as a control.

6.2.9 Biochip-based ELISA assay of mAb CEA in buffer solution, FBS and murine blood

Sandwich ELISA on biochips was applied to test the specific activity of the chips with mAb CEA. CEA-bearing biochips were tested for their capacity to detect low concentrations of the CEA antigen. The assembled biochips were
incubated in a 2% BSA solution to block non-specific binding. After the overnight incubation at 4°C, all chips were washed with DI water to remove the BSA. Then, chips were incubated with dilutions of the CEA antigen in the PBS, pH 7.4, or in FBS with CEA ranging from 125 pg/ml to 15.6 pg/ml for 2 h at RT. CEA-biochips incubated with plain solutions were used as blanks. For the CEA antigen in 50% murine blood samples (the same range of CEA dilutions were used), the biosensors were incubated for 2 h at 37 °C to mimic the physiological conditions. Na citrate (1%) was used as an anticoagulant at a 10-fold blood dilution (normalized volume of blood obtained from mice). Chips were washed with DI water, incubated for 2 h at RT with 2 µg/ml of a secondary FITC-labeled IgG antibody, and finally washed to remove any trace of non-specifically bound secondary antibody. Chips were stored in PBS pH 7.4 at 4 °C until the analysis by microscopy.

6.2.10 ELISA assay with blood samples of CEA-antigen injected mice

CEA biochips were tested for the CEA antigen detection level in the blood of 6-8 weeks old Balb/C mice injected with known concentrations of the antigen. 100 µl of CEA antigen in PBS pH 7.4 was injected via the tail vein.

Each dose was calculated by considering two variables: 1) dilution factor based on estimated mouse blood volume ca. 2 ml/mouse, and 2) the dilution in 0.9% NaCl before the incubation with chips. At 1-2 mins post-injection, the blood was collected by cardiac puncture in Na citrate. Blood samples were diluted (50:50 v/v) in NaCl to obtain a final CEA concentration ranging from 12.5 to 31.25
pg/ml. The samples were incubated with chips as described in the previous paragraph.

6.2.11 Detection of CEA in tumor-bearing mice

Female Balb/c mice were inoculated with breast cancer cells (4T1) suspended in 100 µl of PBS pH 7.4 (100,000 cells/mice). The blood was withdrawn by cardiac puncture from anesthetized mice at days 1, 3 and 7. The blood samples (0.3 to 0.7 ml) contained 50 ul of 1% sodium citrate to prevent coagulation and were stored at 4°C. CEA-biochips were tested as described earlier, imaged and quantified.

6.2.12 Biosensor imaging using fluorescence microscope technique

To quantify the detection of NS and CEA antigens, fluorescent images were processed using the Image J software (NIH, Bethesda, MD, USA). Antibody-loaded biochips were imaged using a Nikon Optiphot 200 Fluorescence microscope equipped with a CCD camera. Images in both bright-field and dark field (green channel fluorescence) were collected for each chip. The fluorescence imaging was performed using a 30 sec exposure time with the CCD amplifier gain set to 0.6 and stored as 24-bit jpg images. For processing, the background noise was removed by built-in background subtraction, followed by extraction of the 8-bit green color channel. The area of the image corresponding to trenches was manually selected and its mean fluorescence intensity was analyzed. Similar
treatment and analyses were carried out for images acquired from control IgG-biochips.

6.3 Results

6.3.1 Immobilization of mAb 2C5 on PSL nanobeads

As shown in Figure 24, the surface of PSL nanobeads with COOH functional groups was coated with IgG, mAb 2C5, or mAb CEA. The amount of antibody coating/attached to the beads was calculated as the difference between the initial quantity of the antibody and the amount of free/non-adsorbed antibody in the supernatant. Regardless of the type of antibody, the adsorption yield was higher than 50% in all cases (as estimated by the BCA assay).
6.3.2 Size and zeta potential of mAb-coated PSL nanobeads

![Graph showing size and zeta potential of mAb-coated PSL nanobeads](image)

**Figure 25**: Particle size and Zeta potential of antibody coated PSL nanobeads (mean ± S.D.; n=5)

Size and zeta potential are crucial parameters since they affect the assembly process of mAb-coated beads into trenches of the biochip (**Figure 25**). Size of the plain PSL nanobeads is ca. 320 nm (from the provider). The size of mAb 2C5- or mAb CEA-coated PSL nanobeads was 360 ± 3.3 and 324 ±8.3 nm, respectively. IgG-coated nanobeads had a size of 375 ± 4.5 nm. Zeta potential value for uncoated nanobeads was ca -70 mV. After the passive adsorption of mAb 2C5 or mAb CEA, it shifted to -44.6 ± 2.5 mV and -59.5 ± 0.54 mV,
respectively. IgG-coated nanobeads had a zeta potential of -38 ± 2 mV. Based on changes in the measurements before and after coating, the particle size and zeta potential values indicated that the antibodies were coated on to the nanobeads without any aggregation (see on right).

6.3.3 Specific activity of mAb 2C5-coated PSL nanobeads

![Image of specific activity graph]

The specific activity of mAb 2C5-coated PSL nanobeads towards NS antigen was determined by indirect ELISA using IgG-coated PSL nanobeads as a control. As seen from Figure 26, the activity of mAb 2C5-coated PSL nanobeads was close to that of the standard native mAb 2C5. IgG-coated PSL nanobeads did not show any specificity towards NS. Plain PSL nanobeads
showed no cross-reactivity to NS confirming the specificity of mAb 2C5-coated PSL nanobeads towards cancer-related NS antigen.

6.3.4 Assembly of mAb 2C5-, mAb IgG- or mAb CEA-coated PSL particles into nanochips

Following antibody coating and characterization, the nanobeads were ready for assembly into the trenches of the biochip.

Biochips were fabricated and assembled at the CHN, Northeastern University. Figure 27 represents the schematic of the template fabrication process for the biochip. Upon the final layer of PMMA, trenches were written...
using EBL. The antibody coated nanobeads were assembled into the trenches electrophoretically using the above shown instrument setup. Particle suspension of pH (10.7 - 11.1) were used with a voltage of (2-3 DC) for a period of 60-180 sec. Each biochip was checked for assembly efficiency using an optical microscope. Figure 28 shows an SEM image of the antibody-coated particles assembled in 400 nm wide and 10 µm long trenches.

![Figure 28: SEM imaging of biochips assembled with antibody-PSL nanobeads](image)

Only biochips with assembly greater than 90% of trench area were considered for the further experiments.

6.3.5 *In vitro nucleosome detection by mAb 2C5 PSL assembled biochips.*

Following assembly, the efficacy of the mAb 2C5-biochips in detecting the NS biomarker was studied. A direct “ELISA-like” assay was performed with mAb 2C5-coated beads assembled in trenches, to detect known amounts of the fluorescent-labeled NS antigen. In Figure 29, the mAb 2C5-biochips (mAb 2C5-particles assembled into trenches) show an increased fluorescent intensity signal compared to the control IgG-biochip, confirming the specificity of 2C5-biochips to NS (in accordance with the indirect ELISA data in the plate format). For quantification, both kinds of chips with mAb 2C5 and IgG antibodies were
imaged, and their images were processed and analyzed by the Image J Software, as described earlier. The fluorescence intensity data plotted is shown below.

**Figure 29:** Fluorescence image showing detection of nucleosome on 2C5-biochips (top) and quantification (bottom), (mean ± S.D.; n=5).

The fluorescence intensity of the IgG-biochips represents a background while the 2C5-biochips detected NS in a concentration dependent manner. This also
suggests that the assembly process did not compromise the reactivity of mAb 2C5 to NS. The results confirmed that mAb 2C5-beads assembled on the chips were able to specifically detect a NS concentration as low as 62.5 pg/ml in FBS.

6.3.6 Sandwich ELISA assay of mAb CEA on biochip format in buffer solution, FBS and murine blood

The applicability of our device was also confirmed for detection of another biomarker, CEA, using the sandwich ELISA technique as shown in Figure 30.

![Schematic representation of sandwich ELISA testing on biochips](image)

**Figure 30:** Schematic representation of sandwich ELISA testing on biochips

Following the capture of CEA on the biochips, the FITC-secondary antibody reacts with the captured antigen. Using this technique, the initial testing was carried out *in vitro* to study the detection of CEA antigen in different media such as PBS, FBS, and blood.
In PBS and FBS the biochip demonstrated a very high sensitivity being able to detect down to 31.25 pg/ml (data not shown), while in the 50% murine blood it was possible to detect 15.6 pg of the antigen per ml (Figures 31).
6.3.7 *Sandwich ELISA assay in blood samples of CEA-antigen injected mice*

The sensitivity of the biochip was further evaluated in conditions simulating an *in vivo* pathology. The test with mAb CEA-assembled particles was run with blood samples from mice injected with CEA antigen, **Figure 32**. Known CEA antigen concentrations were spiked intravenously in mice, and blood samples were collected 1-2 min post injection. Here, the fluorescent ELISA assay on CEA-biochips demonstrated a very high sensitivity detecting the antigen concentration as low as 31.25 pg/ml.

**Figure 32**: Sandwich ELISA assay in murine blood samples, (mean ± S.D.; n=5).
6.3.8 Sandwich ELISA assay in blood samples from tumor-bearing mice

Figure 33: Fluorescence images of CEA-biochips tested in tumor bearing mice.

Figure 33 shows the fluorescence images of the biochips treated with blood from tumor-bearing mice at day 1, 3 and 7. Increase in the fluorescence was observed that suggests an increase in the amount of the captured CEA antigen.

Figure 34: CEA antigen detection in vivo, (n=3) mean ± S.D.; (*) p < 0.05
After processing the images using Image J software (Figure 34) we observed an increase in the fluorescence intensity from day 1 to day 7. This shows that more CEA antigen is present in the circulation suggesting tumor growth. At days 1 and 3 mice showed no signs of tumor, whereas by day 7 a clear vascularized tumor was observed.

![Graph showing fluorescence intensity versus time](image)

**Figure 35**: Blood samples analyzed using commercial BIOQUANT® CEA ELISA KIT.

The biochips were sensitive to detect low amounts of CEA antigen obtained from mice bearing tumors. Additionally, the same blood samples were tested using a commercially available detection ELISA kit (BIOQUANT®) (Figure 35). No signal from the blood samples was observed that indicated that the levels of CEA antigens were too low to produce any signal. This also suggests that the commercial CEA ELISA kit was less sensitive than the CEA-biochips and did not allow any low detection. This result supports the applicability of such or similar biosensors for early tumor detection.
6.4 Discussion

Detecting biomarkers at early stages can help to identify the responsiveness to chemotherapy of a particular cancer and help in better managing the therapy and assist in making suitable clinical decisions. It can help to detect reoccurrence of cancer following treatment [2; 3]. Along with conventional detection techniques, ELISA is the most widely used due to its advantages such as easy handling, simple and flexible technique and analysis of the sample [4]. At the same time, conventional 96-well plate based assay techniques suffer disadvantages such that they are expensive and laborious techniques and are time consuming. In addition, the ultimate detection limit or sensitivity of is not optimum enough to challenge the biomarker detection. Hence, alternate detection techniques such as nanowire sensor arrays, rolling circle amplification DNA, nanocantilevers, nano-particle based bio-barcodes are being developed [5; 6; 7; 8].

These new platforms for detection of biomarkers allows higher amplification in the signal. However, additional improvement in the sensitivity contributes to the cost of manufacturing and intricate readout devices [4]. To overcome these challenges of recent advancements in biomarker detection, antibody arrays are used in development of a chip based bio-sensor device that has a capacity to detect biomarker at concentrations below the current detection limit of ELISA, without modifying the simplicity of the ELISA technique itself. In
addition, this system is useful and applicable in detection of any biomarker analyze with an existing ELISA setup with a high sensitivity and low cost.

These antibody arrays were fashioned by adsorbing mAb 2C5, mAb CEA or IgG on PSL nanobeads (Figure 24) and assembling them onto the trenches of the biochip. The adsorption of Ab on the PSL nanobeads is a result of the capacity of the IgG molecule to bind to the PSL nanobead and the density and surface area of the PSL nanobeads itself. For adsorption of a monolayer of antibody on the surface of the particles with correct spatial orientation and decreased likelihood of nonspecific binding, the amount of antibody needed was calculated based on the following formula provided by Bangs Laboratory (Fishers, IN, USA) and also suggested by [9; 10]:

\[ S = \frac{6}{\rho D}(C) \]  

(1)

Where

\( S \) = amount of representative protein needed to achieve surface saturation (mg protein/g of microspheres),

\( C \) = capacity of microsphere surface for a given protein, which will vary depending on the size and molecular weight of the protein to be coupled (mg protein/m^2 of polymer surface). For IgG type antibodies \( C = 2.5 \text{ mg/m}^2 \),

\( 6 / \rho D \) = surface area/mass (m^2/g) for microspheres of a given diameter (\( \rho \) = density of microspheres, which for polystyrene is 1.05 g/cm^3), and

\( D \) = diameter of microspheres, in microns.
Based on equation (1), a monolayer antibody molecule was adsorbed on the PSL nanobeads. Other methods of conjugation chemistry are described [11; 12; 13] using agents such as EDC. However, adsorption techniques require no additional reagents and are a simple technique resulting in no aggregation. In addition, the particles were stable in terms of their size and zeta potential, which is a critical factor in the assembly of antibody-coated particles into the nano-trenches (Figure 25).

Prior to assembly, the activity of antibody after adsorption was evaluated using indirect ELISA for mAb 2C5-PSL nanobeads (Figure 26). Our findings were similar to previously reported results by our group when mAb 2C5 was used in conjunction with nanoparticles such as micelles and liposomes [14; 15]. The activity of mAb 2C5 was retained compared to standard 2C5. Indirect ELISA allowed us to determine the activity for mAb 2C5-PSL nanobeads, however this technique was not available to determine the activity for mAb CEA-PSL nanobeads which allowed us to carry out traditional sandwich ELISA on the CEA-biochips.

Assembly of both antibody PSL nanobeads was carried out by our collaborators at CHN, Northeastern University (Figure 27, 28). Post assembly, the mAb 2C5 assembled biochips were tested for their activity with fluorescence-tagged NS. Due to the specific activity of mAb against NS, we were able to detect and observe this binding in a concentration dependant fashion (Figure 29). High levels of NS are detected in individuals suffering from diverse forms of
cancer, particularly lung cancer, breast cancer, renal and prostate cancer and lymphomas as a consequence of elimination systems that are overloaded or impaired in such disease states [16]. As a result, NS is a critical biomarker for additional detection and management of cancer. Normal levels of 16 +/− 8.8 ng/ml, range 8-52 ng/ml were reported using the traditional ELISA method [17], in comparison to which we were able to detect down to 62.5 pg/ml in FBS, suggesting that our bio-sensor was much more sensitive than the conventional 96-well plate assay format.

Similarly, the CEA biomarker was also tested using sandwich ELISA (Figure 30). Low levels of CEA antigen were detected in PBS pH 7.4 and FBS at 37°C. The limit of detection using the biochip was 31.25 pg/ml (data not shown). This is of great advantage considering the baseline levels of CEA are 2.5 ng/ml to 5 ng/ml. Detecting low amounts of CEA in very early stages of cancer can help in better prognosis and effective treatment. Similar results were reported when CEA was detected in saliva using quantum dots based on the nano-bio-chip system [18]. In comparison, 15.6 pg/ml of CEA in spiked 50% murine blood was detected in vitro, suggesting our system was highly sensitive (Figure 31).

Additionally, the detection of CEA was studied ex vivo from antigen injected mice in a dose-dependent manner with a limit of detection down to 31.25 pg/ml (Figure 32). These values were less than baseline levels and can be an advantage to detect minute changes in the biomarker levels during cancer development.
Further, the sensitivity of the nanochip was evaluated by simulating *in vivo* pathology conditions (**Figure 33**). Tests run with blood samples from tumor-bearing mice on CEA-biochips showed increased fluorescence from day 1 to day 7 compared to the background (**Figure 34**). Previous studies in nude mice with colonic-tumor xenografts and tumor weights from 160 mg to 2.16 g reported serum CEA levels of less than 11.4 ng/ml [19]. The biochip was sensitive to detect the CEA antigen in tumor-bearing mice in much lower quantities, i.e. at much earlier stages of tumor growth. In these conditions, the CEA antigen was undetected using the commercial CEA ELISA kit (**Figure 35**).

Making use of chip-based technology, PSL nanobeads coated with different antibodies can be assembled in different trenches on one chip allowing for simultaneous detection of different biomarkers. Apart from parallel multi-biomarker detection, very low volumes of samples were required to do the testing, which results in consumption of low amounts of clinical sample and use of antibodies unlike conventional ELISA kits. Also, these biochips can be easily altered in shape, number of trenches and assembly of antibody-coated PLS particles that can contribute to enhanced detection of biomarkers. Such multiplexed conditions are difficult to develop in a traditional 96-well plate format and can add to the overall cost of the device.

**6.5 Conclusion**

mAb-coated PLS-nanobeads can be assembled in trenches of biochips. This system successfully demonstrated detection of very low concentrations of
NS and CEA antigens in different media. More importantly, it confirmed the potential for antigen detection in pathological-like conditions in murine blood samples. These results suggest a potential broad applicability of developed chips for detection of small quantities of any circulating biomarker for disease. Given its extremely high sensitivity, it should serve as a useful tool for early detection of tumors and control of tumor growth.
References:


CHAPTER 7. IMAGING AND THERAPY OF CANCER USING THERANOSTIC AGENTS

7.1 Introduction

A theranostic agent is a nanosystem that integrates a diagnostic agent and a therapeutic entity within the same scaffold. Besides therapy, effective diagnosis of cancer is a key for treatment. Among the variety of imaging modalities including NIR, MRI, PET, CT, PAI and US [1], MRI is of specific interest. Its principle and application in T2 weighted imaging is discussed in this chapter.

The MRI technique is non-invasive and offers soft tissue contrast with high spatial resolution [2; 3; 4]. During imaging it is essential to differentiate the region of a diseased tissue from the normal tissue. In order to do so, contrast enhancing agents are used. Herein, SPION as a MRI contrast agent is discussed for potential application within a theranostic agent.
7.2 MRI as imaging technique for detection of cancer

MRI is a widely used pharmaceutical research tool that provides three-dimensional anatomic images with high resolution. Unlike nuclear scanning, conventional radiography or CT, MRI often relies on contrast enhancers to improve inherent contrast between normal and diseased tissue [5]. Much like CT, MRI obtains images of the body in thin slices [6]. It is a non-ionizing imaging method that provides physiological and pathological information about living tissues by measuring water proton relaxation rates [2].

In the early 1970’s Damadian introduce NMR for *in vitro* measurement of relaxation times of tumors and subsequently Mansfield *et al.* developed an application to make imaging feasible in human tissue samples. Early work on breast MRI was reported by El Yousef *et al.* and Ross *et al* in the early 1980’s. MRI has a high spatial resolution (25-100 µm) at high magnetic fields and studies have demonstrated its ability to provide accurate diagnosis and extent of disease. It is safe and does not limit to provide any anatomic information of the subject under scan with amazing clarity and high diagnostic accuracy [7].

7.2.1 Principle of MRI

The basis of the MRI is the relaxation of the water hydrogen nuclei following application of a RF pulse at an applied magnetic field [8]. When an organism or a human body is placed in a strong magnetic field, \( B_0 \), *(Figure 36)*, a net tissue magnetization, \( M_0 \), is produced due to the collective effect of hydrogen
nuclei. However, this is not true for all the hydrogen nuclei, some align in the direction of the magnetic field, others opposite to the direction of magnetic field. The opposite spins cancel each other and the remaining nuclei oriented in the direction of the magnetic field produce a net magnetization, $M_0$.

![Net Magnetization](image)

**Figure 36**: Hydrogen nuclei within the tissue align over magnetic field ($B_0$). Those in the direction of $B_0$ produce a net magnetization ($M_0$) [7].

To obtain an image (**Figure 37**), a RF pulse at a particular frequency is applied, following which the $M_0$ flips away from $B_0$. This flip occurs in a transverse plane (perpendicular to $B_0$) where the magnetization can be measured.
Figure 37: Basic steps of signal generation in MRI [7].

Once flipped, the precessing $M_0$ causes a change in the magnetic flux, linked to a receiver coil, thereby inducing a current in the receiver coil. The amplitude of the current is measured and the AC signal is converted to a digital output. Tissue magnetization in the transverse plane can be measured as loss of magnetization (T2) after the RF pulse flips $M_0$ out of alignment with the external magnetic field, $B_0$.

7.2.2 T2 measurements using MRI

As discussed previously, the MR signal is maximum and measurable just after a RF pulse is applied and the $M_0$ is rotated to a transverse plane, perpendicular to $B_0$. Two events occur following the pulse; 1) Longitudinal magnetization (T1) i.e. recovery of longitudinal relaxation and 2) Transverse magnetization (T2) i.e. loss of transverse magnetization, of which the latter will be discussed further.
In tissue, different hydrogen nuclei have subtle differences in their magnetic field and they precess differently, as a consequence the magnetic dipoles begin to diphase and as a consequence, the transverse magnetization is lost.

![Transverse Signal](image)

**Figure 38:** T2 relaxation signal (Modified from [7]).

The exponential loss of the T2-signal can be observed over time (**Figure 38**). As the protons begin to diphase, the transverse \( M_0 \) decays with time. This dephasing decreases the measurable T2 and is responsible for T2-decay. Water and liquids have very long T2 values, especially those of pathological or diseased tissues such as cancers, which are typically edematous and may contain cystic fluids that have very high T2 values of several hundred milliseconds compared to fat tissue. This indicates that it will take a long time for loss of signal resulting in a bright image. Such images can be homogeneous with surrounding tissues making it hard to visualize any infarct or lesions. For this
reason, contrast-enhancing agents are used to allow better discrimination of the diseased condition.

Without a contrast agent, the water molecules move very rapidly and as a result, each proton from the water molecule experiences a uniform magnetic field causing T2 to be very long. In the presence of a contrast agent, this movement of protons is slowed causing non-uniformity in the magnetic field leading to a decrease in signal and a short T2 value.

7.3 SPION as T2-weighted contrast enhancing agents

The most important role of a MR contrast agent is to allow discrimination between tumor and surrounding normal, but edematous tissue. This discrimination depends on factors such as the tumor neovasculature, which is a prerequisite to passage of the contrast agent across the capillary endothelium and subsequent accumulation in the tumor interstitium [9]. Many MR contrast agents, such as chelated paramagnetic metals, including Gadolinium, Manganese or Dysprosium, have been used as MR positive (T1) contrast agents [10]. Over past four decades, the application of ION in in vitro diagnostics has been practiced and recently there is an increase in investigations with several types of magnetic iron oxides for the application in cancer diagnostics as MR contrast agent [11].

Lately, SPION has received great attention due to its various biomedical applications in MR imaging and image-guided drug delivery. As the name
suggests, SPIONs are hematite with the formula \((\gamma-\text{Fe}_2\text{O}_3)\) or magnetite with the formula \(\text{Fe}_3\text{O}_4\) (\(\text{FeO. Fe}_2\text{O}_3\)) that possess paramagnetic properties. When metal ions or organic radicals have unpaired electrons or highly aligned/single-magnetic domains, they have very large magnetic moments and are known as paramagnetic [12]. Magnetite is more commonly used for biological applications [13] because of its high saturation magnetization (80-100 A·m²Kg⁻¹), which is two orders higher than other iron oxides [14].

7.3.1 SPION preparation and chemistry

There are different methods of SPION synthesis (Figure 39) and the most widely used is the co-precipitation technique. Conventionally, magnetite is prepared by co-precipitation of ferric and ferrous salt solutions by addition of a base. The aqueous mixture of \(\text{Fe}^{3+}\) and \(\text{Fe}^{2+}\) chloride in a 2:1 molar ratio is subjected to oxidation and dehydration at a temperature of 60°C while adding a base having a pH between 9 and 14 under a non-oxidizing, oxygen free
environment, a black color precipitate or magnetite is obtained. The overall reaction may be written as follows [16]:

\[
\text{Fe}^{3+} + \text{Fe}^{2+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O}
\]

The magnetite particles formed have hydrophobic surfaces with a large surface area to volume ratio. In addition they also experience van der Waals and magnetic forces. Due to these interactions, particles tend to agglomerate into large clusters [17]. These clusters can further aggregate due to the magnetic dipole-dipole interaction and increase their aggregation. To avoid such circumstances, these particles are surface coated with surfactants such as oleic acid and stearic acid during the precipitation process and then suspended in organic solvent to be further used as MR contrast agents using suitable nanocarriers such as polymeric micelles [18]. Other methods of preparation such as the microemulsion method, sol-gel method, bulk solution method, gas deposition method and vapor method have also been used [19]. Table 5 shows the comparison of different characteristic features of the iron oxide nanoparticles fabricated though different methods.
### Characteristics of the iron oxide

<table>
<thead>
<tr>
<th>Method</th>
<th>Synthesis of iron oxide nanoparticles prepared through</th>
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<tbody>
<tr>
<td>Aerosol/vapour (pyrolysis)</td>
<td>Gas deposition method</td>
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<td>Bulk solution method</td>
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<td>Sol–gel method</td>
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<td>Microemulsion method</td>
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<td>Size and size distribution</td>
<td>About 5–60 nm with broad distribution</td>
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<td></td>
<td>About 5–50 nm with narrow size distribution</td>
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<td>About 10–50 nm with broad size distribution</td>
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<td>About 20–200 nm with broad size distribution</td>
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<td></td>
<td>About 4–15 nm with very narrow size distribution</td>
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<tr>
<td>Morphology</td>
<td>Spherical</td>
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<td></td>
<td>Spherical</td>
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<td></td>
<td>Spherical (large aggregates)</td>
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<td>Spherical with high porosity</td>
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<td></td>
<td>Cubic or spherical (no aggregation)</td>
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<tr>
<td>Magnetization values</td>
<td>10–50 emu/g with desired magnetic property</td>
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<td></td>
<td>&gt;20 emu/g</td>
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<td>20–50 emu/g with superparamagnetic behaviour</td>
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<td>&gt;30 emu/g with superparamagnetic behaviour</td>
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<tr>
<td>Advantages</td>
<td>High production rate</td>
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<td></td>
<td>Useful for protective coatings and thin film deposition</td>
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<td></td>
<td>Large quantities can be synthesized</td>
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<td></td>
<td>Particles of desired shape and length can be synthesized, useful making hybrid nanoparticles</td>
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<td></td>
<td>Uniform properties and also size of the nanoparticles can be modulated</td>
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<tr>
<td>Disadvantages</td>
<td>Large aggregates are formed</td>
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<td></td>
<td>Require very high temperatures</td>
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<tr>
<td></td>
<td>Uncontrolled oxidation of magnetite to maghemite, diamagnetic contribution</td>
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<td></td>
<td>Product usually contains sol–gel matrix components at their surfaces</td>
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<tr>
<td></td>
<td>Surfactants are difficult to remove, only a small quantities of iron oxide can be synthesized</td>
</tr>
</tbody>
</table>

Table 4: Comparison of various preparation techniques of iron oxide nanoparticles [19].

Since ION are intended for \textit{in vivo} imaging, SPION particles must also be biocompatible. In a study conducted by Weissleder \textit{et al.}, SPION particles were shown to be non-toxic. No acute toxicity or lethality was reported in 18 rats and 12 beagle dogs at the highest dose of 3000 \(\mu\text{mol Fe/kg}\). In addition, sub-acute toxicity studies revealed no mortality or morbidity, that proved SPION have a
higher safety margin than conventional MR contrast agents [20]. Large particles (>200 nm) are usually sequestered by the spleen via mechanical filtration followed by phagocytosis, whereas smaller particles (<10 nm) are rapidly removed through extravasation and renal clearance. Particles 10–100 nm are believed to be optimal for i.v. administration [21]. Lin and colleagues reported that iron oxide particles were non-toxic and eventually broken down to form blood hemoglobin [22]. Alternatively, when endocytosed, they are broken down by an array of hydrolyzing enzymes at low pH via endogenous iron metabolic pathways.

7.3.2 Applications of SPION

SPION’s offer a wide range of biomedical applications (Figure 40). For example, Nitin et al. 2004, reported delivery of MION as a MR contrast agent for intracellular measurement of gene expression in deep tissues [23]. Similar studies were carried out by Weissleder and coworkers, who showed evidence that transgene expression could be visualized by non-invasive, whole body, in vivo MR imaging [24].
Polymeric nanoparticles encapsulating SPION provide several advantages over traditional contrast agents, because the high payload of the contrast agent significantly improves detection for MRI, as originally suggested by Mulder et al [25]. In a recent study, Sawant et al. reported SPION particles that were encapsulated within PEG-PE-based polymeric micelles and targeted with the anti-cancer NS-specific mAb 2C5, which recognizes a variety of tumors but not normal cells to serve as the MRI contrast agent for enhanced imaging [26]. Hence, SPIONs may prove to be an ideal candidate to be used as MR contrast agents in theranostic preparations.
7.4 Theranostics and rationale to overcome current chemotherapy challenges

There are differences in molecular heterogeneity between cancers of the same type, between the primary tumor and its metastatic foci and between the cells that constitute an individual tumor. Cancer cells are present in the tumor interstitium, intertwined between blood vessels and collagen rich matrix and occupy less than half the volume of a tumor [27]. Tumor heterogeneity, resistant phenotypes of cancer cells and molecular diversity remain formidable challenges in the treatment of this disease. It is important to realize that cancer therapy requires the ability to address these challenges.

The "one-size-fits-all" approach to cancer therapy has failed to address these issues which constitute a need for more personalized form of therapy. Due to the marked diversity within the cancer types and patients, the best treatments have to be diverse and include minimal toxicity [28]. Moreover, characterization of tumors cannot be carried out simply by analysis of a physical biopsy. The diversity of cells can make such sampling prone to error, which in turn will fail to give a full description of cancer phenotype and the extent to which it exists inside a tumor. In addition, a biopsy also fails to characterize the cancer cells that have metastasized to other locations.

To overcome this challenge, the whole body must be subjected to molecular characterization, which should be performed through an efficient imaging system [4].
Theranostics is a system that integrates diagnostics with therapeutics. The main strategy of this field is to associate the therapeutic test with a diagnostic entity that would help to identify if the patient is most likely to be helped or harmed by a given medication, and accordingly manage drug therapy based on the test results. Since the diagnostic moiety has the ability to define the disease state, a theranostic agent would have “the ability to effect therapy or treatment of a disease state” [29]. Theranostics aims to provide information about the response to a treatment that would help to approach each patient as a unique biological entity [30], hence changing paradigm of “one-size-fits-all” drug therapy.

Patients with serious diseases, such as cancer, and clinicians will widely accept theranostic, since it bridges the gap between the diagnosis, prognosis, treatment and monitoring of disease. Theranostics is much different from traditional therapy in that it is based on recent technologies and superior diagnostic tools and is now receiving much attention as pharmacogenomics moves to applications at the point of patient care. [31].

Theranostics offers drug companies a tool to rapidly introduce newer and more effective chemotherapeutics into clinics. This can result in significant drug cost saving for the healthcare system and increase profits for the pharmaceutical drug companies. This approach would help to reduce risk and cost, potentially speeding market admission and ultimately enhancing commercial success of a medication [32]. If companies use theranostics to increase the safety or efficacy, they may be able to win regulatory approval for more products, in turn this can
help overcome industry’s resistance to change the blockbuster drug model, leading to the development of theranostics in areas of oncology and infectious diseases [33].

It is the next generation of therapeutic system that enables early detection of cancer and other disease states simultaneously giving freedom to monitor and treat cancer with minimal toxicity.

7.5 Current theranostic agents

A number of elegant studies have been published on the use of theranostic agents. A few examples of theranostic nanomedicine used are highlighted. Lu and coworkers [34] described therapeutic and diagnostic functionalities in a polymer conjugate system. In this study, PGA was the polymer carrier, Gd-DO3A was used as a $T_1$ contrast agent and Mce6 was used as a photosensitizer for PDT. They PEGylated the nanocarrier system PEG-PGA-(Gd-DO2A)-Mce6 to prolong blood circulation and investigated the pharmacokinetics of the polymer conjugate using MDA-MB-231 breast tumor xenografts using $T_1$-weighted MRI. Images of the tumor treated with PEG-PGA-(Gd-DO2A)-Mce6 showed contrast enhancement and higher tumor accumulation compared to a non-pegylated formulation. In addition, MRI-guided PDT therapy was carried out on tumor-bearing mice. Tumor size decreased in the first 30 days following treatment with PEG-PGA-(Gd-DO2A)-Mce6 in comparison with tumors treated with PEG-PGA-(Gd-DO2A), which showed much less response.
In a different study, Gao and colleagues [35] formulated polymeric micelles with PEG-PLA encapsulating clusters of SPION and DOXO, which were targeted with $\alpha_v\beta_3$ integrins via cyclic RDG moieties. SPION led to enhancement of T2 relativity. Effective targeting was achieved to $\alpha_v\beta_3$ integrins receptors and significant cytotoxic response to tumor (SLK) endothelial cells was achieved. Later on, MR imaging and anti-tumor efficacy studies were carried out in subcutaneous A549 non-small cell lung tumor-bearing mice [36]. cRDG-PEG-PLA-SPIO micelles showed significant tumor growth inhibition compared to non-targeted micelles and simultaneously T$_2$-weighted MRI imaging was carried out, that showed clear accumulation of cRDG-PEG-PLA-SPIO micelles in A549 tumors.

Bhatia and his group [37] investigated numerous methodologies for targeted delivery of siRNA using QD. While many of these theranostic agents have been reported, only a few of them have been investigated in vivo. However, theranostic agents possess great potential to improve the diagnosis and treatment of cancer, since they enable modification of different functionalities including those needed for targeting, imaging and therapy within one platform.

### 7.6 Conclusion

This integration of a diagnostic imaging agent with a suitable therapeutic intervention in one nano-scaffold is a solution to the challenge of current chemotherapy. This real-time, adaptive treatment and imaging is the ultimate and essential component provided by a theranostic nanomedicine to address the
adaptive resistance of cancer cells. Such a design permits the diagnosis, simultaneous monitoring and treatment necessary to achieve personalized medicine.
References:


[35] N. Nasongkla, E. Bey, J. Ren, H. Ai, C. Khemtong, J.S. Guthi, S.F. Chin, A.D. Sherry, D.A. Boothman, J. Gao, Multifunctional polymeric micelles as cancer-


CHAPTER 8. PEG-PE BASED MICELLAR THERANOSTIC AGENT FOR SIMULTANEOUS DETECTION AND THERAPY OF CANCER

8.1 Introduction

The term theranostic was coined about a decade ago and first used to describe diagnostic tests developed to guide personalized therapies [1; 2; 3].

Although a variety of theranostic agents are now available, very few encapsulating SPIONs have been reported such as, PTX conjugated to Au nanoparticles [4] or to TNF coupled to PEGylated Au nanoparticles [5; 6; 7].

Here, a PEG-PE-based polymeric micellar theranostic agent with SPION as the diagnostic component and PTX as the chemotherapeutic component is described. From the family of taxanes, PTX has been used against a broad range of cancer types [8; 9; 10]. The aim of this study was to formulate, optimize and prepare a theranostic agent incorporating these two components in quantities sufficient for the assessment of its functional activity without an effect of their individual properties. The cytotoxicity of the preparation against 4T1 and B16F10 cancer cells was evaluated along with imaging studies using MRI.
8.2 Materials and Methods

8.2.1 Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000] (PEG2000–PE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron (III) acetylacetonate [Fe(acac)₃] (99.9+%), 1,2-hexadecanediol (tech. 90%), oleic acid (tech. 90%), oleylamine (tech. 70%), phenyl ether (C₁₂H₁₀O), ferric chloride (FeCl₃.6H₂O), potassium thiocynate (KSCN), phosphotungstic acid (PTA), paclitaxel (PTX) and hydrogen peroxide (H₂O₂) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). HEPES buffer saline (HBS) pH 7.4 and was prepared in deionized (DI) water.

Cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell culture media, Dulbecco’s Modified Eagle’s medium (DMEM), Hanks’ balanced salt solution (HBSS), fetal bovine serum (FBS), and penicillin/streptomycin stock solutions were purchased from CellGro (Kansas City, MO, USA). Reverse osmosis-derived and deionized (DI) water were used in all experiments.

8.2.2 Synthesis of SPION

SPION were provided by Dr. Sridhars’ Lab, Northeastern University. Briefly, they were synthesized using a high temperature thermal decomposition method as described [11; 12]. Briefly, 2 mmol iron (III) acetylacetonate [Fe(acac)₃] was mixed with 10 mmol 1,2-hexadecanediol in the presence of 6 mmol oleic acid and oleylamine in 20 ml of phenyl ether and stirred constantly
with a magnetic stirrer at room temperature under argon. This mixture was heated to 473 °K for 30 min followed by further heating at 538 °K for an additional 30 mins. After cooling to room temperature, the iron oxide nanoparticles were precipitated by addition of ethanol. The precipitated SPION were collected, dried and redispersed in organic solvents including toluene, chloroform or hexane.

8.2.3 Preparation of micelles

Theranostic micelles were prepared by the lipid film rehydration method [13]. Briefly, 10 mg of PEG–PE in chloroform, 1 to 5 % w/w of SPIONs in chloroform and 1.5 % w/w PTX (1mg/ml methanol) were mixed together in a glass vial. The organic solvents were removed by rotary evaporation or by rotating under N$_2$ gas. Residual solvents were removed by freeze-drying overnight. The dry film obtained was rehydrated with 1 mL of 10 mM HBS, pH 7.4 and vortexed vigorously to produce micelles. Excess non-incorporated drug and/or contrast agent were separated by centrifugation (13,000 x g) for 10 mins to provide PTX-SPION-loaded PEG–PE (theranostic) micelles in the supernatant. For comparison, controls such as SPION-PEG–PE (SPION) micelles, PTX-PEG–PE (PTX) micelles and PEG–PE (plain) micelles were also prepared by the lipid film rehydration method.
8.2.4 Size measurements

Micelle size measurement and distribution analysis for all formulations was done by dynamic light scattering using a Coulter® N4 plus submicron particle system (Coulter Corporation, Miami, FL, USA). The micelle suspension was diluted with 5mM HBS buffer pH 7.4 until the sample provided a light scattering intensity of $5 \times 10^4$ to $1 \times 10^6$ counts per second. For each sample, particle size distribution measurements were carried out in triplicate.

8.2.5 TEM of theranostic agent

The morphology of micelle formulations was obtained by TEM. Briefly, 5 μL of a micelle sample was placed on a circular copper grid and allowed to sit for 30 sec. Excess micelles were removed with filter paper. To this, 5 μL of 2 % PTA was added for negative staining and the excess was removed by blotting. The grid was examined with a JEOL JEM-1010 electron microscope (JEOL USA, Inc., Peabody, MA).

8.2.6 Quantitative determination of iron content

The SPION present in the micelles were characterized by their iron content. A modified version of the method described [14; 15] was used for this purpose. Briefly, an aliquot of SPION-containing micelles was diluted in acetonitrile to disrupt the micelles and release SPION particles. To this solution, 1ml of 5 % KSCN (prepared in DI water), 10 μl of $H_2O_2$ and 100 μl of concentrated HCl was added and vortexed gently. After 5 mins the blood-red
solution was quantified by measurement of its absorbance at 480 nm using a UV-VIS spectrophotometer (Shimadszu, Japan). Measurements were carried out before and after centrifugation. A standard curve of ferric chloride (FeCl$_3$.6H$_2$O) in HBS pH 7.4 was used to compare and evaluate the iron content in SPION micelles using similar conditions. All measurements were made in triplicate.

**8.2.7 Determination of paclitaxel content**

The PTX in the micellar phase was measured with a reverse phase-HPLC (D-7000 HPLC system, Hitachi, Japan) using a Spherisorb ODS2 column, 4.6 mm x 250 mm (Waters, Milford, MA, USA). A clear aqueous dispersion of PTX-containing micelles was diluted in a solvent mixture of mobile phase (acetonitrile:water (6:4)). The solution was centrifuged at 13,000 rpm for 10 min to remove any SPION (in the case of theranostic micelles) and 10 µl of supernatant was injected into the HPLC system. The column was eluted with mobile phase at 1.0 ml/min. PTX was detected at 227 nm. All samples were measured in triplicate. PTX loading was determined from a standard curve obtained under similar conditions.

**8.2.8 Loading efficiency of paclitaxel and SPION**

The incorporation efficiency was calculated for all micellar formulations based on the results obtained from SPION and PTX assay. The percent incorporation efficiency for SPION was calculated using equation (1) and PTX using equation (2).
% SPION incorporation efficiency = \[ \frac{\text{Absorbance of SPION after centrifugation}}{\text{Absorbance of SPION before centrifugation}} \times 100 \] (1)

% PTX incorporation efficiency = \[ \frac{\text{AUC of PTX after centrifugation}}{\text{AUC of PTX before centrifugation}} \times 100 \] (2)

8.2.9 SQUID Measurement

This analysis was carried out at Dr. Sridhars’ Lab, Northeastern University. Magnetic measurements were carried out on a Quantum Design MPMS XL-5 SQUID magnetometer. Briefly, aqueous samples were air dried on 0.6 x 0.6 cm silicon wafers and introduced into the instrument. The magnetization was measured at 300°K as a function of external magnetic field at 10,000 Oe.

8.2.10 Magnetic Resonance Imaging

MRI was carried out in Dr. Craig Ferris’ lab, Northeastern University. MR images were obtained at ambient temperature (~ 298°K) using a Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, MA, USA) and a 20-G/cm magnetic field gradient insert (ID =12cm) capable of a 120-µs rise time (Bruker). Briefly, micelles samples were aliquoted in a 96-well plate and covered with plastic film. The plate was placed in the magnet, and radiofrequency signals were sent and received with quad electronics (INSL, Leominster, MA, USA). A T2-weighted image was acquired using a multi-slice multi-echo (MSME) pulse sequence. The echo time (TE), was 11 ms, and 16 echoes were acquired during imaging with a recovery time (TR) of 2500 ms. Images were acquired with a field...
of view (FOV) = 4 × 4 cm², data matrix = 256 × 256, slice thickness = 2 mm with 2 signal averages.

8.2.11 T-2 relaxation measurements

T2 relaxation values were obtained from a ROI in areas of the T2-weighted image obtained using ParaVision 5.1 software. The T2 values were automatically computed using the equation; \( y=A+C\times\exp(-t/T2) \) (S.D. weighted). Where, \( A \) = absolute bias, \( C \) = signal intensity, \( t \) = echo time and \( T2 \) = spin-spin relaxation time.

8.2.12 Cell cultures

Murine mammary carcinoma 4T1 and murine melanoma B16F10 cells were grown and maintained in DMEM culture medium at 37°C, 5% CO₂. DMEM was supplemented with 10% FBS, 1 mM Na-pyruvate, 50U/mL penicillin, and 50 µg/ml streptomycin.

8.2.13 Cytotoxicity assay

Cytotoxicity of micelles was evaluated using a CellTiter 96® AQueous Non-reactive Cell proliferation Assay (Promega, Madison, WI USA). Briefly, 4T1 or B16F10 cells were plated at a density of 5 x 10³ cells per well in 96-well plates (Corning Inc., Corning, NY, USA). After 24 hours incubation at 37°C, 5% CO₂, the medium was replaced with medium containing free drug dissolved in methanol at concentrations ranging from 4.68 to 600 ng/ml with PTX-PEG–PE micelles, theranostic micelles, SPION-PEG-PE micelles or empty PEG-PE micelles. As a
supplementary control, methanol was used at a concentration of 0.012% v/v. After an additional 24 and 48 hour incubation at 37°C, 5% CO₂, micelle and free-drug containing media were removed. Each well was washed twice with DMEM and cell viability determined by measurement of the fluorescence intensity with excitation/emission wavelengths of 525/590 nm using a BioTek Synergy HT multimode microplate reader (BioTek Instruments, Winooski, VT, USA).
8.3 Results

8.3.1 Formulation optimization and particle characterization

Figure 41: Schematic illustration of a theranostic agent.

PTX and SPION were solublized in the micelle core according to the scheme (Figure 41). Initial optimization of the formulation was carried out by varying the % by weight of SPION to the weight of PEG-PE (SPION micelles). For our final theranostic formulation, we chose a mix of 3% w/w SPION and 1.5% w/w PTX for the preparation of the theranostic agent based on the incorporation efficiency of SPION and the T2-weighted imaging results.

The average particle size of formulations (1 to 5% w/w SPION micelles) was 16.1 ± 0.3 nm. Addition of 1.5% w/w PTX to these formulations did not affect their particle size.
Figure 42: Micelle size and size distribution of (a) empty PEG-PE micelles; (b) PTX-loaded PEG-PE micelles; (c) SPION-loaded PEG-PE micelles and (d) co-loaded PTX/SPION PEG-PE theranostic micelles. (n=3)

The average size (Figure 42) for empty PEG-PE micelles was about 16 nm in diameter, with a narrow size distribution (12 – 20 nm). Loading micelles with SPION, PTX or both SPION and PTX had little effect on the average size or size distribution pattern.
8.3.2 TEM analysis

Figure 43: Transmission electron micrographs of (a) plain micelles; (b) PTX-micelles; (c) SPION-micelles and (d) PTX/SPION PEG-PE theranostic micelles (magnification: 75,000x).

Negative staining with 2% PTA confirmed the presence of SPIONs in SPION-micelles and theranostic micelles (Figure 43). Both formulations had a dark core of SPION in the micelle, encircled by an unstained micellar corona seen as a halo with a thickness of 2-3 nm. Plain micelles and PTX-micelles lacked the dark center core. Images were captured at a magnification of 750,000x. The diameter of the micelles determined by TEM ranged from 10 to 15 nm. These images confirmed that the incorporation of SPION, PTX, or both within the micelle core had no effect on micelle size.

8.3.3 Drug loading of micelles

<table>
<thead>
<tr>
<th>PEG&lt;sub&gt;2000&lt;/sub&gt;PE micelle Formulation</th>
<th>Final Concentration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paclitaxel (mg/ml)</td>
<td>SPION (Fe) (mg/ml)</td>
</tr>
<tr>
<td></td>
<td>mean ± S.D.</td>
<td>mean ± S.D.</td>
</tr>
<tr>
<td>Plain</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ 1.5% w/w Paclitaxel</td>
<td>0.144 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>+ 3% w/w SPIONs</td>
<td>-</td>
<td>0.06 ± 0.001</td>
</tr>
<tr>
<td>+ 1.5% w/w Paclitaxel + 3% w/w SPIONs</td>
<td>0.144 ± 0.000</td>
<td>0.05 ± 0.003</td>
</tr>
</tbody>
</table>

Table 5: Paclitaxel and iron content of PEG-PE micelle preparations, (n=5, mean ± S.D.).
The amount of PTX solublized in the micelles was determined by the reversed phase HPLC. The calibration curve for the quantification of PTX over the concentration range of 0-100 µg/mL had a correlation coefficient (R²) of 0.9993 (Figure 44).

![Graph showing the standard curve of PTX](image)

**Figure 44:** Standard curve of PTX, (n=3, mean ± S.D.).

Relative to the initial amount of PTX (0.150 mg/ml), about 96 % incorporation was achieved in PTX-micelles (Table 6). When compared to theranostic micelles, no difference in PTX loading was observed in the presence of SPION.
8.3.4 Iron content

**Figure 45:** Iron content and % incorporation efficiency of SPION in PEG-PE micelles, (n=3, mean ± S.D.).

The micelles were further characterized for their iron content, since the micelles must possess sufficient amount of the SPION load in order to respond the external magnetic field. The iron content increased nearly linearly as SPION concentration was increased from 1 to 5% (Figure 45). A small decreasing trend in % incorporation efficiency was observed as SPION increased. However, the iron content in the theranostic micelles showed no significant differences when compared to the iron content of SPION-micelles (Table 6), indicating that the micelles just as fully solubilized the contrast agent in presence of the drug. The decrease in incorporation efficiency with increase in SPIONs concentration (1 %w/w and 1.5 %w/w) can be explained by the limited hydrophobic area available for SPION solubilization. Iron content was calculated from a standard curve plot using FeCl₂ with a $R^2$ of 0.9999 (Figure 46).
**Figure 46**: Standard curve of FeCl₂, (n=3, mean ± S.D.).

### 8.3.5 Magnetic susceptibility using SQUID

**Figure 47**: SQUID analysis.

SQUID analysis was carried out for each preparation to determine if the SPION load was sufficient to respond to external magnetic field (Figure 47). The
analysis was carried out at 300°K to determine their magnetic susceptibility. A typical hysteresis curve was observed with high saturation magnetization values for SPION-micelles (2.0 × 10⁻⁵ emu) and theranostic micelles (1.5 × 10⁻⁵ emu) which was higher than plain micelles (0.25 × 10⁻⁵ emu) and PTX-micelles (0.5 × 10⁻⁵ emu). This result suggested that the SPION-micelles and theranostic micelles exhibited superparamagnetic behavior due to the absence of a hysteresis loop. Additionally, MRI was used to observe the T2-weighted images of various concentrations of SPION-micelles, with/without PTX and the final micellar theranostic agent.

8.3.6 Magnetic resonance imaging and T-2 relaxation time

The formulations containing SPION produced a darker image compared to the controls and DI water used as a reference (Figure 48a). The addition of the PTX (Figure 48b) did not affect the T2-weighted imaging. The contrast observed was SPION concentration-dependent in both cases. Further, the T2 relaxation times, (Figure 48c), confirmed that increasing the amounts of contrast agent decreased the relaxation time. This effect was also observed when PTX was added to SPION-micelles.
Figure 48: T2-weighted MR image of formulations (a) with or (b) without 1.5 % w/w PTX; and (c) T2 relaxation time of SPION micelles with and without PTX, (n=3, mean ± S.D.).
Figure 49: (a) T2-weighted MR images and (b) T2 relaxation time of the SPION/PTX theranostic agent, (n=3, mean ± S.D.).

With double-loaded theranostic micelles, (Figure 49a), contrast properties were comparable to those of drug-free SPION-micelles. The T2 relaxation time of the micelles, (Figure 49b), decreased significantly with incorporation of SPIONs and remained unchanged in the presence of PTX.
8.3.7 Cytotoxicity

Next, the cytotoxicity studies of SPION-micelles (Figure 50) and theranostic micelles (Figure 51) were carried out against cancer cells (4T1 and B16F10 cells) in vitro using a cell viability assay. SPION-micelles produced no
significant decrease in cell viability after incubation for 24, 48 or 72 hours in either cell line.

Figure 51: *In vitro* cytotoxicity of Plain, SPION, PTX, theranostic micelles and free drug (PTX) at (A,C) +24 hours and (B,D) + 48 hours incubation. Data represent mean ± S.D. (n=3) (*) p < 0.05 between PTX-micelles and free PTX; (#) p < 0.05 between theranostic micelles and free PTX (Student’s t-test).

Likewise, no significant cytotoxicity was observed with plain PEG-PE micelles. However, in the presence of free PTX, PTX-micelles and double-loaded theranostic micelles, a 20 % decrease in cell viability was observed in both cell
lines after 24 hours. SPION-micelles were not toxic at a concentration of 0.015 to 0.25 µg/mL. No differences in cytotoxicity were observed among the free PTX, PTX- micelles, and theranostic micelles. After 48 hours of incubation, a clear difference between free PTX and PTX-micelles and theranostic micelles was observed. The micellar preparations led to increased toxicity. A 30 to 40% decrease in cell viability was observed with free drug in 4T1 and B16F10 cells and a 60 % decrease in cell viability was seen with PTX-micelles and double-loaded theranostic micelles in both cell lines. Additionally, methanol by itself did not show any cytotoxicity at 0.012 % v/v concentration, similar to the concentration present in free PTX.
8.4 Discussion

Interest has grown in the combination of a diagnostic and a therapeutic agent to form a theranostic system with the aim of improved cancer treatment and detection of cancer. Using a contrast agent as a part of the theranostic preparation allows imaging to be performed before, during and after the treatment and thereby provides for a more personalized form of chemotherapy, by bridging the gap between cancer diagnosis and therapy [16]. For example, some theranostic agents are prepared by chemical immobilization of drug molecules, such as MTX on PEGlyated ION via an amide bond [17; 18; 19] or electrostatic coupling of DOXO to a negatively charged site on anti-biofouling thermally crosslinked PEGlyated ION [20; 21], and linking of PTX through a phosphodiester moiety [22]. DOXO and PTX were also partitioned in pluronic-stabilized nanoparticle-containing ION [23]. These modifications are restricted to surface chemistry via conjugation or covalent coupling.

Very few studies have reported encapsulation of both a contrast and a chemotherapeutic agent. Early studies described the use of polymeric micelles of PEG-PLA encapsulating water-soluble DOXO and SPION along with a cancer-targeting cyclic-RGD moiety [24]. Recently, encapsulation of hydrophobic ION into the core of targeted polymeric vesicles was observed [25]. However, the DOXO was conjugated with a pH-sensitive bond. Given that many chemotherapeutic agents possess poor water solubility, additional techniques
have been used to encapsulate these agents in hydrophobic compartments of various drug carriers and contrast agents such as ION.

For instance, ION stabilized using surfactants such as oleic acid or stearic acid are frequently dispersed in organic solvents such as hexadecane [26] and could also be solublized in a carrier’s hydrophobic compartment. Some water soluble ION have also been reported [27; 28] that are solublized in the hydrophilic regions of some polymeric systems. In this study, both PTX and SPION were encapsulated simultaneously in polymeric PEG-PE micelles and characterized.

Initial optimization of the formulation was carried out by varying the % by weight of SPION to the weight of PEG-PE (SPION micelles). These formulations (1 to 5 % w/w SPION micelles) had an average particle size of 16 nm. Other micellar systems prepared with ION of different particle sizes, and with varying micelle composition and SPION load had sizes ranging from 17 to 110 nm [29]. To formulate the theranostic agent, we incorporated 1.5 %w/w of PTX into each SPION-containing micelle formulation [30]. The addition of PTX did not affect the particle size. We have chosen a mix of 3% w/w SPION with 1.5% w/w PTX for preparation of the theranostic agent based on the incorporation efficiency of SPION and the T2-weighted imaging results. No significant difference in particle size was observed when plain PEG-PE micelles were loaded with either SPION, PTX or both suggesting that significant quantities of SPION and PTX could be
entrapped within the micelle core without affecting the micelle size (Figure 42). Small size promotes their accumulation in various pathological zones including tumors via the EPR effect [31; 32; 33].

The presence of SPION in SPION-micelles and theranostic micelles was confirmed by TEM analysis using negative staining with 2% PTA, similar to what was reported previously [34; 35]. The SPION-containing micelles produced a corona around the dark core of ION due to its electron-density, suggesting the incorporation of SPION particles within the micelle (Figure 43). Plain micelles and PTX-micelles lacked the dark core. The size 10 to 15 nm of the micelles by TEM confirmed that the incorporation of SPIONs, PTX, or both within the micelle core had no effect on micelle size.

Next, the iron content was determined in each SPION formulation. Samples were treated with strong acids, HCl and H₂O₂, to reduce any ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions and to promote reaction with SCN⁻ from KSCN to form an intense blood red colored iron (III) thiocyanate ion complex (FeSCN^{2+}). A similar technique was employed, with some modifications, for the estimation of the cell-bound iron [36]. We observed a trend towards saturation, indicating the limitation of solublization of SPION within the micelle core as evidenced by the incorporation efficiency that decreased as the SPION increased (Figure 45). This tendency can be explained by the limited hydrophobic area available for SPION solublization, which is in good agreement with other data indicating similar
behavior as the SPION concentrations increased [37]. However, no difference in the iron content of the theranostic micelles and SPION-micelles was observed, indicating that the micelles just as fully solublized the contrast agent both in the presence and absence of the drug. Similarly, when PTX loading was compared between theranostic micelles and PTX micelles, the presence of SPION had no significant effect on PTX incorporation (Table 5).

The SPION load in micelles was sufficient to generate a response to an external magnetic field as observed from the SQUID analysis (Figure 47). Magnetization curves (M vs H loop) at 300 °K showed high saturation magnetization of SPION-micelles and theranostic micelles that can be attributed to the presence of the SPION, as reported by others [38; 39; 40]. This also indicates that SPION are completely encapsulated within the inner core of the micelles, which is in good agreement with the TEM results. Plain micelles and PTX-micelles showed a pattern similar to diamagnetic materials with minimal background. The absence of the hysteresis loop in case of SPION-micelles and theranostic micelles confirms the superparamagnetic behavior of SPION and their potential usefulness for MRI application.

Further, MRI was used to observe the T2-weighted images of various concentrations of SPION-micelles, with/without PTX and with the final micellar theranostic agent (Figure 48). ION typically generate intravoxel dephasing, resulting in decreased signal and enhance the T2-weighted contrast image. The
T2-weighted MR images of formulations containing SPION micelles clearly demonstrated loss of the signal intensity in a concentration-dependent fashion at 1 to 5% w/w SPION. Similarly, other groups reported that an increase in the ION from 16.25 uM to 1040 uM in DSP-PEG5K micelles increased the signal intensity observed from the T2-weighted image [29]. Importantly, similar results were observed when 1.5%w/w PTX was added to SPION-containing micelles, indicating that the addition of PTX did not affect the MRI or T2 relaxation parameters (Figure 49). This result suggested an optimized formulation of our theranostic agent with 1.5%w/w of the PTX and 3% of SPION.

From the T2-weighted MR imaging, it was evident that theranostic and SPION-micelles drastically reduced the T2 signal intensity, confirming the presence of SPION in the theranostic agent and its good contrast properties. Most importantly, the signal intensity from SPION-micelles and theranostic micelles was similar without any effect of PTX load. T2-weighted images had a high T2 relaxation for plain micelles and in formulations without contrast agent (averaging around 500 ms). This is due to the hydrogen atoms in the water that move rapidly and experience a uniform magnetic environment causing the T2 to be very long. However, in the presence of the contrast agents, SPION-micelles and theranostic micelles, the T2 relaxation shortened to 20 ms without any interference of PTX in the MRI signal (Figure 49).
Some concern has been associated with the possible toxicity of SPION. SPION alone at 0.005 to 0.8 mg/ml did not influence cell viability of different cancer cell lines, including MCF-7 [40], LNCap and PC3 [41]. However, at much higher SPION concentrations (500 to 1000 mg/mL), cell viability of MDA-MB-435 decreased to about 40% after 3 days [39]. At the SPION concentration reported here (0.015 to 0.25 µg/mL), no decrease in viability of 4T1 or B16F10 cells occurred over a period of 72 hours suggesting little likelihood of direct SPION toxicity (Figure 50). With theranostic micelles containing PTX, free PTX and PTX-micelles however, the cell viability decreased compared to plain micelles and SPION micelles at 24 hours with no clear viability differences among free PTX, PTX-micelles and theranostic micelle treatment (Figure 51). After incubation for 48 hours, the viability decrease was even more pronounced. It is important to note that no difference in theranostic micelles and PTX-micelles was observed indicating no effect of the co-loaded SPION on the toxicity of the micellar PTX.

8.5 Conclusion

A PEG-PE-based micellelar theranostic agent that accommodates PTX and SPION was prepared and optimized within a micelle core without any alteration in micellar properties or the functional activity of either incorporated agent. This theranostic showed excellent T2-weighted contrast in MRI and definite cytotoxicity against 4T1 and B16F10 cancer cell lines. This theranostic preparation was then tested for its application in vivo.
References:


CHAPTER 9. T2-WEIGHTED CONTRAST PROPERTIES AND TUMOR APOPTOTIC ACTIVITY OF LIPID-BASED THERANOSTIC AGENT IN VIVO USING 4T1 AND B16F10 TUMOR MODELS

9.1 Introduction

A theranostics agent that incorporates both, PTX and SPION within a single PEG-PE-based micelle was described previously in chapter 8.

The in vitro observations suggested that our micellar theranostic agent was suitable for in vivo applications. Here, a theranostic agent with SPION and PTX and its ability to produce a strong T2-weighted contrast and simultaneous anti-tumor effect on melanoma and breast tumor models in vivo is described and evaluated.
9.2 Materials and Methods

9.2.1 Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000] (PEG2000–PE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron (III) acetylacetonate [Fe(acac)₃] (99.9+%), 1,2-hexadecanediol (tech. 90%), oleic acid (tech. 90%), oleylamine (tech. 70%), phenyl ether (C₁₂H₁₀O), ferric chloride (FeCl₃.6H₂O), potassium thiocyanate (KSCN), paclitaxel (PTX) and hydrogen peroxide (H₂O₂) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Phosphate buffer saline (PBS) pH 7.4 was prepared with deionized (DI) water.

Cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell culture media, Dulbecco’s Modified Eagle’s medium (DMEM), Hanks’ balanced salt solution (HBSS), fetal bovine serum (FBS), and penicillin/streptomycin stock solutions were purchased from CellGro (Kansas City, MO, USA). Reverse osmosis-derived and DI water was used in all experiments.

9.2.2 Preparation of micelles

Theranostic micelles were prepared by the lipid film rehydration method [1]. Briefly, 10 mg of PEG–PE in chloroform, 3 % w/w of SPIONs in chloroform and 1.5 % w/w PTX (1mg/ml methanol) were mixed together in a glass vial. The organic solvents were removed by rotary evaporation or by rotating under N₂ gas. Residual solvents were removed by freeze-drying overnight. The dry film
obtained was rehydrated with 0.15 mL of 10 mM HBS, pH 7.4 and vortexed vigorously to produce micelles. Excess non-incorporated drug and/or contrast agent were separated by centrifugation (13,000 x g) for 10 mins to provide PTX-SPION-loaded PEG–PE (theranostic) micelles in the supernatant. For comparison, controls such as SPION-PEG–PE (SPION) micelles, PTX-PEG–PE (PTX) micelles and PEG–PE (plain) micelles were also prepared by the lipid film rehydration method.

9.2.3 Size measurement and characterization of micelles.

Micelle size measurement and distribution analysis for all formulations was done by dynamic light scattering using a Coulter® N4 plus submicron particle system (Coulter Corporation, Miami, FL, USA) at a light scattering intensity of $5 \times 10^4$ to $1 \times 10^6$ counts per second. For each sample, particle size distribution measurements were carried out in triplicate. Micelles were also characterized for PTX and SPION loading as described in chapter 8.

9.2.4 Cell cultures

The murine mammary carcinoma 4T1 and murine melanoma B16F10 cells were grown and maintained in DMEM culture medium at 37°C, 5% CO₂. DMEM was supplemented with 10% FBS, 1 mM Na-pyruvate, 50U/mL penicillin, and 50 µg/ml streptomycin.
9.2.5 Animal models

All animal experiments were conducted as per the animal protocol approved by The Division of Laboratory Animal Medicine and Northeastern University – Institutional Animal Care and Use Committee. We used two different animal models. Approximately $1.5 \times 10^5$ 4T1 and B16F10 cells (100 µl PBS pH 7.4) were inoculated in 6-8 weeks-old female Balb/C mice and 6-8 weeks-old male C57 mice respectively by the subcutaneous injection into the left flank. Prior to injection of cells, mice were anesthetized with 1.5% isoflurane and depilated. Fourteen days after tumor inoculation, when the tumor volumes reached ~ 150 to 200 mm$^3$, the mice were injected intratumorally with different formulations equivalent to 5mg/kg of PTX. After 48 hrs, the tumors were harvested, embedded in tissue freezing media and stored at −80 °C for further analysis.

9.2.6 T-2 weighted in vivo MRI.

MR images were obtained before and immediately after the intratumor injection of the formulation. MRI was carried out at ambient temperature (~ 298°K) using a Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, MA, USA) and a 20-G/cm magnetic field gradient insert capable of a 120-µs rise time (Bruker). Mice were placed in the magnet under constant flow of 2% isoflurane for the duration of imaging. Respiration rate was monitored throughout the scan.

Radiofrequency signals were sent and received with the quad electronics (INSL, Leominster, MA, USA). A T2-weighted image was acquired using a multi-
slice multi-echo (MSME) pulse sequence. The echo time (TE), was 22 ms, and 16 echoes were acquired during imaging with a recovery time (TR) of 2500 ms. Images were acquired with a field of view (FOV) = 4 × 4 cm$^2$, data matrix = 256 × 256, slice thickness = 1 mm with 2 signal averages.

**9.2.7. Microscopic analysis of tissue samples**

Tumor tissues were cryo-sectioned as 4-μm-thick sections using a microtome and mounted onto glass slides (Superfrost Plus®, ThermoFisher Scientific, Waltham, MA, USA). Sections were stained with the Terminal Deoxynucleotidyl Transferase BiotindUTP Nick End Labeling (TUNEL) assay for apoptosis using the manufacturers' protocol. SPION particles were identified in the tumor tissue using a HT20 Iron Staining kit (Sigma-Aldrich, St. Louis, MO, USA).
9.3 Result

9.3.1 Particle characterization

The average particle size of plain micelles was 15 ± 3 nm. PTX, SPION and theranostic micelles showed no difference in size compared to plain micelles.

9.3.2 T2 weighted MRI imaging of mice tumor models

![MRI images of mice bearing 4T1 tumor. T-2 weighted images were taken of mice before (A,C) and after injection of SPION-micelles (B) and theranostic micelles (D). Arrows indicate tumor area.](image)

**Figure 52:** MRI images of mice bearing 4T1 tumor. T-2 weighted images were taken of mice before (A,C) and after injection of SPION-micelles (B) and theranostic micelles (D). Arrows indicate tumor area.
The imaging capability of theranostic micelles was compared to SPION micelles \textit{in vivo}. Following intratumoral injection, both breast (Figure 52) and melanoma (Figure 53) tumor-bearing mouse demonstrated an enhancement of the T-2 weighted image contrast. The images were obtained following a dose of 100\micro l of SPIONs (equivalent to 3mg of Fe/Kg).

\textbf{Figure 53:} MRI images of mice bearing B16F10 tumor. T-2 weighted images were take of mice before (A,C) and after injection of SPION-micelles (B) and theranostic micelles (D). Arrows indicate tumor area.
Theranostic micelles showed the ability to produce contrast in a manner similar to SPION micelles. This data suggests that theranostic micelles retained the imaging property \textit{in vivo} and this property was not affected by the presence of PTX.

\textbf{9.3.3 Iron staining}

![Figure 54: Prussian blue staining of breast (4T1) and melanoma (B16F10) tumor tissue sections](image)

Histological analysis confirmed the presence of SPION particles in both tumor models. Prussian blue staining (Figure 54) clearly demonstrated clusters of iron particles in tumors of mice treated with theranostic and SPION micelles. However, this was not observed in mice treated with either plain or PTX micelles.
9.3.4 **Tumor apoptosis**

Therapeutic activity of the theranostic agent is determined by the presence of PTX. The apoptotic activity of theranostic nanopreparation was evaluated in both tumor models by TUNEL assay (Figure 55).

![Figure 55: TUNEL assay of (A) breast and (B) melanoma tumor sections 48 hours post intratumor injection.](image)
The presence of the apoptosis or nuclear DNA fragmentation in tumor sections confirmed that cell death took place in mice treated with PTX and theranostic micelles regardless of the tumor model. Nuclei were stained with DAPI, and apoptotic cells were fluorescently labeled green. These images suggest that theranostic micelles show anti-tumor activity \textit{in vivo}.

\textbf{9.4 Discussion}

Of the various imaging technologies available, MRI is the most widely used for detection of cancer because unlike nuclear scanning, conventional radiography or computer tomography, MRI often permits the use of contrast enhancers to improve inherent contrast between normal and diseased tissue by altering the longitudinal and transverse relaxation rates of tissue protons [2]. By employing contrast agents, the detection can be enhanced allowing for better discrimination of a tumor or infarcted tissue. From the many contrast agents available, SPION are a prime choice in theranostic preparations due to their high magnetization values. They yield much stronger \textit{in vivo} transverse and longitudinal relaxation effects compared to Gd-based contrast agents [3]. In a theranostic agent, it is important to have a balance between the imaging or diagnostic capabilities and the therapeutic efficacy of the chemotherapeutic agent. For the most effective performance, neither of the agents must interfere with each others’ activity.

We have developed and characterized a PEG-PE-based micellar theranostic agent and showed that neither PTX nor SPION altered each others’
properties *in vitro*. Herein we showed the applicability of this theranostic agent *in vivo* in two tumor models.

The MR images were clearly distinguishable pre- and post-injection of theranostic micelles in both tumor models ([Figure 52, 53]). To avoid any unwanted biodistribution and to study only the contrast properties of the theranostic agent, the formulations were injected intratumorally. It is known that intratumor administration has various advantages such as diffusion of the formulation to a certain degree primarily in the interstitial space within the tumor [4]. The T2-weighted image of the animals injected with theranostic and SPION micelles were highly contrasted. These results are in agreement with our *in vitro* studies that showed no difference in the imaging properties between theranostic and SPION micelles. Similar results were reported when magnetite/silica core-shell nanoparticles were injected intratumorally in a breast tumor model [5]. Additionally, intratumorally administered magnetic cationic liposomes also showed enhanced contrast in SCID mice [6]. Some contrast was also observed around the tumor area in the interstitium. This can be explained by the relatively high intratumor pressure and the multiple injections made to ensure complete delivery of the formulation [4; 7]. The dose of SPIONs in the theranostic micelles administered was equivalent to 3 mg Fe/Kg, which is much less than the 20 mg Fe/kg of Fe [8] or 12.5 mg Fe/Kg [9] used *in vivo* to produce T2-weighted contrast image. However, up to 10-30 mg Fe/Kg have been typically employed in animal MR studies [3]. It is important to note that PTX within the theranostic
micelles did not alter the imaging properties. These images are concurrent with the in vitro study that also showed no effect of PTX in formulations containing SPIONs during MRI imaging.

The presence of SPION particles in the tumor was confirmed at the end of 48 hours by Prussian blue staining of excised tissue slices that clearly demonstrated the presence of iron within the tumor tissue in case of SPION and theranostic micelles (Figure 54). Similarly, iron deposits were observed in tumor sections obtained from pancreatic tumor [10] and from squamous cell carcinoma [11] model. Additionally, in vitro localization of iron was observed in LNCaP, PC3 [12] and HeLa [13] cells incubated with SPION particles by Prussian blue stain. Animals treated with control, plain or PTX micelles did not show the presence of iron particles. Staining of iron due to SPION appears differently from the intracellular iron that can stain blue during the same process [14]. However, this situation was not encountered in our case. The presence of iron after 48 hours suggests that the formulations remained localized within the tumor area. It has been reported that even after 15 days PE-DSPPE-coated SPION were detectable in Balb/c mice [13]. The localization of theranostic agents on cancer cells is significant for cancer detection and further treatment.

The therapeutic potential of the theranostic agent was evaluated by studying its in vivo anti-tumor activity using a TUNEL assay DNA fragmentation kit (Figure 55). The kit helped us to identify and differentiate apoptotic from necrotic cells by the DNA fragments that are produced by the apoptotic cells,
identified by Fluorescein FragEL™ [15]. We observed no differences between mice treated with PTX and theranostic micelles suggesting significant apoptotic cell death at the end of 48 hours in both mouse tumor models. Background fluorescence was observed in the case of plain or SPION-micelles, and similar results were observed in a breast tumor model when mice received intratumor injection of PTX micelles [16]. In comparison, mice treated with plain or SPION micelles did not show anti-tumor activity. These in vivo results are in good agreement with our in vitro cytotoxicity data that suggest the presence of SPION within the theranostic agent was not itself toxic and did not interfere with the cytotoxic property of PTX.

9.5 Conclusion

Theranostics (the fusion of therapeutic and diagnostic approaches) aims to personalize treatments with chemotherapeutic drugs. The theranostic agent described here is applicable for both diagnostic and therapeutic purposes. We observed a strong T2-weighted effect in animals injected with SPION-containing preparations, which was confirmed by histological studies. PTX also produced an apoptotic effect in both mouse tumor models.

The advantage of such a theranostic approach is that it can provide early feedback of the therapeutic efficacy of a chemotherapeutic agent. Hence, theranostic agents can overcome the current challenges of the “one-size-fits-all” form of chemotherapy leading to a more personalized form.
References:


STATISTICAL ANALYSIS

Data reported in the chapters 3, 4, 6 and 8 were reported as mean ± standard deviation. Comparison between groups was made using the Student’s t test. The p value calculated was either < 0.05 or <0.005. Statistical analysis was carried out using standard SPSS software.
OVERALL CONCLUSIONS

Nanopreparations that address some of the current challenges of cancer imaging and chemotherapy were developed. Lipid-based micelles for imaging of tumors were formulated and tested using quantum dots as a diagnostic tool for near infrared cancer imaging. These agents proved better than the conventional available quantum dots. Further, by attachment of monoclonal antibody (2C5) in the preparation of quantum dot-immunomicelles, an increase in signal from the tumor area compared to quantum dot-micelles was observed. These findings are a step further in development of systems for rapid detection of cancer. Additionally, a highly sensitive in vitro biochip-based tool was fashioned for identification of tumor biomarkers in detecting cancer at early stages by identifying minute changes in biomarker levels. This device identified nucleosomes and carcinoembryogenic antigens at low picogram concentrations in vitro and from blood samples obtained from tumor-bearing mice. In pursuit of a combined therapeutic and diagnostic approach, a PEG-PE micelle-based theranostic agent was developed and characterized that incorporated both paclitaxel and superparamagnetic iron oxide nanoparticles in sufficient quantities. A strong T2-weighted effect was observed in animals injected with the theranostic agent and also produced apoptotic effects in in vivo tumor models. Thus, three different nanopreparations were developed, characterized and tested for cancer imaging, biomarker detection and simultaneous imaging and therapy of cancer.
Quantum dot loaded immunomicelles for tumor imaging

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Abstract

Background: Optical imaging is a promising method for the detection of tumors in animals, with speed and minimal invasiveness. We have previously developed a lipid coated quantum dot system that doubles the fluorescence of PEG-grafted quantum dots at half the dose. Here, we describe a tumor-targeted near infrared imaging agent composed of cancer-specific monoclonal anti-nucleosome antibody 2CS, coupled to quantum dot (QD)-containing polymeric micelles, prepared from a polyethylene glycol/phosphatidylethanolamine (PEG-PE) conjugate. Its production is simple and involves no special equipment. Its imaging potential is great since the fluorescence intensity in the tumor is twofold that of non-targeted QD-loaded PEG-PE micelles at one hour after injection.

Methods: Para-nitrophenol-containing (5%) PEG-PE quantum dot micelles were produced by the thin layer method. Following hydration, 2CS antibody was attached to the PEG-PE micelles and the QD-micelles were purified using dialysis. 4T1 breast tumors were inoculated subcutaneously in the flank of the animals. A lung pseudometastatic B16F10 melanoma model was developed using tail vein injection. The contrast agents were injected via the tail vein and mice were depleted, anesthetized and imaged on a Kodak Image Station. Images were taken at one, two, and four hours and analyzed using a methodology that produces normalized signal-to-noise data. This allowed for the comparison between different subjects and time points. For the pseudometastatic model, lungs were removed and imaged ex vivo at one and twenty four hours.

Results: The contrast agent signal intensity at the tumor was double that of the passively targeted QD-micelles with equally fast and sharply contrasted images. With the side views of the animals only tumor is visible, while in the dorsal view internal organs including liver and kidney are visible. Ex vivo results demonstrated that the agent detects melanoma nodes in a lung pseudometastatic model after a 24 hours wash-out period, while at one hour, only a uniform signal is detected.

Conclusions: The targeted agent produces ultrabright tumor images and double the fluorescence intensity, as rapidly and at the same low dose as the passively targeted agents. It represents a development that may potentially serve to enhance early detection for metastases.

Background

Near infrared (NIR) imaging is a particularly promising method of imaging since it is not invasive, requires relatively simple and easy-to-use equipment, and can take place in real time. The detection limit can be as low as in other imaging modalities, and it is much less hazardous compared to radionuclide or magnetic resonance imaging as it does not make use of radioisotopes that have special handling and storage requirements, both for their use and their disposal [1]. It is also very versatile and affordable. Instruments are not as expensive or complicated as those for nuclear or magnetic imaging [2]. In the NIR, the light penetrates much further through the body compared to imaging in the visible part of the spectrum [3]. This ‘absorbance window’ allows for the visualization of various phenomena deep inside the body. Using targeted contrast agents, the fluorescence signal can be highly localized. Examples
INTRODUCTION

Ordered arrays of nanoparticles have attracted a tremendous amount of attention because of their potential applications in various emerging fields such as nanophotonic crystals, nanoelectronics, and nanosensors.1-7

Because of their high surface area and size compatibility with biological elements such as enzymes, antigens, and antibodies, nanoparticles can carry on important functions such as the immobilization of biomolecules and the catalysis of electrochemical reactions. Unlike commercially available enzyme-linked immunosorbent assay (ELISA)8 sensors, it has been shown that employing uniformly distributed mAb-coated nanoparticles on the sensor surface increases the orientation and distribution of antibodies,9-11 thus increasing the antibody-antigen binding events and hence the sensitivity of the sensor. This makes the nanoparticles a strong candidate for fabricating biosensing devices.12-15 The simultaneous detection of multiple biomarkers has attracted tremendous interest in the field of microsensors.16-20

Nanoparticle-based sensors can potentially enable multiplex detection. To achieve multiple detection capability, particles with different diameters can be coated with different biomarker proteins and size-selectively assembled at the desired locations on surfaces. Also, the size-selective assembly of nanoparticles has potential applications in multiplexing the detection of vapor sensors21 and miniaturizing nanoparticle-based chemical nose sensors,22 enabling in vivo detection. For these applications, precise and selective positioning of nanoparticles is challenging.

Methods for assembling nanoparticles suspended in a solution onto prefabricated patterns on a template can be broadly classified into self-assembly23-26 and directed assembly.27-33 Previous attempts to position nanoparticles on a template according to their size have been limited to template-assisted self-assembly (TASA) by controlling either the chemical properties30-34 or topography35 of the template. Topographically patterned substrates have been used as 3-D templates to guide crystalline colloidal assembly, Yin and Xia35,36,38 used patterned topography and capillary interactions to assemble monodisperse polystyrene particles into a variety of complex aggregates (such as polygonal and polyhedral clusters) that have well-controlled sizes, shapes, and structures, and their work has been recently reviewed.40 Recently, Kuehne et al.41 showed that it is possible to employ TASA to trap 200, 350 nm, and 500 nm particles on carefully designed topographic features. Relying only on the wetting of the particle suspension and employing a single evaporation/dip-coating step, Fan et al.42,43