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TITLE: Microenvironment-Sensitive Multimodal Contrast Agent for Prostate Cancer Diagnosis

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14. ABSTRACT The goal of this work is to develop the materials and methods to non-invasively monitor the aggressiveness of prostate cancer. Prostate cancer is the second leading cause of death among men in the United States, yet no reliable diagnostic tools or effective therapies are currently available. There is increased focus on the development of tools for improved detection and monitoring of prostate cancer. In this work, we have developed magnetic nanoparticles that are able to detect and bind to PC3 prostate cancer cells. Using the particles' magnetic properties, they can be localized in the body using MRI. The addition of a fluorescent marker on the nanoparticle that is sensitive to proteolytic activity then provides a means to detect and quantify the aggressiveness of the cancer.					
15. SUBJECT TERMS Prostate cancer, nanomedicine, diagnosis, cancer staging, proteolytic activity; non-invasive detection; MRI					
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1. Introduction

Prostate cancer (PCa) is the most diagnosed non-skin malignancy, and the second leading cause of death, among men in the United States. On January 1, 2007 there were approximately 2,276,112 men alive in the United States who had a history of prostate cancer, and 1 in 6 men born today will be diagnosed with prostate cancer during their lifetime. In order to achieve more effective therapy, focus has recently shifted to the early detection of PCa. While prostate-specific antigen (PSA) monitoring has enabled earlier detection of prostate cancer, the high variability in PSA levels has limited its diagnostic value. Additionally, dynamic changes in molecular, cellular and tissue level processes occur during disease progression and remission, and their quantitative assessment is important for treatment planning and monitoring of treatment efficacy. Measurement techniques currently in use (e.g. biopsy) are invasive, and provide limited spatial and temporal sampling. Improved molecular imaging techniques, that are both quantitative and sensitive, and that can detect processes deep within the human body are required to monitor these changes which may be predictors of treatment outcomes. Hence, the need for a non-invasive, highly sensitive and accurate diagnostic tool for measuring the aggressiveness of prostate cancer is both acute and imperative. Taking all these facts into account, the U.S. Preventive Services Task Force (USPSTF) has recommended that, “research is urgently needed to identify new screening methods that can distinguish nonprogressive or slowly progressive disease from disease that is likely to affect the quality or length of life.” Improved molecular imaging techniques, that are both quantitative and sensitive, and that can detect processes deep within the human body are required to monitor these changes which may be predictors of treatment outcomes. The primary objective of this application is to confirm the feasibility of a microenvironment-responsive multimodal contrast agent (MMCA) for non-invasive, 3D measurement of prostate cancer aggressiveness.

2. Keywords

prostate cancer; cancer staging; imaging; MRI; nanomedicine; proteolytic activity; non-invasive detection

3. Accomplishments

The goal of the funded project is to develop multimodal and bioprocess-sensitive “smart” magnetic nanoparticles (MNP) capable of detecting dynamic changes in molecular and tissue level processes that occur during prostate cancer progression. Specifically, we are developing MNPs that respond to and are able to monitor and quantify overexpressed proteolytic activity in the tumor microenvironment. Proteolytic processes have been shown to be important for cancer growth, progression, extracellular matrix remodeling and metastasis. The ability to non-invasively monitor this activity would not only enable better treatment planning, but also provide a means to monitor therapeutic effect. The

specific aims of this project are: **Aim 1** – Synthesize the microenvironment responsive multi-modal imaging agent (MMIA) and characterize its physical properties (e.g. magnetic susceptibility, particle size distribution, zeta potential, etc); **Aim 2** – *In vitro* characterization of the target selectivity, protease specificity, and detection sensitivity of the MMIA using low- and high proteolytic activity cell lines; and **Aim 3** – Conduct a pilot *in vivo* animal study to demonstrate the utility of this system for monitoring tumor progression.

According to our statement of work, the objective for this period of work was to complete *Task 1 – Synthesis and characterization of the microenvironment-responsive MMIA*. The subtasks to be done included:

- a. Synthesis of the MMIA components
- b. Optimization of MMIA stability
- c. Assembly of the MMIA/fluorescent probe/targeting agent complex
- d. Characterize the physical properties of the MMIA

The accomplishments for each of these subtasks are briefly summarized below.

3a. Synthesis of the MMIA components

The MMIA to be developed in this project is comprised of magnetic nanoparticles (MNP), which serve as a contrast agent for Magnetic Resonance Imaging (MRI), coated with a biopolymer (i.e. starch) to improve biocompatibility, and tagged with prostate cancer-targeting ligands. A significant challenge to translation of nanomedicine from lab bench to the clinical setting arises due to variations in particle behavior *in vivo* as a function of nanoparticle size distribution and surface properties. The vast majority of research in nanomedicine is conducted using particles having a broad size distribution; leading to uncertainty in the pharmacokinetics, biodistribution, and safety risks of the formulations. In order to reduce this barrier, we have developed synthesis and purification procedures that would produce MNPs of a relatively narrow size distribution.

Our approach was to utilize the size-dependent magnetic properties of the MNPs and the drag force exerted on a particle, which is also size dependent, to separate a MNP population with a wide size distribution into sub-populations with a more narrow distribution. The feasibility of this approach was first evaluated using a simulation developed in Matlab (MathWorks, Natick, MA). Using this simulation, we were able to determine the conditions required to “purify” particles of a particular size. **Figure 1** provides the predicted traces of MNP ranging in size from 40 nm to 400 nm, under the influence of a magnetic field. Using these results, a prototype separate process was established to take a sample of MNP having a broad size distribution and to produce MNPs with tighter size control, as shown in **Figure 2**.

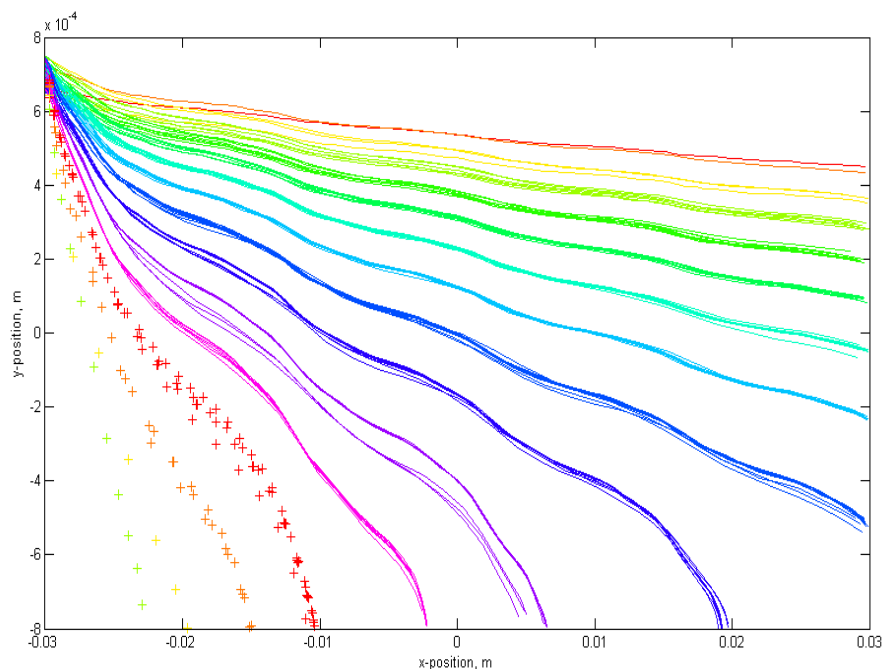


Figure 1. Predicted particle trajectories for magnetic nanoparticles in the size range of 44 nm (top, orange trace) to 400 nm (bottom, green markers) under the influence of drag and magnetic forces.

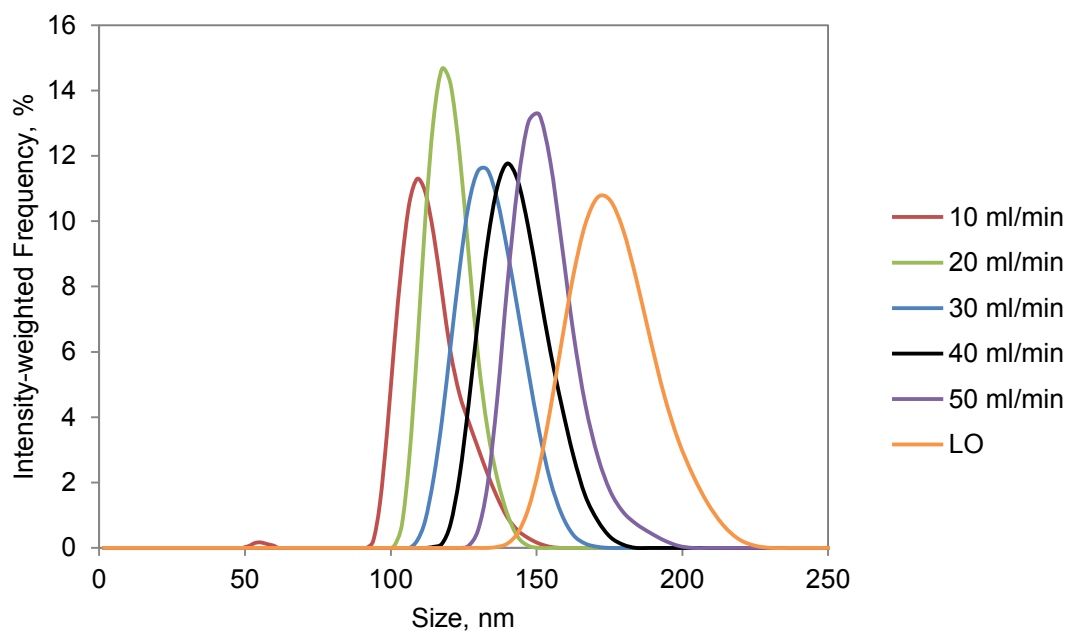


Figure 2. Average MNP size distributions of six fractions obtained using a prototype device to size-selectively separate MNPs from a sample having a broad size distribution.

3b. Optimization of MMIA stability

For the probe to be effective, it has to be long circulating; accumulate selectively at the tumor site; and respond sensitively to a specific protease activity. To enhance the pharmacokinetic (PK) properties of the probe, we modified the surface of the MNP with polyethylene glycol, a polymer frequently utilized to improve stability of nanoparticles. We assessed size stability of the PEGylated MNP (PEG-MNP) in complete culture media, which contains proteins and other components found in blood plasma, using dynamic light scattering and performed *in vitro* experiments to mimic the first two steps (protein adsorption and macrophage uptake) of the reticuloendothelial system (RES) clearance process. Our results (not shown) indicated that PEG-MNPs showed significantly better size stability (i.e. resisted aggregation) and lower protein binding than the unmodified MNP. The MNPs were also incubated for varying time periods with macrophages. As shown in **Figure 3**, the unmodified MNP were rapidly taken up by the RAW 264.7 macrophage cells, while only limited uptake of PEG-MNP was observed even after 24 hours. Among “normal” cells found in the body, macrophages have one of the highest rates of particle uptake. The synthesized particles are therefore expected to be very stable in physiological media and uptake of PEG-MNPs by healthy cells is expected to be negligible.

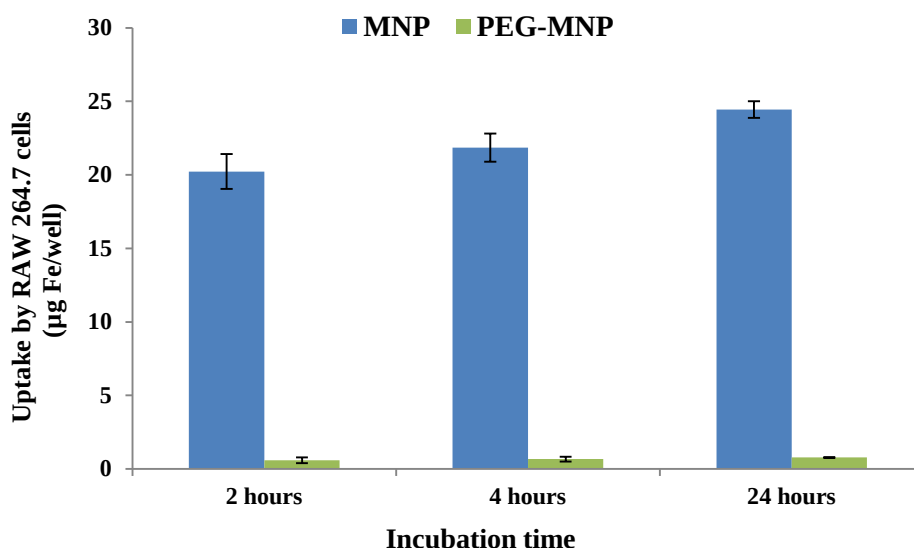


Figure 3. Macrophage (murine RAW 264.7) uptake studies were done to mimic the RES clearance process in the body. PEG-MNPs displayed significantly reduced macrophage cell uptake for up to 24 hours.

3c. Assembly of the MMIA/fluorescent probe/targeting agent complex

While the previously state results showed that stable MNPs have been developed and their uptake by “normal” cells minimized, it is also necessary that these particles target and accumulate in cancer cells. To induce uptake of MNPs by prostate cancer cells, we functionalized the surface with a tumor-targeting

peptide (F3 peptide), which selectively binds to nucleolin present on the surface of tumor cells and endothelial cells of angiogenic tumor blood vessels. While nucleolin is present in all cells, it is only found on the extracellular surface of cancer cells – providing a means to target these cells. In order to determine whether the density of the targeting peptide affects the targeting efficiency, MNPs with a high density of F3-peptide, an intermediate density, and no targeting peptide were incubated with PC3 prostate cancer cells and the particle uptake measured after 1 and 4 hours. As shown in **Figure 4**, the F3-labelled MNPs should significantly greater targeting of the PC3 cells compared to the non-targeted, PEG-MNP. It should be noted that the F3-MNP did not show significant uptake by “normal” cells (data not shown), demonstrating our ability to maximize the target selectivity of the MNP to cancer cells.

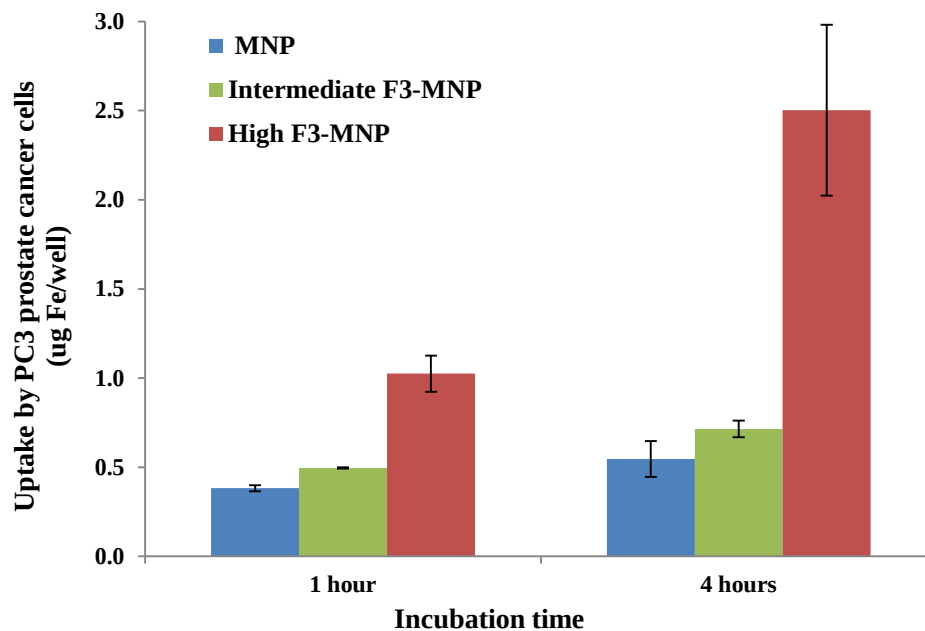


Figure 4. The conjugation of F3 tumor targeting peptide enhances MNP uptake by PC3 prostate cancer cells compared to the non-targeted PEG-MNP.

In addition to the targeting of the MNP to cancer cells, an important component of the proposed work is to label the particles with a fluorescent dye whose release is triggered by proteolytic activity. The fluorescently labeled peptide 5-TAMRA-SGKGPRQITAGGC-amide was conjugated to the surface of the MNP using maleimide chemistry. To determine its response to proteolytic activity, the MNP-peptide was incubated with varying concentrations of the protease TrypLE Express (Life Technologies, Grand Island, NY) for 15 minutes, centrifuged, and the fluorescence of the supernatant measured at 546/579 (characteristic of 5-TAMRA fluorescent dye). As seen in **Figure 5**, the concentration of the protease correlated with the release of 5-TAMRA from the surface of the MNP. Based on these preliminary results, we are currently evaluating the ability of this system to

detect activity of legumain and matrix metalloproteinase 9 (MMP-9). Legumain and MMP-9 are important tumor-associated proteases, overexpressed in the prostate cancer microenvironment, and the level of which is associated with metastatic potential of the cancer.

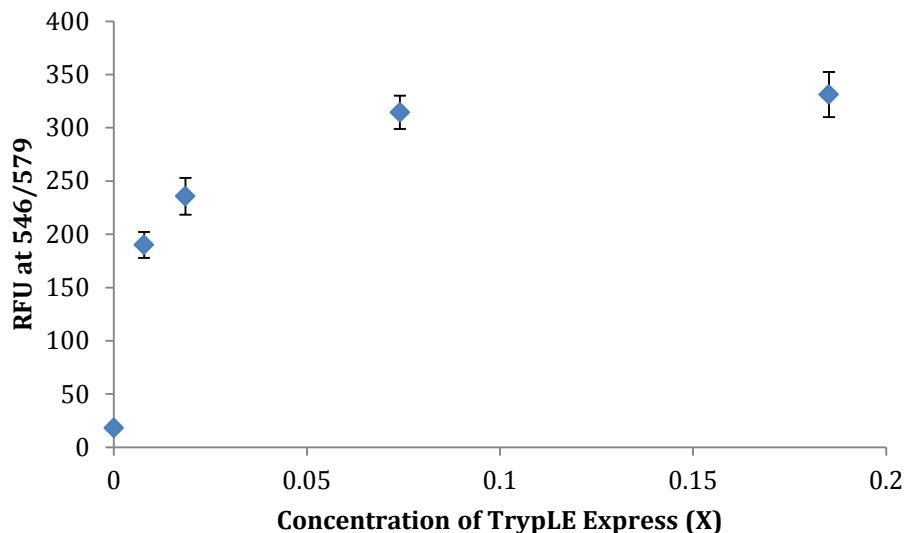


Figure 5. The conjugation of F3 tumor targeting peptide enhances MNP uptake by PC3 prostate cancer cells compared to the non-targeted PEG-MNP.

3d. Characterize physical properties of the MMIA

The physical properties of the particles are continuously measured as part of the synthesis process. This has included measurement of particle size distribution by dynamic light scattering (DLS) and transmission electron microscopy (TEM), quantification of the PEG and peptides conjugated to the MNP surface, and determination of particle stability under various conditions.

Opportunities for training and professional development

During the past period of this project, two graduate students, a Master student and one PhD student, have been trained in the synthesis and characterization of MNPs, as well as in the techniques of cell culture work. The graduate students have also attended several conferences, including the 2013 AIChE Annual Meeting, San Francisco, CA where they presented their work and attended talks. Additionally, three undergraduate students have been mentored in the lab during the funded period. One undergraduate student has since graduated and is currently attending a medical school. Two other undergraduate students continue to work in the lab.

Dissemination of results to communities of interest

Nothing to report

Plan for the next report period

Much of the work proposed for Task 1 of the project (i.e. synthesis and optimization of the MNP) has been completed and some of the work for Task 2 has been initiated (e.g. *in vitro* studies of cancer cell target specificity and protease specificity). During the next report period, we will continue to evaluate the MNP with *in vitro* assays by extending the proteolytic activity response to legumain and MMP-9 proteases and conduct more rigorous evaluation using prostate cancer and normal prostate cell lines. **The project is currently on schedule with the timeline provided in the statement of work.**

4. Impact

Nothing to report.

5. Changes/Problems

Nothing to report

6. Products

Rogers HB, Anani T, Read C, David AE. Magnetic nanoparticles as multimodal contrast agents for the diagnosis of prostate cancer (poster). 2013 AIChE Annual Meeting, San Francisco CA. 2013 Nov.

Anani T, Read C, Rogers H, Choi YS, David AE. Magnetic nanoparticles as multimodal contrast agents for measuring prostate cancer aggressiveness (poster). 2013 AIChE Annual Meeting, San Francisco CA. 2013 Nov.

7. Participants & Other Collaborating Organizations

Individual Participants:

Name:	Allan E. David
Project Role:	PI
Person month worked:	1
Contribution to project:	Overall planning, data analysis, trouble shooting, and monitoring of graduate students

Name:	Tareq Anani
Project Role:	Graduate student
Person month worked:	12
Contribution to project:	Synthesis of MNPs and cell culture studies
Funding support:	Partially supported by Auburn University Research Initiative in Cancer Fellowship

Name:	Hunter Rogers
Project Role:	Graduate student
Person month worked:	12
Contribution to project:	Synthesis of MNPs and development of method for size-selective purification of the MNPs
Funding support:	NSF Fellowship

Active other support of PI

Nothing to report

Involved organizations as partners

Nothing to report

8. Special Reporting Requirements

Nothing to report