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PRINCIPAL INVESTIGATOR: **Suzy V. Torti, Ph.D.**

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Farmington, CT 06030-3806

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Suzy Torti, Ph.D.

Email:storti@uchc.edu

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14. ABSTRACT The objective of this proposal is to test targeted carbon nanomaterials for their ability to thermally ablate kidney cancer. Carbon nanotubes (CNTs) and other forms of carbon nanomaterials, such as graphene, are efficient transducers of near-infrared radiation (NIR). We have shown that carbon nanotubes are effective in laser-induced thermal therapy of kidney cancer in mouse models. Our goal is to improve the anti-tumor efficacy of CNTs by targeting them uPAR, a surface receptor overexpressed in kidney cancers. We will use D5, a peptide designed in the laboratory, as the targeting ligand. In the past year, we made progress in evaluating materials for delivery of D5 and also performed microfluidic studies that will be important in optimizing delivery of our particles to tumors. We published two papers. In the upcoming year we hope to complete our conjugation studies, pursue microfluidic studies to evaluate particle characteristics that will enhance delivery, and perform in vivo efficacy studies. This grant is a mentor/predoctoral award that also focuses on training of a predoctoral candidate. The predoctoral fellow carried out the microfluidic experiments described in this progress report and presented his work at the annual Society of Rheology meeting.

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Table of Contents

INTRODUCTION

The overall goal of this proposal is to test targeted carbon nanotubes for their ability to thermally ablate kidney cancer. Carbon nanotubes (CNTs) have been shown to be efficient transducers of near-infrared radiation for laser-induced thermal therapy of kidney cancer in animal models. However, the current generation of carbon nanotubes lacks the ability to selectively target cancer cells following systemic administration. In this proposal, we will develop carbon nanotubes designed to bind to uPAR, a surface receptor overexpressed in kidney cancers that is involved in growth, migration, proliferation, metastasis and angiogenesis. Binding of peptide fragments of kininogen (D5) to uPAR induces apoptosis in endothelial cells and inhibits tumor growth and metastasis. We will combine the anti-tumor properties of CNTs with those of D5 into one combined treatment by conjugating D5 peptides to several types of multiwall carbon nanotubes. *In vitro* experiments will be performed to test the specificity of binding of these conjugates to uPAR in human endothelial cells, kidney cancer cells, and kidney cells. Thermoablative properties, proliferation, survival, apoptosis, and downstream signaling of uPAR will be examined in these cell lines following treatment with D5 nanotubes. Tthermoablation will be studied initially. Human kidney cancer cells will be injected into the kidney capsule of nude mice. The effect of D5 nanotube injection and thermoablation on tumor growth and survival will be determined. The effect of D5 nanotubes on tumor angiogenesis will be studied by repeating these experiments using co-injection of human kidney cancer cells with human endothelial cells in a matrigel plug. We believe that these conjugated nanotubes will be able to demonstrate enhanced tumor ablation via targeting compared to thermal ablation alone.
In July of 2012 we moved the laboratory from Wake Forest School of Medicine in North Carolina to the University of Connecticut Health Center. This move caused a substantial disruption in research activities. This was partly due to the time it took to transfer the grant from one institution to the other: the award was not fully transferred until May 2013. Nevertheless, we were able to make some progress on the Aims of the grant, as described below. We are applying for a no-cost extension to enable us to complete these experiments.

BODY:

Specific Aim 1. Synthesize nanotube-based particles ligated to D5s.

Progress. We performed exploratory studies to test the suitability of graphene as a carbon backbone for D5 delivery. We found that fluoridation of graphene conferred hydrophilicity and enhanced biocompatibility. Further, fluorinated graphene oxide (FGO) was very effective in photothermal therapy (Figure 1). FGO is a broad wavelength absorber, with high charge transfer and strong non-linear scattering is optimal for NIR laser-induced hyperthermia. FGO acted as a magnetically responsive drug carrier that could serve both as a magnetic resonance imaging (MRI) and photoacoustic contrast agent, under preclinical settings, and as a type of photothermal therapy. This work was published in *Advanced Materials*.

The conjugation of peptides to nanotubes is also part of this aim. It is led by Dr. King, a collaborator on this project. We have developed a linker to amidate carbon nanotubes and conjugated the linker to nanotubes using EDAC. Application of this methodology to conjugation of the D5 peptide to carbon backbones has been awaiting funds transfer.

Specific Aim 2. Test binding, cytotoxic and thermoablative properties of D5s-nanoparticles *in vitro*.

Progress: We tested the binding of D5s to the uPAR receptor, and determined that the peptide exerts a cytotoxic effect on cultured kidney cancer cells, as anticipated. We also determined that the carbon particle backbone exerts a substantial thermoablative effect on cultured kidney cancer cells. We summarized work on thermoablative properties of carbon nanotubes in an article that was recently published in *Advanced Drug Delivery Reviews*.

Specific Aim 3. Examine accumulation and anti-tumor effect of particles in mice.

Progress: This Specific Aim is directed at the delivery of nanoparticles to tumor sites in vivo. While awaiting approval to conduct these experiments in mice, we conducted microfluidic studies to simulate the passage of these nanoparticles in blood vessels. This should be of enormous assistance in planning effective in vivo experiments.

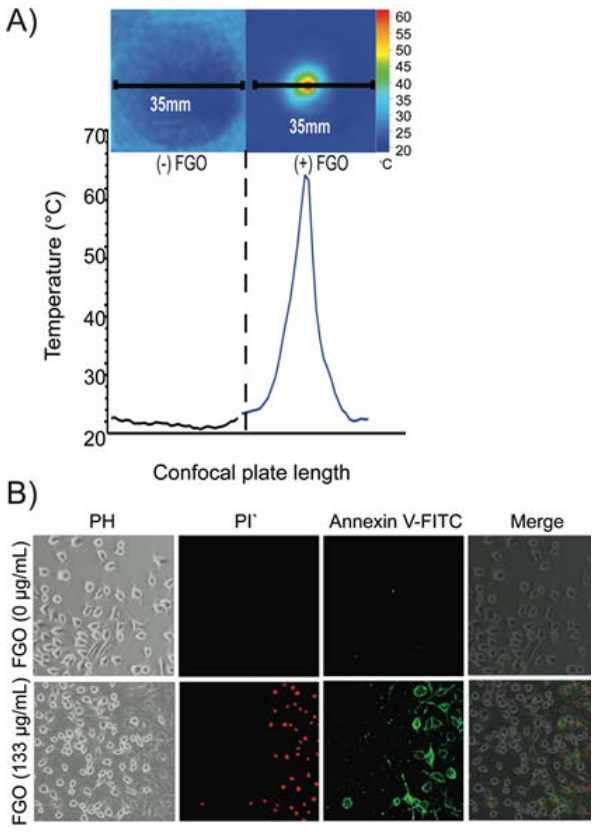

Figure 1 Hyperthermia cell ablation facilitated by FGO. (A) NIR-laser-induced hyperthermia of DMEM medium (-) FGO and of (+) FGO. Each confocal plate length is 35mm. (B) *In vitro* NIR-laser induced differential photothermal ablation of cells (GI-1) incubated with 0µg mL^{-1} of FGO (top) or 133µg mL^{-1} of FGO (bottom) using an apoptotic/necrotic staining and observed by optical microscopy.

When injected intravenously, nanoparticles must be able to tolerate flow in blood vessels and resist aggregation, and must also penetrate and stay within the tumor region. These particles are believed to accumulate in tumor cells due to the Enhanced Permeability and Retention (EPR) effect, as a direct result of the irregular and "leaky" vascular structures typically found in tumor sites.[1,2]. Normal endothelial cell interstitial spacing is on the order of 1-2 nm, whereas tumor-affected vasculature exhibits a pore size of anywhere from 100 to 780 nm. Many studies postulate that this abnormally large pore size will allow for enhanced uptake of small, drug-containing nanoparticles by the tumor, as compared to regular tissue. However, the interstitial fluid pressure in a tumor is higher than the capillary pressure of blood vessels as a result of protein buildup in the tumor, thereby creating a pressure gradient that leads to an outward convection and low hydraulic conductivity, both of which oppose inward diffusion. As a result, it is questionable as to the exact nature of the EPR effect and whether even small particles, such as drug-containing solid lipid nanoparticles, will be able to successfully diffuse into and remain in the tumor.

However, there also exists the possibility that nanoparticles may exhibit margination, wherein they are moved closer to the periphery of the blood vessels as a result of the forces resulting from blood flow. Margination is responsible for the movement of white blood cells towards the blood vessel periphery and red blood cells towards the center of the blood vessel and is a result of hydrodynamic forces, inertial forces, and particle shape and size. It is vital to better understand this phenomena before exploring the EPR effect, as margination has an enormous impact in drug delivery because it affects the proximity of drug carriers to the periphery of blood vessels and, by extension, the diffusion of the drug into tissue cells from the blood stream.

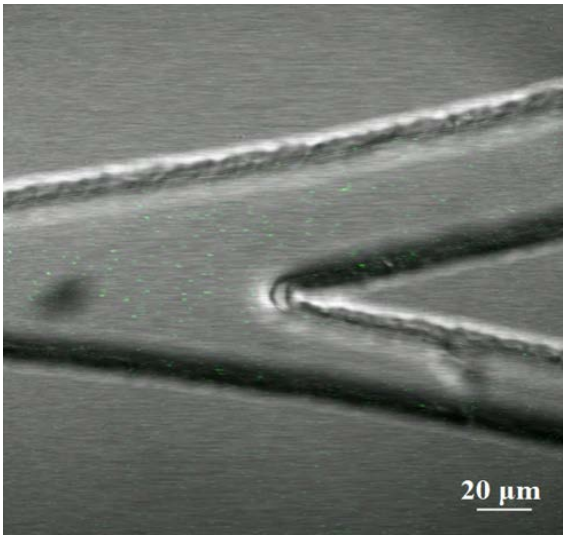

Figure 2 Confocal image of 0.1 micron (100 nm) polymer beads (Fluoresbrite® YG Microspheres 0.10 µm, Polysciences Inc.) in a polydimethylsiloxane microfluidic device. The device's main channel has 50 micron width and 62 micron depth and bifurcates into a channel of identical dimensions (top channel in figure) and a channel of 30 micron width and 62 micron depth (bottom channel).

Because of their large aspect ratio, it is unknown how nanotubes will behave in blood flow and whether or not they will exhibit margination. We therefore investigated margination behavior of various shapes and sizes of particles through the use of microfluidics. Microfluidics utilizes micron-scale devices, typically fabricated using polydimethylsiloxane polymer via soft lithography techniques. By creating such a device, a simulated channel can be created and the margination of carbon nanotubes in a flowing media, such as blood, can be observed and studied. The goal is to achieve a better understanding by observing the margination effect in vitro and to ultimately characterize and accurately predict and model this effect via experimental trials and the use of image-analysis and particle tracking software.

To begin, a procedure for making microfluidic devices was developed with the help of the Shor lab at UConn. Once devices were easily reproducible, a simple system was used consisting of spherical 100 nm diameter polystyrene beads (Fluoresbrite® YG beads; Polysciences Inc.) in water in a microfluidic device (Figure 1). These particles were imaged using a confocal microscope but they were very difficult to track as an initial, model system. For this reason, 0.5 micron beads were used in order to develop particle tracking procedures.

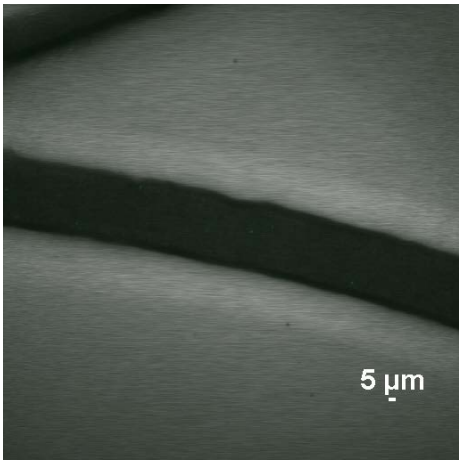

Figure 3 Confocal image of fluorescent, 0.5 micron polystyrene beads (Fluoresbrite® Carboxylated YG Microspheres 0.5 µm, Polysciences Inc.) diluted 1:3000 times in bovine blood and flowing through a 50 micron wide and 62 micron deep channel in the microfluidic device.

It was important to ensure a physiologically relevant flow rate before introducing blood into the system. A flow rate of 0.6 microliters per minute was calculated, in order to achieve the physiologically relevant flow rate of 0.328 mm/s. This flow rate was achieved via the use of a syringe pump, which was hooked up to the microfluidic device while the device was imaged via the use of the confocal microscope. In order to check that the pump

was working properly, beads were tracked using ImageJ software and an average velocity of 0.623 mm/s was calculated, which is comparable to the expected syringe pump flow rate of 0.328 mm/s.[3]. This flow rate is therefore physiologically relevant.

Finally, a trial was conducted using 0.5 micron beads in bovine blood. As can be seen in Figure 2, the beads can be easily visualized in blood and, therefore, are trackable. By using the capability of the confocal microscope to image only a very thin depth of the device, margination near the walls of the device can be observed and particle motion and margination propensity can be quantified. Future trials will observe particle behavior at the bifurcation of the device and also the effects of size, shape, and other properties on nanoparticle margination propensity.

KEY RESEARCH ACCOMPLISHMENTS:

- Tested the suitability of fluoridated graphene as a delivery vehicle for D5
- Developed microfluidic technology to study flow properties of particles and their margination with a view to optimizing their delivery in vivo.

REPORTABLE OUTCOMES: The trainee, Erik Carboni, attended the 84th Annual Meeting of the Society of Rheology, where he presented his results on nanoparticle microfluidics.

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CONCLUSION:

In the past grant year, our progress was substantially delayed by a relocation of my laboratory. Nevertheless, we made progress in evaluating materials for delivery of D5 and also performed microfluidic studies that will be important in optimizing delivery of our particles to tumors. In the upcoming year we hope to complete our conjugation studies, pursue microfluidic studies to evaluate particle characteristics that will enhance delivery, and perform in vivo efficacy studies.

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