Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 - 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814 935 1081

5. **Grant SAP Number:** 4100050904

6. **Project Number and Title of Research Project:** 33. Tumor Associated Receptor Targeted Neuroimaging for Effective Diagnosis of HGA

7. **Start and End Date of Research Project:** 9/1/2010 - 6/30/2012

8. **Name of Principal Investigator for the Research Project:** Achuthamangalam B. Madhankumar, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 61,800

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Madhankumar, A.B.	Assistant Professor, Principal Investigator	10%	$9,110
Webb, Becky	Technician	10%	$4,710

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Liu, Xiaoli	Postdoc	10%
Weston, Cody	Graduate student	5%
Duck, Kari	Graduate Student	5%
Sheehan, Jonas	Associate Professor of Neurosurgery	2%
Yang, Qing	Associate Professor of Radiology	5%
Connor, James	Distinguished Professor, Department of Neurosurgery	5%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Targeted MRI Contrast for Image-guided drug delivery in Glioma (NIH R01)	☒NIH ☐Other federal (specify:_____) ☐Nonfederal source (specify:_)	2012	$547,457	Not Funded

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

With the liposome project, we are planning to propose studies using multifunctional liposomes carrying doxorubicin and Gd-DTPA and will evaluate them for the MRI guided imaging and therapy.

With the quantum dot project, we are proposing to detect certain oncogenic receptors present in the soluble form and in the form of extracellular vesicles in the biological fluid. This will aid in the earlier detection of the tumor metastasis process and also to study the biology of the tumor progression and therapy.

12. Future of Research Project. What are the future plans for this research project?

With the help of Tobacco CURE funded research, we were able to demonstrate the potential of IL-13 conjugated quantum dots and contrast agent loaded liposomes to detect the tumor associated receptor. Interestingly, we observed the ability of the IL-13 conjugated quantum dots to detect the IL-13Rα2 receptor in GBM stem cells and also the soluble IL-13Rα2

receptor in a medium. The next step would be to extrapolate this idea to CSF and serum from GBM and other brain metastatic cancer which are known to express the IL-13Rα2 receptor as evidenced by our preliminary experiments by Western blots and ELISA.

With the quantum dot project, we started to see exciting results. IL-13QD is sensitive enough to detect the IL-13Rα2 receptor in glioma stem cells and in a media containing soluble IL-13Rα2 receptor. We propose to utilize this technique to detect the traces of soluble receptor in the serum or cerebrospinal fluid by the tendency of the quantum dots to bind and form a larger complex, which can be quantified. Future plans are to submit an R21 grant and Department of Defense grant proposal based on our preliminary data. This will find potential to develop into translational research.

Although here we utilize IL-13 receptor as a target, the concept is translatable to all the other cancers like HER2 expressing breast cancer, GD2 expressing melanoma and other cancers with specific markers.

Furthermore, to achieve the in vivo tumor targeting and tumor boundary determination, we will propose to utilize quantum dots conjugated to IL-13 and another ligand which can mediate the transport of the particles across the blood brain barrier will be utilized. Based on our in vitro BBB transport studies, the transport of the targeted quantum dot across the endothelial cell culture model is insignificant. However, since the BBB is compromised in the glioma tumor model, this may not be an issue. To facilitate the rapid transport for enhanced visualization of the tumor, we will propose to utilize transferrin or transferrin receptor or ApoE4 conjugated quantum dots, which aids in the transendothelial migration of the quantum dots and will determine their transport in vivo.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			1	
Female			1	1
Unknown				
Total			**2**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			2	1
Unknown				
Total			**2**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White			2	
Black				
Asian				1
Other				
Unknown				
Total			**2**	**1**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes___X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This research project has provided training opportunities for two predoctoral students and a postdoctoral fellow in the area of nanotechnology and its application to cancer research which has further strengthened the research environment at the Penn State Hershey Cancer Institute.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

Recently we established collaboration with investigators at the Cleveland Clinic to utilize our techniques to detect the soluble receptors and extracellular vesicles released from glioma stem cells and to determine their significance.

16(B) Did the research project results in commercial development of any research products?

Yes_____ No__X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research leads to new involvement with the community?

Yes_____ No___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes.
Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Glioblastoma Multiforme (GBM), which is classified as a high-grade astrocytoma (HGA), is considered the most devastating type of brain tumor. These tumors grow rapidly and infiltrate normal brain tissue. As a result, high-grade tumors can be difficult to treat surgically and many are resistant to current chemotherapeutic and radiation treatments. We have developed a nanotechnology strategy to target a liposomal delivery system selectively to high grade astrocytomas. Our overall goal is to successfully use the receptor targeted nanoliposomes and nanoparticles to diagnose and treat brain tumors.

***Specific Aim 1**: Determine the efficacy of using gadolinium loaded IL-13 conjugated liposomes to target and retain in the glioma tumors*

The ultimate goal of our project is to effectively image the glioma tumors in intracranial glioma tumor bearing mouse models in an enhanced way so that the tumor formation can be detected at a much earlier stage and also tumor boundaries can be marked distinctly to differentiate them from the normal healthy tissues. In order to achieve our goal of enhanced and tumor specific imaging, we set forth the following aims to obtain preliminary data which can lead to sustainable long term funding for the project.

We were able to achieve the goal of encapsulating the gadolinium complex in liposomes and target them to GBM in a receptor specific manner. A contrast enhancement was also observed in the tumor region. Attached are the data corresponding to a manuscript based on the IL-13 – Gadolinium liposomes. Once the data is organized and formatted this will be submitted to the journal "Neuro-Oncology."

In the objective 2, we were not able to perform the radiation necrosis as there was a problem in generating the radiation induced necrosis animal model. In our attempt the animal could not survive higher radiation and died.

Objective 1: To demonstrate that IL-13 conjugated nanoliposomes encapsulated with gadolinium can effectively target intracranial glioma tumors and improve the imaging efficacy

A detailed description of the experiments performed and the results are described under materials and methods in the discussion section below.

Objective 2: To differentiate radiation induced necrosis and tumor growth using tumor targeted gadolinium enhancement

As reported earlier, we were unable to complete this objective as the athymic nude mice were not able to withstand a higher radiation dose appropriate for inducing radiation mediated necrosis and almost all the animals were dead after exposing them to radiation

Objective 1: To demonstrate that IL-13 conjugated nanoliposomes encapsulated with gadolinium can effectively target intracranial glioma tumors and improve the imaging efficacy

To achieve this objective the liposomes were formulated and characterized thoroughly. Subsequently the material was used for in vitro and in vivo diagnosis of the glioma tumor as

detailed below in the materials section:

Preparation of Inteleukin-13-Liposomes-Gd-DTPA (IL-13-Liposome-Gd-DTPA). A lipid mixture consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine(DPPC) or L-α-phosphatidylcholine, hydrogenated (HSPC), Cholesterol,1,2-distearoyl-*sn*-glycero-3 phosphoethanolamine -N-[carboxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-Mal) in the mole ratio of 10:5:5:0.5 was dissolved in a mixture of methanol/chloroform 1:1 (v/v). Subsequently, the organic solvent was evaporated using a rotatory evaporator (Buchi) to form a film. Using a bath type sonicator, the film was reconstituted in a saturated solution mixed by gadopentetate dimeglumine (Magnevist^{R}, Bayer, Inc.) (Gd-DTPA) with poly-L-lysine to formulate the multilamellar vesicles. The multilamellar vesicles were extruded through the polycarbonate membranes; with four passes through a 200 nm, subsequently, with four passes through a 100 nm. Then liposomes-Gd was conjugated with human IL-13 protein to generate the contrast agent IL-13-Lip-Gd. Next, IL-13-Lip-Gd was passed through the Sepharose CL-2B column to remove the free IL-13 protein. For further purification, IL-13-Liposomes-Gd-DTPA was exhaustively dialyzed against PBS at 4°C to remove the free Gd-DTPA. IL-13-Liposomes-Gd-DTPA was concentrated by centrifuge at 4°C using Regenerated Cellulose 30,000 MWCO (Millipore Cooperation Billerica, MA).

Characterization of IL-13-Liposomes-Gd-DTPA.

(a)*Measurement of the size and distribution of IL-13-Gd-Lip particles.* To measure the size and the distribution of IL-13-Gd-Lip, a light scattering analysis, ALV/DLS/SLS-5022F compact goniometer system (ALV) was utilized. The PALS Zeta Potential Analyzer measured the polydispersity index (PDI) of IL-13-Lip-Gd (Ver. 3.16; Brookhaven Instruments Corp.). The PDI represents the particle of the distribution of particle size of the IL-13-Liposomes-Gd-DTPA in a given medium or buffer in which they are suspended.

(b)*Determination of the morphology of IL-13-Liposomes-Gd-DTPA.* To envision the morphology of IL-13-Liposomes-Gd-DTPA, a negative stain transmission electron microscopy (TEM) was applied. The samples were stained using the standard drop method whereby a suspension of the liposomes were applied to a 300 mesh copper grid with a freshly glow discharged continuous carbon film, rinsed with distilled water wash and stained with a drop of 0.75% Uranyl Formate. After drying, the samples were examined in a JEOL 1400 TEM at 120kV and imaged with a Gatan Orius SC1000 CCD.

(c)Qualitative analysis of the IL-13 on the liposomes. A slot blot was performed with free IL-13 protein, thiolated IL-13 protein after imminothiolane treatment and the final product IL-13 conjugated liposomes using a slot blot apparatus. The protein transferred blot was blocked in 5% milk and then with a goat polyclonal antibody for human IL-13 (1: 1000)(Santa Cruz Biotechnology, Inc. Cat. No. sc-1292) followed by HRP conjugated secondary anti-goat antibody (1:15000). After 3 times washing with PBS, the blot was exposed to chemiluminscent substrate for HRP and exposed to an imager (Fuji).

(d)*Evaluation of the loading efficiency of the gadolinium into IL-13-Liposomes-Gd-DTPA.* To The loading efficiency of the gadolinium was determined after digesting the liposomes with 20%

nitric acid and analyzing the elemental gadolinium using inductively coupled plasma-atomic emission spectroscopy (ICP-AES)(Perkin Elmer spectrometer (Model Perkin-Elmer Optima 5300)).

(e) *Determination* of the cytotoxicity of IL-13-Lip-Gd. In vitro cytotoxicity was determined by plating the glioma stem cells in the geltrex coated plates and adding the IL-13-Liposomes-Gd-DTPA at various concentrations. The cell proliferation was monitored by MTS assay.

(f)To assess the systemic toxicity IL-13-Liposomes-Gd-DTPA was injected into the athymic nude mice. Two hours later the kidney and liver enzyme levels were monitored by measuring total bilirubin, blood urea nitrogen (BUN), creatine, aspartate amino transferase (AST, also called SGOT), alanine aminotransferase (ALT, also called SGPT), and alkaline phosphatase

In Vitro Studies.

(a)Uptake of the IL-13-Liposomes-Gd-DTPA complex by human glioma stem cells (GSC) The binding and internalization of the IL-13-Liposomes-Gd-DTPA was added to the U251, U87 and 3691 glioma cells cultured (20,000 cells /well) on the chamber slides at a concentration of 0.4 mg phospholipid/mL and 1 h later the cells were visualized using a confocal microscope (Leica TCS SP2; Leica Microsystems)

(b) BBB transport of *IL-13-Gd-Lip:* Bovine brain microvascular endothelial cells (BBMVEC) were coated on the transwell filter at a density of 100,000 cells per well for 2 days and serum starved for 48 hours with 50 M of hydrocortisone. On day 5, IL-13-Gd-Lip was added to the apical chamber at a concentration of 4.6 ug/mL and the aliquots were withdrawn periodically from the basal chamber. The basal chamber media was analyzed for gadolinium content using ICP-AES.

(c)The in vitro relaxivity of IL-13-Liposomes-Gd-DTPA in T1-weighted MRI. To ensure the quality of IL-13-Gd-Lip, the *in vitro* relaxivity of each batch of IL-13-Lip-Gd has been determined by T1 weighted MRI image on a 7T system using a 5 mm NMR tube along with free Gd (Magnevist) suspended in PBS of same concentrations (0, 100, 150, 250 µg/mL) as IL-13-Lip-Gd referred [1]. IL-13-Lip-Gd displayed a stable trait. Each batch of IL-13-Gd-Lip was tested for its *in vitro* relaxivity.

In Vivo Studies

(a)IL-13-Liposomes-Gd-DTPA penetration of brain tissue in vivo in living mice with intact brains was studied by enhancement of signal noise ratio (SNR from MRI). To evaluate whether IL-13-Gd-Lip could penetrate an intact BBB, *in vivo* relaxivity was measured. Adult athymic nude mice were separated into two groups (n=3 for each group) and anesthetized with 2% isoflurane/oxygen. Subsequently Magnevist (Bayer Healthcare, Wayne, NJ) or the IL-13-Lip-Gd was injected through tail vein. The relaxivity in pituitary gland *brain* parenchyma region were acquired using Axial T1-weighted MRI images using 7T MRI system (Bruker Biospin 7/20a, Ettlingen, Germany), with 540/11ms TR/TE, 8 NEX, 192x192, 3.2cm FOV, 0.5mm thickness with 0.5mm gap at following time-points: a pre-dose and 5 post-dose scans at 10, 30, 60, and

120-minutes and 24-hours.

(b)Image Data Analysis: The signal-to-noise ratios (SNR) of pre- and post-injection images were evaluated from two region of interest (ROIs): 1) from posterior pituitary gland on the same slice due to the posterior pituitary gland and from the brain parenchyma.

(c)Histological chemistry. H*istological chemistry* was performed on brain tissue following the injection of the liposomes. Adult mice were injected with FITC-labeled IL-13-Liposomes-Gd-DTPA (with 736 µg of Gd) through the tail vein Axial T1-weighted images were acquired on a 7T MRI system (Bruker Biospin 7/20a, Ettlingen, Germany) at following time-points: the pre-dose and 3 post-dose scans at 10, 30 and 60-minutes, then the mouse was sacrificed and the brain tissue was sectioned with 12 micron thickness. The sections were stained with DAPI 1% (w/v) to identify cell nuclei, mounted and then dried overnight at room temperature. The images were captured through confocal microscopy.

(d)Study the contrast agent IL-13-Liposomes-Gd-DTPA in an intracranial tumor model.

Intracranial tumor model. For testing IL-13-Liposomes-Gd-DTPA as a contrast agent, an Intracranial glioma was induced by implanting 50,000 human glioma stem cells T3691 in the caudate putamen region after the female athymic nude mice weighing 20-30g (5-8-weeks old) were anesthetized by intraperitoneal injection of ketamine-xylazine 10mg/kg-100 mg/kg body weight. The tumor formation and volume was routinely monitored using MRI with Magnevist enhanced T1-weighted MRI at 2 weeks post tumor induction. When tumor reaches to 5-10 mm in diameter within 3-4 weeks, it is ready for the experiment.

Detection of an intracranial glioma tumor by IL-13-Liposomes-Gd-DTPA. To assess whether IL-13-Liposomes-Gd-DTPA could generate effective contrast and detect the small intracranial glioma (size < or = 2mm).An athymic nude mice with an intracranial tumor was pre-imaged with T1- and T2-weighted MRI along with a stereotactic head-frame with 3 orthogonal fiducially marker sets. At the same time, the same mouse was injected with Magnevist and imaged again. The next day, the same mouse was injected with FITC-IL-13-Gd-Lip and re-imaged.

Anatomic analysis by histological chemistry. Following the procedure 2.7.2, the mouse was sacrificed 1 hours post injection of the contrast agent FITC-IL-13-Liposomes-Gd-DTPA and the brain tissue was frozen at -20°C. Then brain tissue was sectioned to 12 micron thickness the next day. Subsequently, the nucleus of the slides with an even number was stained with DAPI 1% (w/v), mounted and then dried overnight. The images were captured through microscopy.

Detection of glioma in smaller size < or = 2 mm by IL-13-Liposomes-Gd-DTPA To test whether IL-13-Gd-Lip is able to detect the glioma in smaller size, the slides were prepared (refer to 2.7.3) and separated into two groups; 1) the slides were prepared with H&E staining, 2) the nuclei were stained with DAPI, both groups were imaged by microscopy.

Results and discussion

Formulation and Characterization of IL-13-Lip-Gd-DTP . *T*he average size of IL-13-Liposomes-Gd-DTPA was 137 nm (four different batches) and ranged from 80-150 nm. The particles were

evenly distributed with PDI 0.1-0.2 nm as shown in Figure.1A. The morphology of IL-13-Liposomes-Gd-DTPA was revealed by electron microscopy as a spheroid structure shown in Figure 1B. The loading efficiency of Gd into IL-13-liposomes was confirmed by the measurement of the concentration of Gd in IL-13-Liposomes-Gd-DTPA (from four batches), which was in the range of 4.0-10.0 mg/L quantified by ICP-AES. In addition, the expression of IL-13 on the surface of the liposomes was also confirmed through immunoblotting with IL-13 Ab shown in Figure 2E. To compare the three bands on the blot, the band of lane 1 showed stronger signal than lane 2 and 3 because lane 1 represents the original recombinant IL-13 protein (pure 10 µg), lane 3 represents the thiolated IL-13 and lane 2 represents the final product IL-13-Liposomes-Gd-DTPA. The purpose of this experiment is to prove that IL-13 was conjugated effectively on the liposomes

The size range of our contrast agent encapsulated liposomes is 80-160 nm with a mean size 137 nm compared to our targeted liposomes with doxorubicin alone which has a mean size of 104 nm [2]. The mean size from our particles was about 33 nm larger. Keeping the size of the liposomes in a range was important because larger liposomes will experience greater uptake by the reticuloendothelial system and, therefore, the drug will be metabolized faster[3].

The contrast agent IL-13-Liposomes-Gd-DTPA was non-toxic in either vitro or in vivo models. MTS assay in our *in vitro* model of U251 cells and glioma stem T3691 cells did not reveal cytotoxicity even at the concentration of 60 µg/mL of Gd as shown in Figure 3A & B. In addition, the index of the blood chemistry did not reveal any sign of liver or kidney toxicity as evidenced by the levels of Alkaline Phosphatase AST U/L, Alkaline Phosphatase U/L, SGPT AST U/L, SGOT AST U/L, Total Bilirubin mg/dL, Creatine mg/dL shown in Figure 3C & 3D.

IL-13-Liposomes-Gd-DTPA was up taken by human glioma U251, U87 cells and glioma stem cells T3691. FITC labeled IL-13-Liposomes-Gd-DTPA could be detected in the cytosol of glioma U251, U87 and GSC T3691 cells (Figure 4 A, B & C). The liposomes conjugated with IL-13 (FITC-IL-13-Lip-Gd-DTPA) or without IL-13 (FITC-Lip-Gd-DTPA) were compared as shown in Figure 4 (q and t). GSC T3691 retained intense fluorescence from FITC-IL-13-Liposomes-Gd-DTPA Figure 4C (t) than from FITC-LIP-Gd-DTPA Figure 4B(q).

The contrast agent IL-13-Liposomes-Gd-DTPA facilitates crossing the BBB in our in vitro model. To determine if liposomes enhanced the transport of Gd across the BBB we used our established cell culture of the BBB. Either magnevist or IL-13-Gd-Lip was placed in the apical chamber of the BBB model. After 4 hours the level of Gd in the basal chamber was 2000 ppb of Gd in the magnevist treated wells compared to 6000 bp in the liposome treated wells. In 24 hours, there was twice the amount of Gd in the basal chamber in the liposome group compared to the magnevist group (Figure 5). Rhodamine labeled dextran was present in all experiments to serve as a control for the integrity of the tight junctions[4].

The MRI relaxivity in athymic nude mice with an intact brain after administration of IL-13-Liposomes-Gd-DTPA. Signal to noise ratio (SNR) calculated based on the relaxivity data at the location of pituitary gland demonstrated that there is about 25% increase in SNR. On the contrary, SNR remained unchanged and stayed at the base line from the location of brain parenchyma shown in Figure 6B. It indicated Magnevist alone was not able to reach the brain

parenchyma in the mice with intact brains. In contrast, for the injection of IL-13-Liposomes-Gd-DTPA, SNR increased 10% from the location of the brain parenchyma 30 minutes post injection shown in Figure 6A. It indicated IL-13-Liposomes-Gd-DTPA penetrated the brain parenchyma in a living athymic nude mouse with an intact brain. SNR from *brain* parenchyma illustrated that only IL-13-Liposomes-Gd-DTPA was able to cross an intact BBB in living athymic nude mice, in contrast, the Magnevist was not.

Image of confocal microscopy presented direct evidence that IL-13-Liposomes-Gd-DTPA penetrated brain tissue *in vivo* in living *athymic nude mice.* The confocal images from sectioned brain tissue slides after the injection of FITC-IL-13-Liposomes-Gd-DTPA revealed that the FITC labeled liposomes was localized in the brain tissue, which provided direct evidence that IL-13-Liposome-Gd-DTPA crossed an intact brain and entered the brain tissue as shown in Figure 6C (images of b, c, h and i). In contrast no FITC was observed in the control, untreated mice in Figure 6C (images of e and f).

As a conventional methodology, employment of Gd-DTPA, Gd-DOPA and Gd-BOPTA in MRI for detection of glioma, when its size ≥ 2 mm, is not complicated due to BBB leakage generated by brain cancer. However, the cancer boundary produced by, for example, Magnevist-enhanced MRI reflects only the extent where BBB is compromised instead of a true cancer-normal tissue boundary. The complication arises at the early stage glioma when its size is < 2 mm where the BBB leakage is relatively too small to hold a sufficient amount of a conventional contrast agent to induce a clear detectable signal in MRI. Facing this challenge, we realized that we have to test our novel contrast agent with an intact BBB in living mice in order to examine whether IL-13-Liposomes-Gd-DTPA could cross an intact BBB for detection of a glioma at its early stage. To search the evidence, IL-13-Liposome-Gd-DTPA was injected into the normal mice with an intact BBB. The relaxivity of MRI and the histological images from confocal as shown in Figure 6A and C favors our hypothesis that IL-13-Liposomes-Gd-DTPA possesses characteristics capable of crossing an intact BBB. Based on the results, IL-13-Liposomes-Gd-DTPA as we predicted, was able to detect a glioma at an early stage. As solid evidence from MRI, SNR increased to 5% in brain parenchyma post injection of IL-13-Gd-Lip contrary to SNR, which remained at base line post injection of Magnevist. More direct evidence was seen in the histological images sectioned brain tissue, where fluorescence indicates the accumulation of liposomes Figure 6C (h).

The contrast agent IL-13-Liposomes-Gd-DTPA was able to detect the intracranial glioma. Axial T1-weighted images acquired on a 7T MRI system for the athymic nude mice with intracranial tumor after receiving the Magnevist or IL-13-Gd-Lip indicate that IL-13-Liposomes-Gd-DTPA is a potential contrast agent similar to free Magnevist as shown in Figure 7.

The contrast agent IL-13-Liposomes-Gd-DTPA was able to detect a small intracranial glioma tumor in the size < or =2mm, while Magnevist was not. The section of the MR from Axial T1-weighted images acquired on a 7T MRI system could not detect the small tumor shown in Figure 7B-a. In contrary, IL-13-Gd-Lip did detected the signal in Figure 7B, which was further proved by H&E staining that differentiated the tumor from the normal tissue. IL-13-Gd-Lip in Figure 7B-b confirms the tumor initiated by T3691 glioma stem cells in the mice by MRI as shown in Figure 7B (c and e). In addition, the FITC green color observed from the image of confocal

further confirmed that IL-13-Liposomes-Gd-DTPA crossed the BBB and entered the brain. IL-13-Liposomes-Gd-DTPA based contrast agent was able to detect glioma at its early stage in MRI as array pointed shown in Figure 6 B(b), which was further validated by the fluorescence detected on the histological image shown in Figure 7B(d) and H & E staining shown in Figure 7 B(c and e).

Figure 1

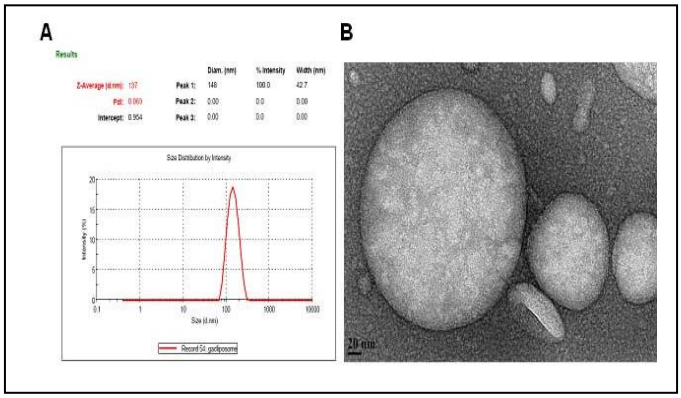
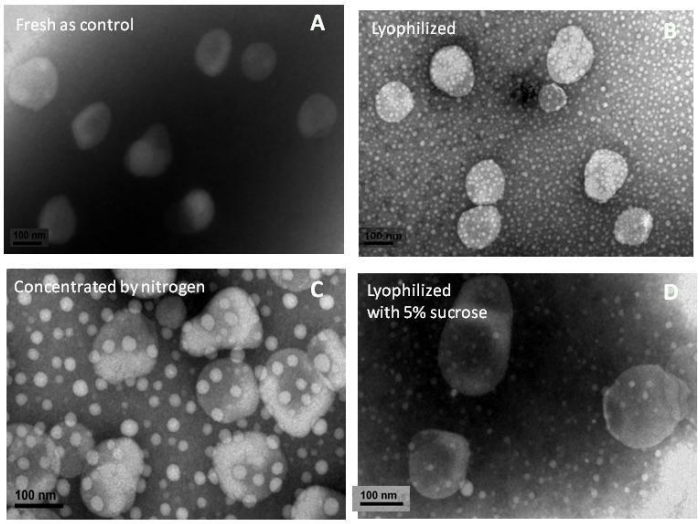


Figure 1. A. Particle size analysis performed on IL-13-LIP-Gd-DTPA B, Morphology of IL-13-Lip-Gd-DTPA as evidenced by Transmission Electronic Microscopy.

Figure 2



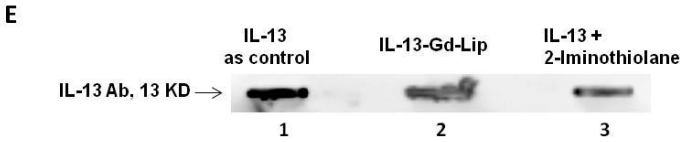


Figure 2. Morphology of IL-13 conjugated liposomal gadolinium as observed by TEM after lyophilization (B), concentration by nitrogen (C) and after lyophilization with 5% sucrose. Figure 2E indicates the images of slot blot Lane 1, recombinant protein IL-13 as control, lane 2, IL-13-Lip-Gd-DTPA, lane 3, IL-13-2-thiolated product an intermediate product of the conjugation.

Figure 3

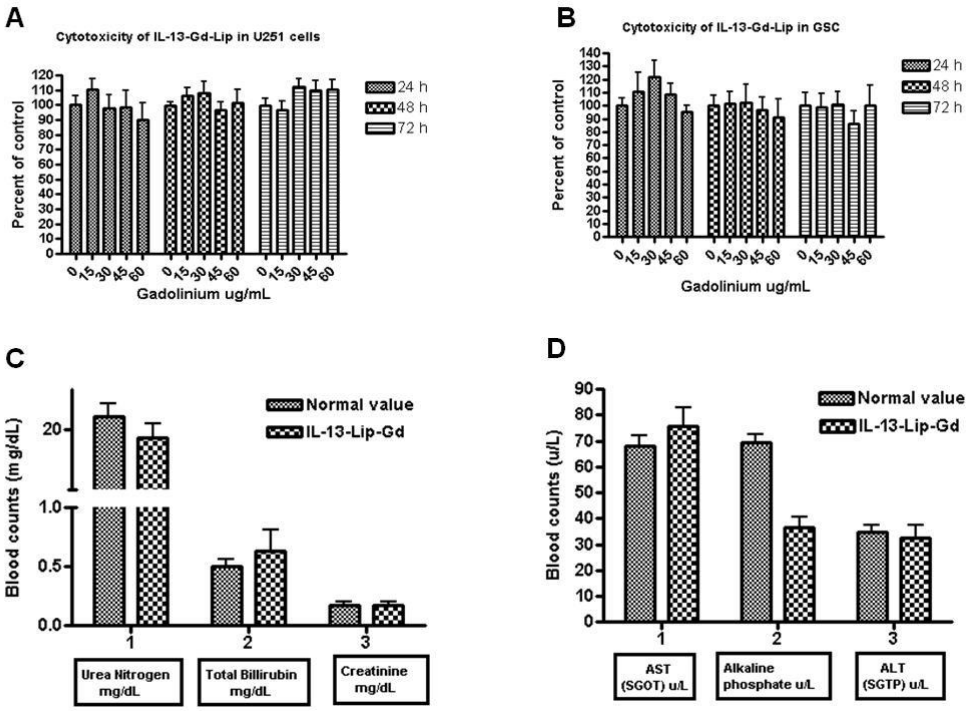


Figure 3. In vitro cytotoxicity assay of IL-13-Lip-Gd-DTPA *in vitro* A, in U251 cells. B, in GSC T3691. C, D, the graphs representing liver and kidney enzymes in the serum of the mice compared with the untreated mice as control. No significant increase was observed in five of the toxic markers index compared with the untreated mice as control (n=3 each condition).

Figure 4

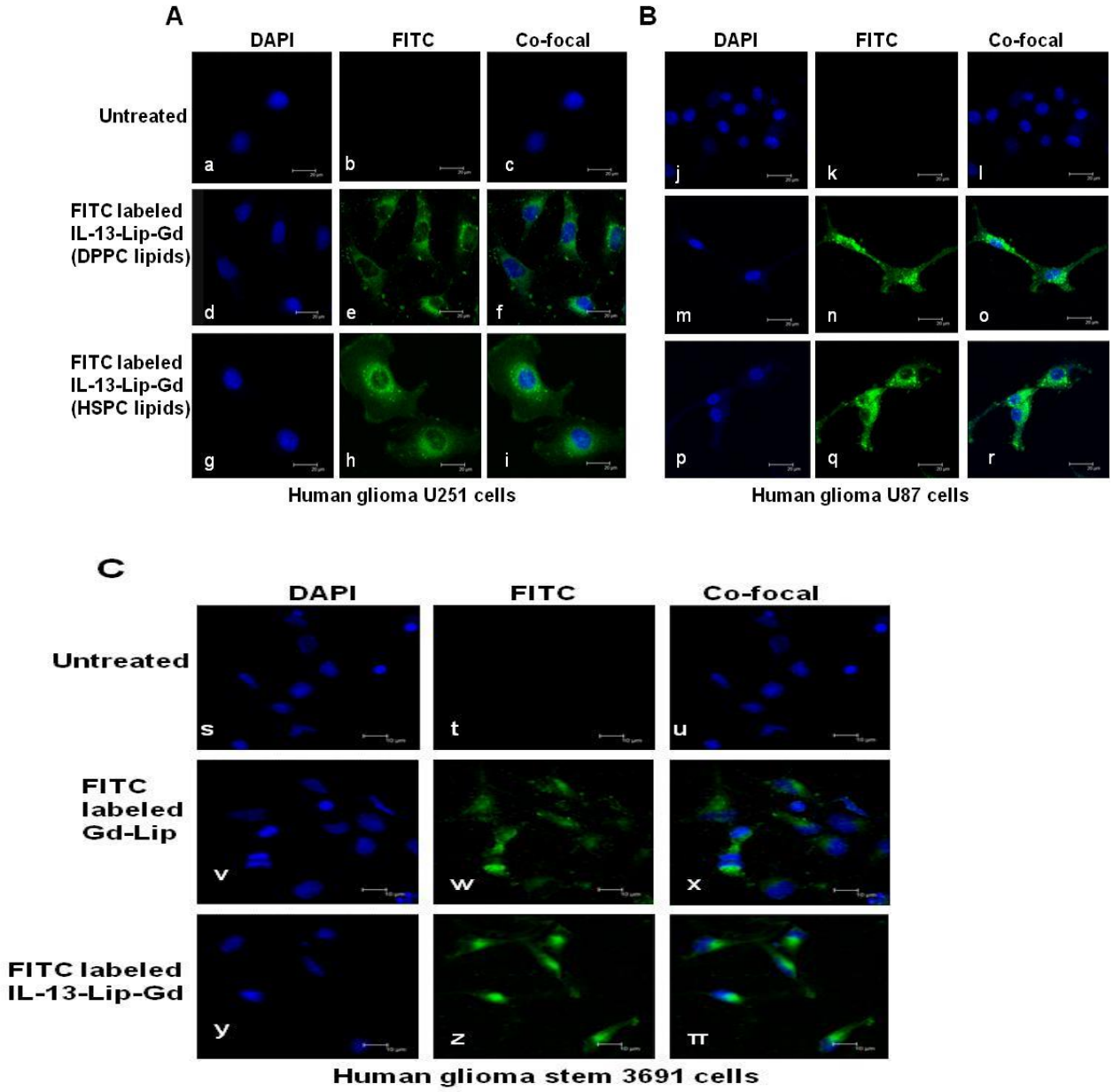


Figure 4. Internalization of the IL-13-Gd monitored by confocal microscopy. IL-13-Lip-Gd-DTPA complexes were internalized by human glioma cells (A, U251), (B, U87) and (C, T3691). Images of a, b, c, g, h, I, m, n and o represented untreated cells as control. images of (U251-e), (U87-k) and (T3961-g, t) represented that IL-13-Lip-Gd-DTPA was internalized as evident from green fluorescence. Blue color in images of a, d, g, j, m, p and s represented the nucleus stained with DAPI.

Figure 5.

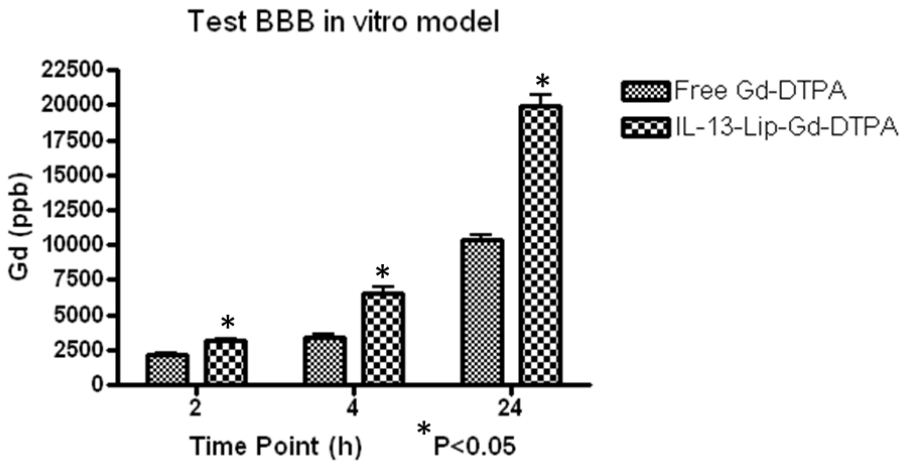


Figure. 5. IL-13-Lip-Gd-DTPA facilitated Gd crossing the BBB in an *in vitro* model viewed by the measurement of the concentration of Gd by ICP. The graph represented the concentrations of Gd at the bottom of the well at 2, 4 and 24 hour time point. The concentration of Gd was doubled 4 and 24 hours post exposure of IL-13-Lip-Gd-DTPA compared with Magnevist. Student-t test was conducted and the significant difference, *P<0.05, was observed for all three time points.

Figure 6

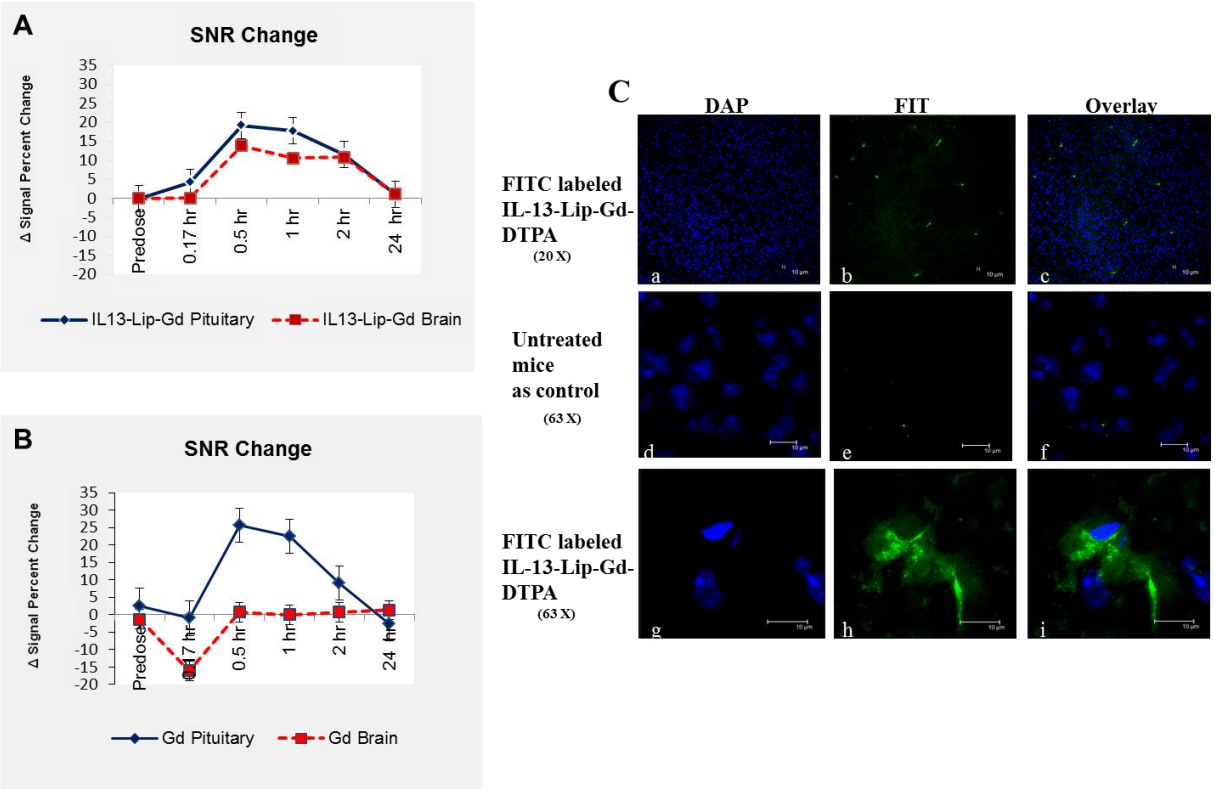


Figure 6. The SNR transformed by relaxivity in MRI illustrated penetration of brain tissue. . SNR in the location of pituitary gland (n=6). SNR in the location of brain parenchyma (n=6) A, IL-13-Lip-Gd-DTPA. B, Magnevist. Both IL-13-Lip-Gd-DTPA and Magnevist successfully entered the blood vessel and reached the A, SNR Increased for 5% captured in brain parenchyma indicating that IL-13-Lip-Gd-DTPA crossed an intact BBB and reached the parenchyma 30 minutes post the injection of contrast agent. B, Unchanged SNR captured in brain parenchyma indicated that Magnevist as a control did not cross the BBB and failed to reached brain parenchyma of an intact BBB 30 minutes post injection. C, IL-13-Lip-Gd-DTPA crossed an intact BBB of athymic nude mice examined by images of confocal microscopy. The images of the sectioned brain tissue from an intact BBB one hour post the IV injection of FITC-IL-13-Lip-Gd-DTPA (e, k, q and t) and FITC was observed indicating that IL-13-Lip-Gd-DTPA crossed an intact BBB. No FITC was observed in the images of untreated mice (b, h and n). Blue color in images of a, d, g, j, m, p and s represented the nucleus stained with DAPI.

Figure 7

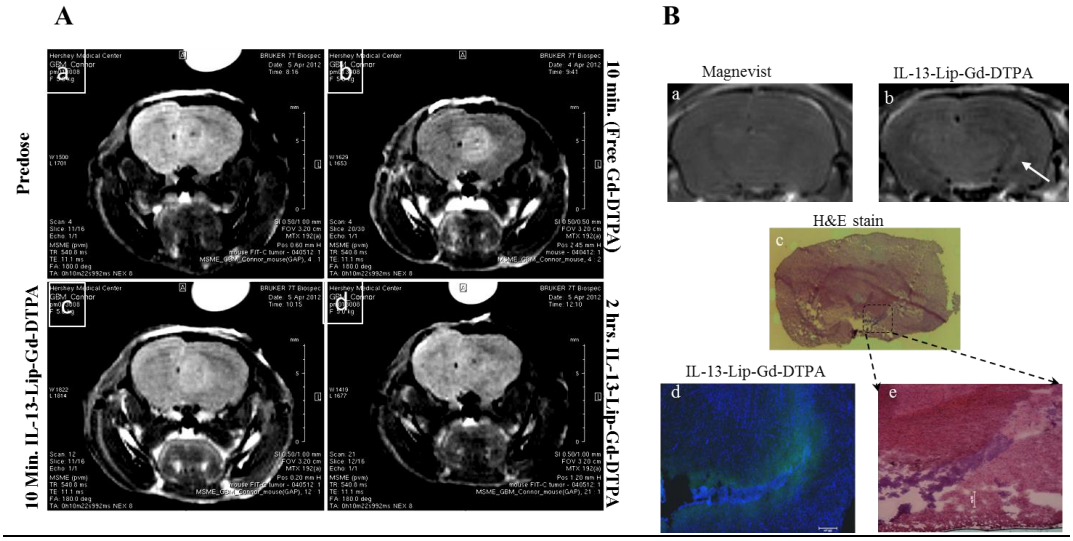


Figure 7. IL-13-Lip-Gd-DTPA detected glioma by T1-weighted images acquired on a 7T system MRI and H&E staining. A, image of pre-dose as a control, 10 minutes post injection of Magnevist as a positive control at day 1 and 10 minutes post injection of IL-13-Lip-Gd-DTPA at day 2. B, glioma cells was detected by IL-13-Lip-Gd-DTPA that was further confirmed by H&E staining. a, injection of Magnevist, b, injection of Glioma cells was detected by IL-13-Lip-Gd-DTPA. c and e, images of H&E staining and d, image of electron microscopy. The purple color in image of c, e and FITC color in image of d corresponded with the glioma cell shown with array in image b. The blue color in images of d represented the nucleus stained with DAPI and FITC represented the liposomes.

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Specific Aim 2: *Investigation of the ability of IL-13 linked semiconductor quantum dots to mark the tumor boundaries in the established glioma tumors*

It is strongly evident that the high grade brain tumors including GBM predominantly overexpress IL-13Rα2 receptor. The receptor expression level is not only restricted to the tumor, but also frequently observed in the cerebrospinal fluid and serum of the brain tumor patients exhibit an abnormal protein expression pattern. The soluble total protein in CSF has been reported to be elevated in about 71% GBM patients as observed by a study, in particular when the tumor is present in cerebral hemisphere (Merrit et al). Expression of several other protein biomarkers were identified previously in the serum of highly invasive breast and cervical cancer[1]. Certain growth factors like VEGF were found to be elevated in the serum of GBM patients [2]. The total concentration of the protein in CSF is only about 0.5% of that in plasma, due to the presence of blood CSF barrier. The nature and composition of such protein may shed light on the disease progression in the central nervous system. It is important to identify such soluble protein or receptor in the cerebrospinal fluid in the highly invasive cancer patients in a much more sensitive manner to minimize the metastasis to brain.

Nanoparticles are the particles in the size range of 1-200 nm, which has a variety of application in our physiological systems ranging from drug delivery to disease diagnosis. Nanoparticles possess unique physicochemical properties in solution and in biological system, which enables them to function as multifunctional agent. Recently there are several studies focusing on utilizing nanomaterials for imaging the tumor and the tumor vasculature. Although it is important to image them for initiating a therapeutic regimen, the treatment course in certain invasive and malignant cancers fails. This is mostly attributed to the metastasizing phenomena of the aggressive cancers to the central nervous system. It is inevitable to detect such cancers before metastasis to CNS from the peripheral site. During and before metastasis process, the tumor cells secrete certain soluble factors and lipid vesicles called exosomes in the serum and cerebrospinal fluid. If a nanotechnology based method is devised to identify them and these soluble factors are detected at the premalignant or benign state, it is possible to control the progression. IL-13Rα2 receptor is an oncogenic receptor expressed in several malignant cancers like GBM, ovarian

cancer and renal cell carcinoma. IL-13Rα2 receptor expression itself marks the tumor malignancy and invasiveness. In our own study earlier, we demonstrated the therapeutic potential of this marker in the form of IL-13 linked nanovesicles[3,4]. It is highly plausible that such invasive cells secrete soluble receptors either from the circulating tumor cells or from the exosomes. The goal of our present investigation is to utilize quantum dot based ligand targeted nanoparticles to assess the expression of receptors in the cells and in the soluble media which could find immense application in the detection and characterization of tumor cells and the soluble factors secreted by them in the biological fluid.

Materials and Methods

Materials

CdSe quantum dots surface modified with carboxylic acid functional groups used for our studies were purchased from Ocean Nanotechnology, Inc. Interleukin-13 protein used for our studies was expressed and purified in our laboratory following the method as described earlier. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), a water soluble carbodiimide was purchased from Sigma chemicals. Sephadex G25M column was purchased from Amersham Pharmacia, Inc. YM-30 centrifugal concentrators were from Amicon, Inc. Human glioma cells U251 was from ATCC and the glioma stem cells T3691 was a gift from Dr. Jeremy Rich, (Lerner Research Institute, Cleveland Clinics).

Conjugation and characterization of IL-13 quantum dots

The carboxylated quantum dots were conjugated to IL-13 protein by EDC chemistry. Briefly 8 µM quantum dots were reacted with IL-13 protein in the presence of EDC for 1 hour at room temperature. The reaction was stopped by treating them with these quantum dots were enclosed with CdSe in the core and ZnS covering them protecting the leaching of Cd from the quantum dot. The surface of the quantum dots was decorated with polyethylene group for enhanced circulation half-life in the biological system (Figure 1). The quantum dots were purchased from Ocean Nanotech Inc. Human recombinant IL-13 protein was expressed in E.Coli and purified in our laboratory using a plasmid encoding the protein and purified using Ni column as described by us previously.

IL-13 protein was conjugated to the carboxylic acid groups by EDC chemistry using water soluble carbodiimide (EDC) as conjugating agent. After conjugating IL-13, the particles were confirmed for their conjugation by agarose gel electrophoresis method. The particle sizes before and after conjugation was also verified using zetasizer. Surface morphology was also characterized using Atomic Force Microscopy.

AFM imaging

Surface morphology

The images of the quantum dots were obtained using a Multimode AFM with a Nanoscope IIIa control system (software version 5.12r3, Digital Instruments, Santa Barbara, CA) operating under tapping mode (intermittent contact). The image acquisition was performed under ambient conditions. For the tapping mode images, a moderate tapping force of r_{sp}= 0.75 was applied between the probe and the particles where r_{sp} =A/A0 (set point amplitude/free amplitude of oscillation where the free amplitude was set to 20nm in our experiment). Using a standard silicon

tapping probes (spring constant, k= 20-75 N/m, NSC15, MikroMasch, Wilsonville, OR) topographic images were recorded in ambient conditions.

Force map investigation

IL-13 conjugated quantum dots were covalently coupled to AFM probes which bears si3N4 triangular cantilevers (Veeco Instruments, Santa Barbara, CA). Before performing the conjugation, the probe was functionally modified to bear amino groups by treating them in a 1% (v/v) solution (in ethanol) of aminopropyltriethoxysilane (Gelest, Inc., PA) for 1h at room temperature. The probes were then extensively washed with Millipore water and treated with 10% glutaraldehyde for 1h at room temperature. The probes were then again washed with Millipore water to remove the non-specifically bound glutaraldehyde. The activated probe was then incubated in IL-13 conjugated quantum dots at a concentration of 20nM for 1h. It has been previously reported that this method of attachment of protein provides sufficient flexibility for the protein to orient themselves for proper binding.

Similarly to determine the functional integrity of the IL-13 protein conjugated to quantum dots, a force of interaction between the IL-13QD and the glioma cells were determined. For this method quantum dots were bound to the AFM probe and the U251 glioma cells were cultured on a coverslip in complete medium. After 24 hours of culture when the cells were about 90% confluent the force of interaction was studied using the Atomic force microscopy by tapping mode contact method.

In vitro studies

Binding of IL-13QD to the glioma cells

In vitro binding experiments were performed using targeted (IL-13 conjugated quantum dots, IL-13QD) and non-targeted quantum dots on U251 human glioma cells (ATCC Inc.) and T3691, a glioma stem cell (gift from Dr. Jeremy Rich, Cleveland Clinics). Previously all the cell lines used for the study were confirmed for the expression of IL-13Rα2 receptor by immunoblots of the respective cell lysates. For the binding studies 20,000 cells per well were plated in a chamber slide which was cultured overnight at 37°C. These cells were exposed to blocking buffer to remove any non-specific binding followed by 100 fold excess of IL-13 protein for 1h. These cells were then exposed to quantum dots at 1-20 nM concentration at 4°C for 10 minutes. After the DAPI staining the slides were gel mounted and dried. The media was aspirated and fixed on coverslips and dried overnight before microscopic observation.

Agarose gel retardation assay to monitor the presence of soluble receptor

To determine the ability of the IL-13 conjugated quantum dots to complex with the soluble IL-13Rα2 receptor, a complexation assay was performed in the presence of IL-13QD and IL-13Rα2 receptor protein. In this experiment 20nM concentration of the IL-13QD and non-targeted (unconjugated) quantum dots were treated with the IL-13Rα2 receptor protein and were subjected to gel electrophoresis using 1% agarose. Uncomplexed IL-13QD and non-targeted QD served as negative control. The electrophoresed gel was imaged using Fuji imager.

Flow cytometry

For determining the *in vitro* potential of the IL-13QD to detect the soluble receptors present in an aqueous media, flow cytometry assay was performed. To mimic the biological fluid containing

the soluble IL-13 receptor, a simulated medium was created by suspending IL-13Rα2 receptor protein at various concentrations in phosphate buffered saline. The simulated media was subsequently exposed to 20 nM concentration of IL-13QD and incubated at room temperature for 30 minutes. A flow cytometry was performed to determine the aggregation property of the IL-13 conjugated quantum dots and the gated population where the aggregates occur were determined and quantified using the flow cytometry software.

In vivo studies

Subcutaneous glioma tumor mouse model

Subcutaneous glioma tumor was established in the athymic nude mice by injecting 15x106 cells in the flank and allowed to grow for 3 weeks. Once the palpable tumor started appearing, IL-13 conjugated QD (20nM) was injected into the tail vein of the subcutaneous glioma tumor bearing athymic nude mice. About 48 hours after injection, the mouse was anesthetized by administering ketamine-xylazine mixture and imaged through IVIS imaging system in various positions.

Intracranial glioma tumor mouse model

Intracranial glioma tumor mouse models were established in the athymic nude mouse by injecting T3691 glioma stem cells (50,000 cells) in a volume of 10µl and injected in the caudate putamen region as described in our earlier publication. IL-13QD was injected through tail vein and the animals were imaged by IVIS system.

Results

Protein conjugation and characterization of quantum dots

IL-13 protein was conjugated effectively on the pegylated quantum dots by EDC chemistry as evidenced by gel electrophoresis (Figure 1). The size of the quantum dots were uniformly distributed as evidenced by particle size analyzer (zeta sizer) and by atomic force microscopy (Figure 2). On an average the mean diameter of the IL-13 conjugated quantum dots was about 24 nm whereas that of the non-targeted quantum dots was 12 nm.

Binding of IL-13QD with the glioma and glioma stem cells

IL-13 QD was able to bind effectively and selectively to the glioma stem cells as observed under fluorescence microscopy (Figure 3A & 3B); whereas the sham control and CD20 conjugated quantum dots were not able to bind to the cultured glioma cells. When the receptor sites on the tumor cells were blocked with 100 fold molar excess of recombinant IL-13 protein, no binding of IL-13QD was observed. Similarly even in the glioma stem cells (T3691) the quantum dots were able to bind and be internalized in the cytoplasmic region as seen in the figure 3B but not in the unconjugated QD.

The force of interaction between the IL-13QD and the glioma cells

The atomic force microscopic investigation revealed significant interaction between the IL-13QD which is bound to the AFM probe and the U251 glioma cells cultured in the coverslips even at a minimum time of exposure of 1 second (Figure 5A). In contrast the force curve generated in the presence of non-conjugated quantum dots were smooth without significant force of interaction between the quantum dots and the glioma cells (Figure 5B)

In vitro binding of IL-13QD in the cell lines and in the patient derived glioma stem cells:

When observed under fluorescence microscopy binding of IL-13QD to the glioma cells and T3691 glioma stem cells were evident (Figure 3), whereas the sham control and CD20 conjugated quantum dots were not able to bind to the glioma tumors. When the receptor sites on the tumor cells were blocked with 100 fold excess of IL-13 protein, no binding of IL-13QD was observed. The binding study clearly demonstrated the receptor specific binding of IL-13 conjugated quantum dots to the glioma cells. The binding study clearly demonstrated the receptor specific binding of IL-13 conjugated quantum dots to the glioma cells. These binding is also receptor mediated which is evident from the fact that the IL-13QD could not bind the stem cells when the receptor sites are either blocked with 100 fold excess of IL-13 protein or when the cells were treated with non-conjugated quantum dots.

Detection of soluble receptor in the media by IL-13QD

Based on the gel retardation assay IL-13 conjugated QD tends to retard the mobility of the electrophoretic band upon complexation with IL-13Rα2 receptor protein compared to that of the uncomplexed QD. However in the case of unconjugated QD, the shift in the band between the complexed and uncomplexed form is minimum as evident from figure 6A &6B.
In addition, based on the flow cytometry assay on quantum dots after exposure to various concentrations of IL-13Rα2 chimeric protein (recombinant), a concentration dependent aggregation pattern of the IL-13QD is evident from the gated population R1(figure 4). Here a simulated medium was prepared by suspending the IL-13Rα2 receptor protein at various concentrations in the presence of 20μM of quantum dots. The percentage of gated population increases with increasing concentration in the picomolar range.

In vivo distribution of IL-13quantum dots

Nude mice injected with IL-13 conjugated (non-pegylated) quantum dots in the tail vein showed random distribution after 24 Hours (Figure 8) (panel A). Mice injected with IL-13 conjugated pegylated quantum dots in the tail vein showed random distribution after 24 hours with some limited accumulation in the tumor (Panel B). Mice injected with IL-13 conjugated (non-pegylated) quantum dots intraperitoneally (left) and intratumorally (right) showed limited distribution of the particles after 3 days in both cases.

When the pegylated IL-13 conjugated QD was injected through tail vein, the quantum dots were observed to accumulate around the tumor after 2-6 hours (Figure 9). But significant accumulation in the liver and spleen was also observed. The top and bottom panel represents the IL-13 QD distribution in different heat map visualization.

Discussion

One of the major tasks in the area of cancer therapy is to determine the cancer progression at the premalignant and early stage. Although several markers exist for cancer, very few are specific for malignant glioma. Identification of a marker at the tissue and cellular level is a common phenomenon followed universally by monitoring the protein or gene expression in the tumor tissues which can be either obtained during the biopsy or surgery from the operating room. However, very few studies were focused on the cancer associated markers in the biological fluids like blood, urine and cerebrospinal fluid, in particular for malignant cancers like GBM. IL-13Rα2 is one such marker which is expressed in several malignant tumors and a distinct marker for the malignancy and invasiveness.

One of the salient features that prompted us to utilize quantum dots for our present investigation is the optimal size distribution 10-30nm and its distinct fluorescence properties which would allow us to monitor their dynamics in the membrane and in solution form. The size of the quantum dots in our case increases upon conjugation with IL-13 protein from 12 nm to 24 nm, which is about 100% increase from their original size. The larger size also means their decreased cellular toxicity as evidenced previously with quantum dots. Furthermore unlike the lipid based nanoparticles which possess certain degree of non-specific interacting nature, these semiconductor based quantum dots virtually possess no affinity towards the cells. This is evident from our studies where the IL-13 conjugated quantum dots possess selective binding affinity to the glioma cells and glioma stem cells expressing the oncogenic IL-13Rα2 receptor. The specificity is more evident from the negligible binding of the unconjugated quantum dots to the glioma cells and also by the fact that when receptors were blocked with 100 fold excess of its ligand (IL-13 protein), the binding affinity of the quantum dots to the glioma cells was completely abrogated. Our second line of evidence arises from the fact that CD20-Ab conjugated quantum dots were not able to bind to the glioma cells. As CD20 is a B-lymphocyte antigen predominantly activated in lymphoma but not in other cancers, this observation is not surprising as one cannot anticipate CD20-Ab to bind to glioma tumors which does not express CD20. In the in vivo experiment in the GBM mouse models a tumor specific binding in the subcutaneous tumor was not observed as evidenced by intravital imaging spectroscopy (IVIS) (Figure 8). IL-13QD was able to locate the intracranial glioma tumor with significant localization in the spleen (Figure 9). In spite of its specificity, the serum chemistry panel of the IL13QD injected mice indicates increased levels of aspartate aminotransferase (AST) and blood urea nitrogen(BUN) indicating an elevated kidney and liver toxicity of the quantum dots. Possibly this is caused by the presence of cadmium in the composition of quantum dots. Recent literature indicates a possibility of synthesizing non-toxic quantum dots using silicon. Utilization of such non-toxic quantum dots to target the tumor would presumably eliminate the in vivo toxicity.

These semiconductor nanoparticles by nature possess ability to aggregate in the presence of certain physicochemical changes of the environment in which they are suspended. This property can be exploited in the detection of traces of certain antigens in a media if the quantum dots were linked to suitable antibody.

IL-13-QD + IL-13Ralpha2 (soluble) → aggregates (Figure 6B)

(at pM and fM concentration)

IL-13QD acts as a host in the biological medium and in the circulation when they intercept IL-13Rα2 receptor, which also acts as a guest, these semiconductor nanoparticles aggregate around the receptor (figure 6) leading to the formation of host-guest complex. This behavior is evident from the enhanced aggregation potential of the IL-13QD in the presence of the soluble IL-13Rα2 receptor protein which was also confirmed by gel retardation assay, where the IL-13QD after complexation with the soluble IL13Rα2 protein moves slower in the 1% agarose gel compared to the uncomplexed IL-13QD (quantum dots without treatment with IL13Rα2 receptor protein) (Figure 6A).Interestingly, with the non-targeted quantum dots there is not much difference in the gel shift pattern between the uncomplexed and complexed quantum dots, indicating that

conjugation of IL-13 improves the QD's aggregation in the presence of the IL-13Rα2 receptor protein. We determined the presence of IL-13Rα2 receptor (soluble) in some of the CSF samples from the brain tumor patients by immunoblot (figure 7B) and ELISA (Table 1). ELISA method was able to detect the presence of soluble receptor only in few patients samples but non-detectable in others. But the presence of the receptor in the CSF is evident after differential centrifugation of the CSF and subsequent immunoblotting. This demands the development of more sensitive method to detect the presence of the soluble IL-13Rα2 receptor protein in the biological fluid at subnanomolar concentration. Based on our flow cytometric assay it is possible to identify the presence of the receptors in the sub nanomolar concentration.

The in vivo experiments indicate that it is harder to reach the tumor when the location of the tumor is subcutaneous. This is evident from the random distribution of the pegylated IL-13 conjugated quantum dots as observed under IVIS. However, in the case of intracranial glioma tumor mouse model, accumulation of the quantum dots in the tumor was much more efficient than subcutaneous tumor model. However, the distribution of quantum dots was also observed to some extent in the liver and spleen. Also based on the serum chemistry analysis with the quantum dot treated mice, the liver and kidney enzymes like Aspartate aminotransferase (AST), Alaninine aminotransferase (ALT) and blood urea nitrogen (BUN) were elevated compared to the untreated control mice indicating the possibility of toxicity of quantum dots. However this toxicity is most probably attributed to the presence of cadmium in the quantum dots. It is also possible to design similar quantum dots by using alternate non-toxic semiconductor materials like silicon in the place of cadmium.

Thus in conclusion our investigation with the quantum dots confirms the specific binding property of the IL-13QD towards the tumor cells expressing IL-13Rα2 receptor and also with tumor initiating glioma stem cells, by means of which it is possible to detect the tumor initiating stem cells with high selectivity and sensitivity. The tumor targeted quantum dots demonstrate potential to utilize them as a tumor marker agent in the intracranial mouse model. Future plans would be to develop non-toxic silicon based quantum dots to mark the tumor much more effectively. Secondly, even when the receptors are present in the soluble form, it is possible to detect them using IL-13QD exploiting the property of these quantum dots to aggregate. Optimization of this method would find immense potential to detect the soluble IL-13Rα2 receptor in the CSF of the patients at the sub-nanomolar concentration which would serve as a prognostic marker.

Figures

Figure 1

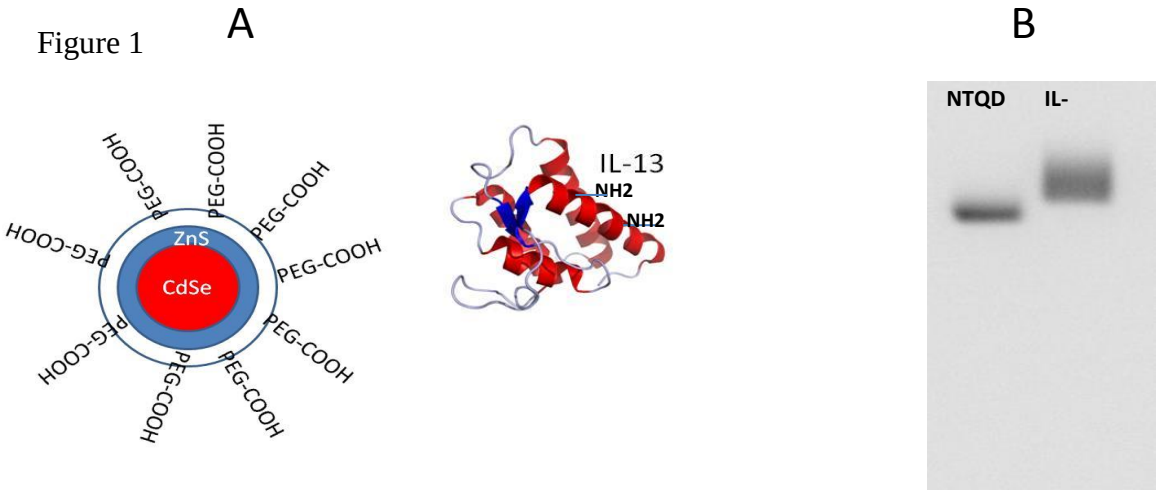


Figure 1. A. Pegylated quantum dots were conjugated to recombinant IL-13 protein by EDC chemistry by utilizing the carboxylic acid groups in the quantum dot and the amino groups on the lysine residues in the IL-13 protein. B. The conjugation of IL-13 to the quantum dots was confirmed by agarose gel electrophoresis.

Figure 2

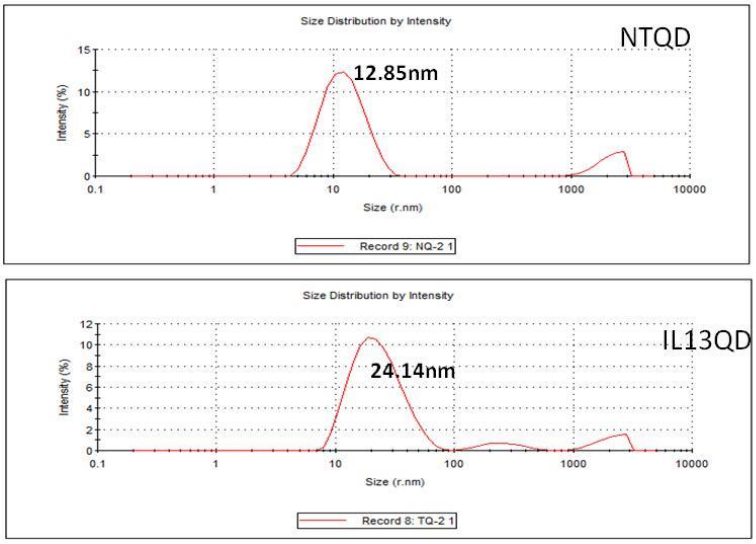


Figure 2. IL-13 conjugated quantum dots had uniform particle size distribution, with an average particle size of 12.8nm for non-targeted quantum dots and 24.14 nm for the IL-13 conjugated quantum dots.

Figure 3A.

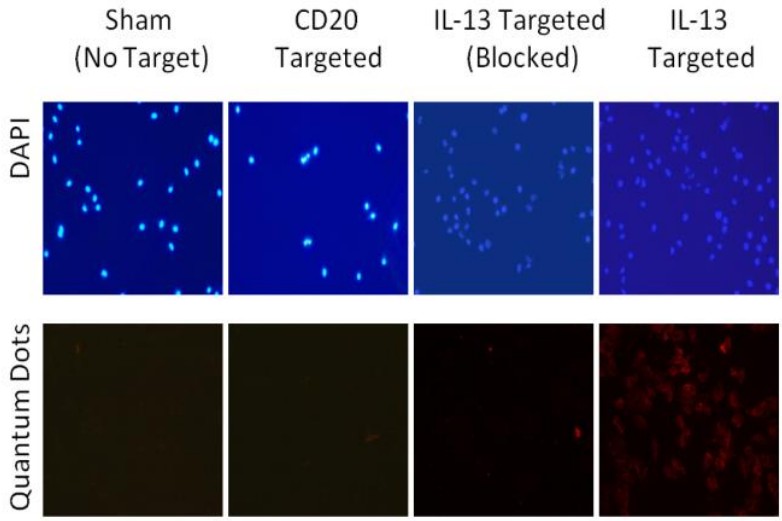


Figure 3A. Fluorescence Microscopy: Three control conditions demonstrate specific binding of IL-13 targeted quantum dots to U251 glioma cells. Sham quantum dots with no targeting ligand, CD20-targeted quantum dots, and IL-13-targeted quantum dots with blockade using 100-fold excess IL-13, all showed virtually no binding due to competitive binding by excess IL-13. In contrast, IL-13-targeted quantum dots bind avidly to U251 cells when the receptor was available for binding.

Figure 3B.

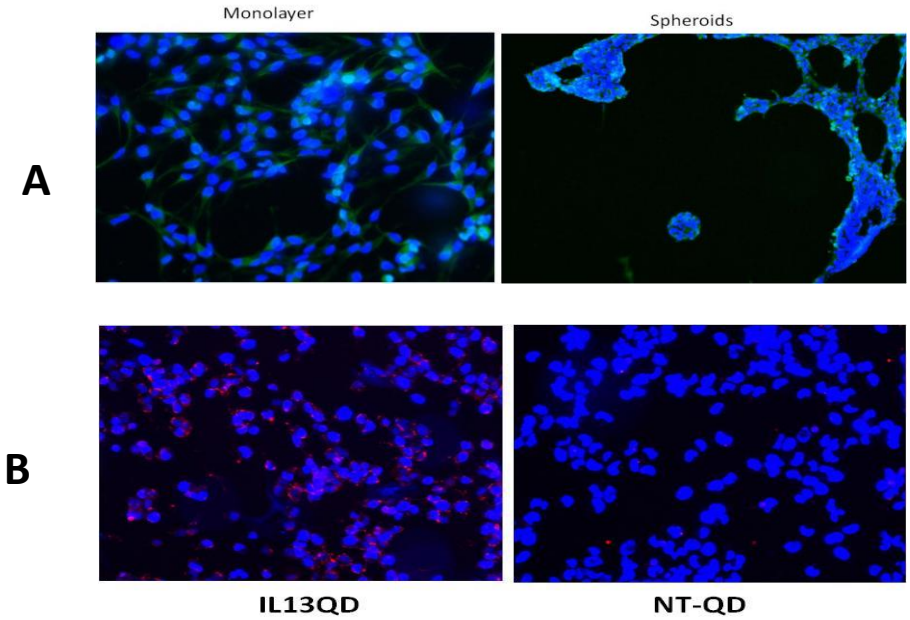


Figure 3B. IL-13QD selectively binds the glioma stem cells which were confirmed to express IL-13Rα2 receptor as evidenced by immunohistochemistry. Panel A-T3691 glioma stem cells in the monolayer and spheroid form express IL-13Rα2 receptor (green) and the DAPI stains the nucleus (blue).Panel B-Binding of IL-13QD to T3691 glioma stem cells but non-targeted quantum dots were not able to bind to the glioma stem cells.

Figure 4.

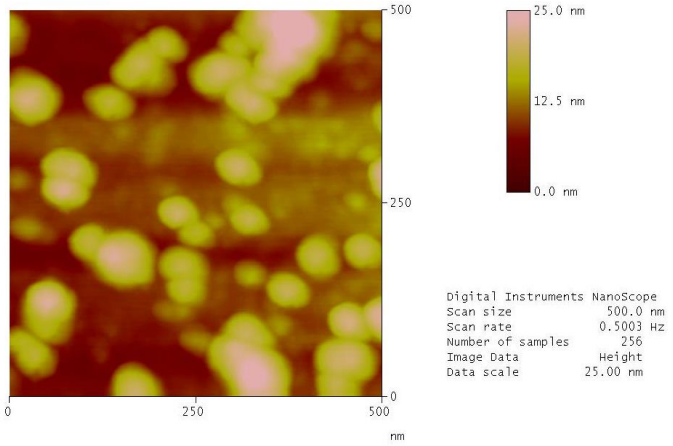
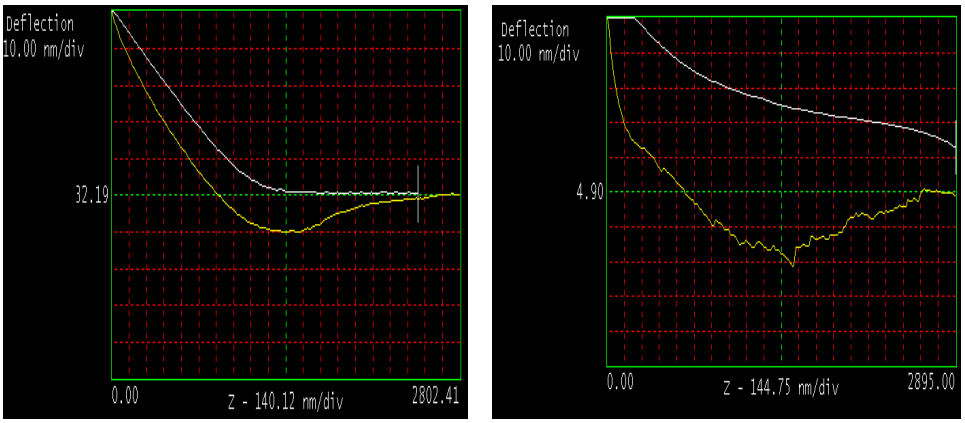


Figure 4. The spherical morphology of the IL-13QD as evidenced by Atomic Force Microscopic imaging (tapping mode)

Figure 5A.

A B

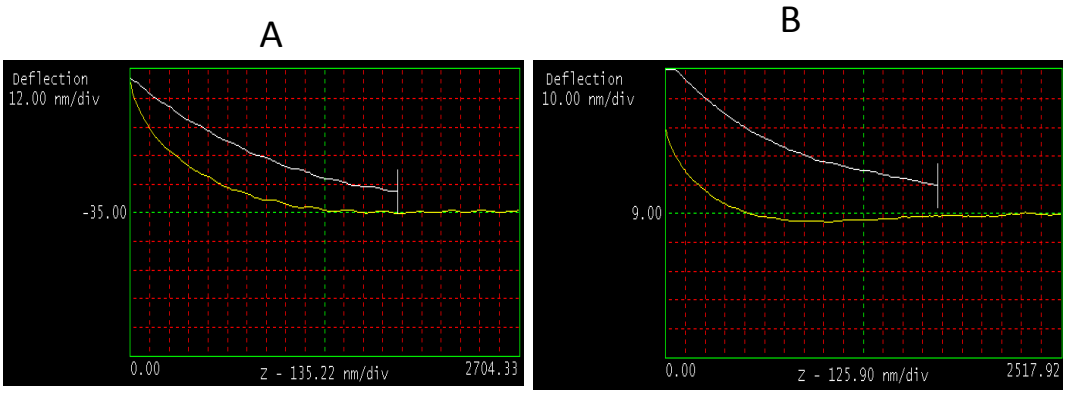


IL-13QD and U251 cells (time of exposure 0 secs)

IL-13QD and U251 cells (time of exposure 1 sec)

Figure 5A. Force curve generated after permitting the interaction of the IL-13 QD's fixed on the atomic force microscopy probe (AFM) with the U251 glioma cells without (0 secs)(A) and with 1 sec exposure (B) demonstrates an increased force of interaction between the probe and the cells.

Figure 5B.



Non-targeted QD and U251 cells (time of exposure 0 secs)

Non-targeted QD and U251 cells (time of exposure 1 sec)

Figure 5B. Force curve generated after interaction between the non-targeted QD's fixed on the atomic force microscopy probe (AFM) and the U251 glioma cells without (t=0 secs)(A) and 1 sec exposure (B). A smooth curve demonstrates negligible force of interaction between the AFM probe and the receptors on the cells. It is also clear that there is a time dependent increase in the force of interaction between the IL-13QD and the receptors on the cells.

Figure 6.

A **B**

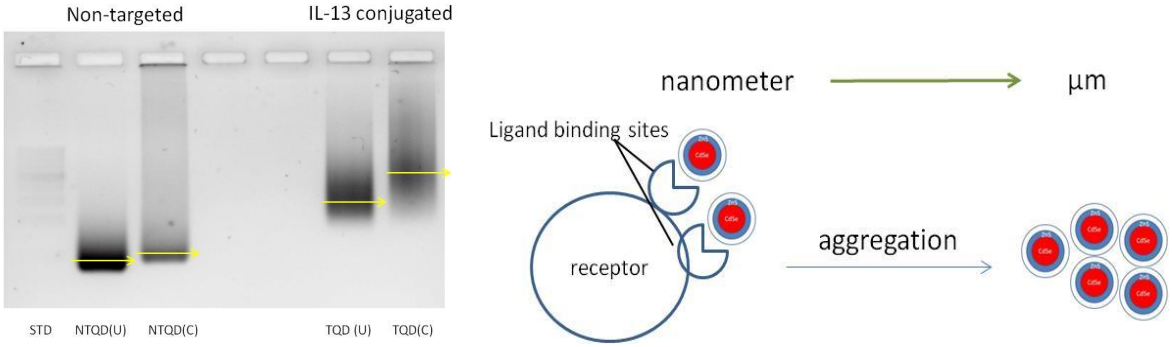


Figure 6. Agarose gel (1%) indicates a diffused pattern of targeted quantum dots indicating a variable extent of aggregation of the IL-13 conjugated quantum dots in the presence of IL-13Rα2. U-uncomplexed quantum dots, T-quantum dots after complexing with IL-13Rα2 receptor protein (nanomolar). B- illustration showing the binding of ligand conjugated quantum dots to a receptor with more than one binding site will lead to aggregation phenomenon.

Figure 7A.

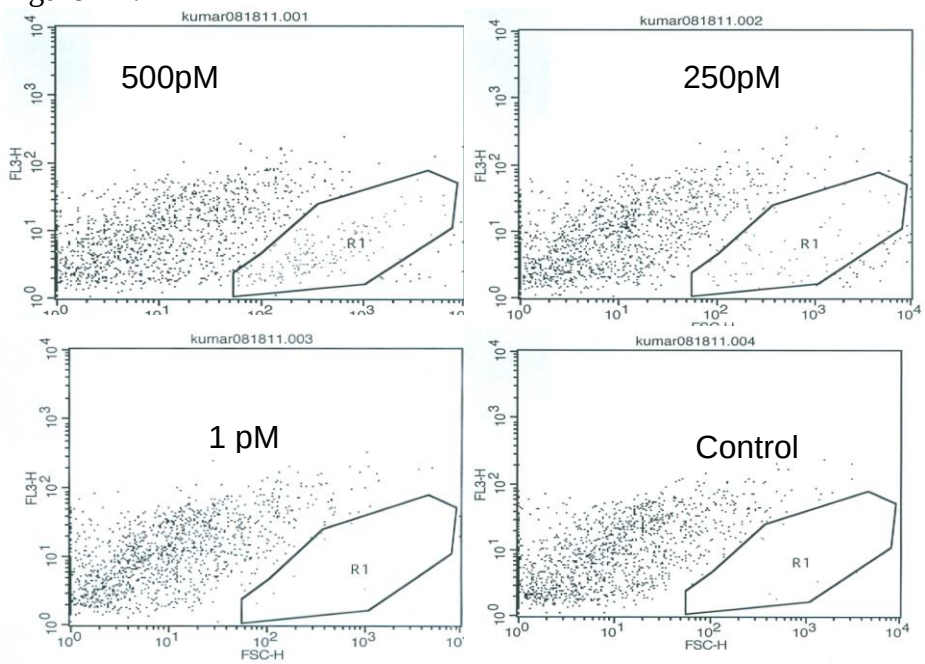


Figure 7A. Flow cytometric images of IL-13QD after exposing them to IL-13Rα2 receptor in various concentrations (1pM, 250 pM, 500 pM) at room temperature for 2h. The gated population (R1) represents the formation of aggregates, which would indicate the presence of soluble receptor.

Figure 7B

Figure 7B. IL-13Rα2 expression (52kDa) in the cerebrospinal fluid from several brain metastatic brain tumor patients as evident from the differential centrifugation of the CSF samples. ELISA performed on the CSF was able to be detect the receptor expression in few samples (Table 1).

Figure 8

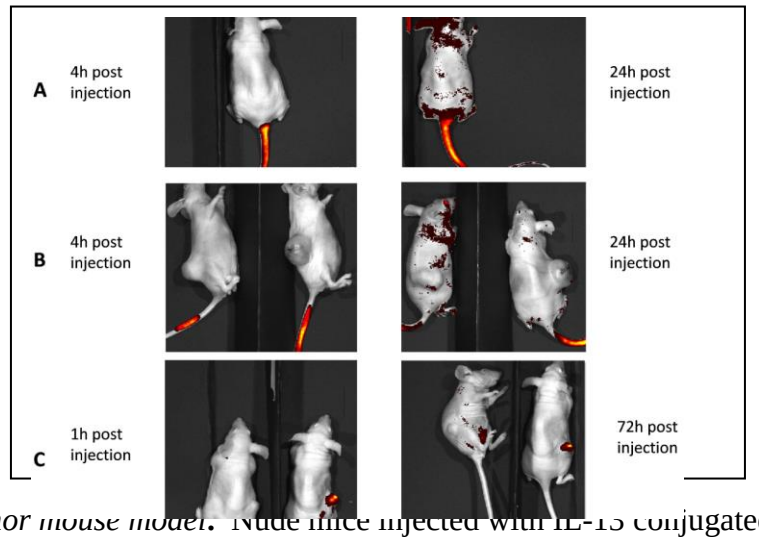


Figure 8. *Subcutaneous glioma tumor mouse model.* Nude mice injected with IL-13 conjugated (non-pegylated) quantum dots in the tail vein showed random distribution after 24 Hours (panel A). Mice injected with IL-13 conjugated pegylated quantum dots in the tail vein showed random distribution after 24 hours with some limited accumulation in the tumor (Panel B). Mice injected with IL-13 conjugated (non-pegylated) quantum dots intraperitoneally (left) and intratumorally (right) showed limited distribution of the particles after 3 days in both cases.

Figure 9. IVIS imaging of the glioma stem cell induced orthotopic GBM mouse model (intracranial glioma tumor) after injecting with IL-13 conjugated quantum dots. The mice were earlier confirmed for the tumor formation by MRI.

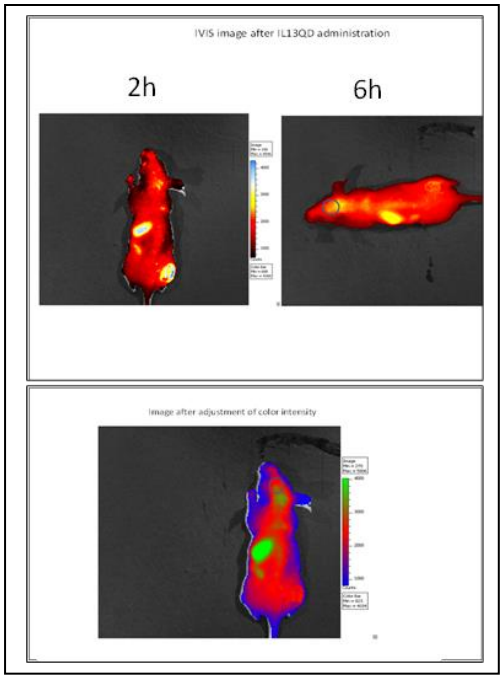


Figure 10.

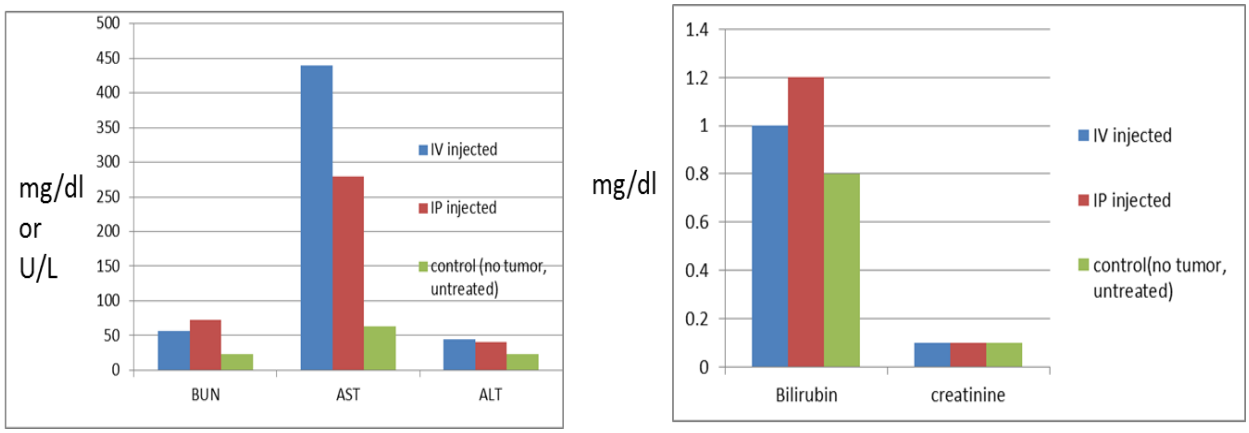


Figure 10. Serum chemistry in the mice 72 hours after injecting the IL-13 QD in normal mice

Table 1. The expression of IL-13Rα2 receptor levels in CSF as quantified by ELISA method.

Patient ID	**Concentration (ng/mL)**
P466	Non detectable
P468	Non detectable
P474	1.969058
P475	0.022291
P476	-0.22858
P477	Non detectable

Presentations or abstracts at national meetings

1. Cody Weston, Achuthamangalam Madhankumar, Jennifer Baccon, Michael Glantz, James Connor, Quantum Dot Probes for Glioma Cell detection", , 87th Annual meeting of American Association of Neuropathologists, June 2011, Seattle, Washington

2. "Direct in vivo evidence for the penetration of blood-brain barrier with the nanoliposomes", Xiaoli X. Liu, Achuthamangalam B. Madhankumar, Patti A. Miller, Kari A. Duck, James R. Connor, and Qing X. Yang, 20th Annual Meeting of the International Society for Magnetic Resonance in Medicine (ISMRM), Australia, May 2012

3. A.B.Madhankumar, Becky Slagle-Webb, Sheehan, J., Cody Weston, Siedlecki, C., Lichong, Connor, R. Development of receptor targeted quantum dots as an in vitro and in vivo diagnostic agent for GBM, Annual meeting of the Society for Neuro-oncology, San Francisco, CA, Nov 2013

References
1. Wang, Y.S., *et al.* Altered protein expression in serum from endometrial hyperplasia and carcinoma patients. *J Hematol Oncol* **4**, 15.
2. Takano, S., *et al.* Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. *Cancer Res* **56**, 2185-2190 (1996).
3. Madhankumar, A.B., Slagle-Webb, B., Mintz, A., Sheehan, J.M. & Connor, J.R. Interleukin-13 receptor-targeted nanovesicles are a potential therapy for glioblastoma multiforme. *Mol Cancer Ther* **5**, 3162-3169 (2006).
4. Madhankumar, A.B., *et al.* Efficacy of interleukin-13 receptor-targeted liposomal doxorubicin in the intracranial brain tumor model. *Mol Cancer Ther* **8**, 648-654 (2009).
5. Park JH, Gu L, von Maltzahn G, Ruoslahti E, Bhatia SN, Sailor MJ. Biodegradable luminescent porous silicon nanoparticles for in vivo applications. *Nat Mater.* 2009;8:331-336

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

____Yes
__X_No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
_X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving

Research Goals, Objectives and Aims. For example, the number of eligible subjects approached the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involves, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

As our project involves 2 different research areas namely IL-13 conjugated liposomes and quantum dots for glioma tumor diagnosis and imaging, we believe that we have sufficient

data for at least two manuscripts. Our first manuscript focuses on formulation, characterization and glioma tumor diagnosis by utilizing IL-13 Liposomes-Gad. As we described above in our progress report we successfully encapsulated gadolinium in IL-13 conjugated liposomes and the resulting liposomes shows improved contrast property and our in vivo data shows the ability of the liposomes to target the tumor crossing the blood brain barrier.

In our second project utilizing the Cadmium and Selenium based quantum dots, we conjugated IL-13 and demonstrated the binding affinity of the resulting quantum dots towards the glioma stem cells and established glioma cell lines like U251. The material was characterized for its ability to detect the receptor qualitatively and quantitatively in the cells and simulated medium containing soluble IL-13Rα2 receptor. A new method of identification of soluble receptor in the biological fluid using the quantum dots was established. A manuscript is in preparation for publication in a journal.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The outcome of the research projects will be a development of novel nano particles that can be used in the detection of glioma tumors in a sensitive manner by in vivo in a preclinical model which will be translated in large animal model. Utilizing our model, it is possible to detect the stage of the disease based on the receptor expression in the biological fluid.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

In our first project, tumor targeted liposomes encapsulated with magnevist, an MRI contrast agent, were formulated and demonstrated to have significant signal to noise ratio compared to free magnevist in vitro. We also demonstrated the tumor specific binding property. There is an increased signal to noise ratio change in the brain compared to pituitary region in the IL-13-Liposome-Gd group compared to free magnevist group in the brain parenchyma indicating the efficacy of the IL-13-Liposome-Gd. Similarly the effectiveness of the IL-13-Liposome-Gd for in vivo imaging was also verified.

In our second aim, IL-13 QD was demonstrated to be an effective agent in increasing the

tumor binding property in the in vitro cell culture models and in vivo mouse model and their ability to be detected by IVIS imaging. We also investigated the tumor associated receptor binding properties of the IL-13 QD soluble media by monitoring the aggregational property of the quantum dots

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes _____ No ___X___

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes___x_____ No_____

If yes, please describe your plans:

We are planning to utilize IL-13 QD as a detection agent for soluble receptor (IL-13Rα2) in the biological fluid based on the aggregation potential of the IL-13QD. For this we will analyze the CSF from number of brain tumor patients and will measure the presence of IL-13Rα2 receptor by conventional ELISA method and will compare the aggregation potential of the quantum dots in those fluids. After optimization this method of detection of soluble receptors would be patented. An invention disclosure preparation is on the way.

We are planning for a second patent based on the formulation of multifunctional IL-13-LIP-Gd-DTPA+ DOX. To achieve this we will generate a second generation gadolinium liposomes containing both gadolinium and doxorubicin targeted to glioma tumors. Our plan is to demonstrate that these multifunctional liposomes can detect and treat the glioma tumors at early stage.

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Madhankumar, Achuthamangalam B.	Assistant Professor
eRA COMMONS USER NAME (credential, e.g., agency login) amadhankumar	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Madras, India	M.S.	09/91	Chemistry
University of Madras, India	Ph.D.	04/99	Chemistry-Drug Delivery System
Penn State University College of Medicine, Hershey, PA	Post-Doc	02/04	Molecular Targeting-Cancer Therapy

B. Positions and Honors

Positions and Employment

Dec1998 - Jan2000	Research Scientist, Center for Protein Engineering and Biomedical Research, Chennai, India
Jan2000 - Oct2000	Scientist, Cadila Healthcare Ltd, Ahmedabad, India
Oct2000 – Feb2004	Postdoctoral Researcher, Department of Neurosurgery, Pennsylvania State University, College of Medicine, Hershey, PA 17033
Feb2004 – Jun 2008	Instructor, Department of Neurosurgery, Pennsylvania State University College of Medicine, Hershey, PA 17033
Jul 2008-	Assistant Professor, Department of Neurosurgery, Pennsylvania State University College of Medicine, Hershey, PA 17033

Awards

1992	Junior Research Fellowship Award from the Council of Scientific and Industrial Research (CSIR), India
1994	Senior Research Fellowship Award from the Council of Scientific and Industrial Research (CSIR), India
2011	Best Poster presentation in the Annual Meeting of Pennsylvania Neurosurgical Society for the presentation entitled, " Tumor selective delivery of doxorubicin for Peripheral Nerve Sheath Tumors", Hershey, PA

Presentations

March 2009	Invited for a presentation in Nanotechnology Colloquium at Lock Haven University, Lock Haven, PA, USA
April 2011	Presented a webinar "Nanotechnology in Medicine" organized by nanotechnology applications and career knowledge center (NACK), PA, USA

Professional Memberships & other activities

1. Member in American Association for Advancement in Science (AAAS)
2. Member in the American Association for Cancer Research (AACR)
3. Member in American Association for Pharmaceutical Scientists (AAPS)
4. Member in the Society for Neuro-oncology (SNO)
5. Member in Controlled Release Society (CRS)
6. Reviewer for the following journals *Neoplasia, International Journal of Pharmaceutics, Journal of Controlled Release, PLOSone, Molecular Pharmaceutics*
7. Participant of NIH sponsored 6 weeks in-house Multimodal Neuroimaging Training Program (MNTP) at University of Pittsburgh-Carnegie Melon with emphasis on PET neuroimaging and compartment models, Pittsburgh, PA, May 2010 to Jun 2010

C. Selected Peer-reviewed Publications

1. Madhankumar AB, Slagle-Webb B, Mintz A, Sheehan JM, Connor JR. Interleukin-13 receptor-targeted nanovesicles are a potential therapy for glioblastoma multiforme. *Mol Cancer Ther*. 2006;5,3162-9.
2. Fisher JL, Devraj K, Ingram J, Slagle-Webb B, Madhankumar AB, Liu X, Klinger M, Simpson IA, Connor J. Ferritin - a novel mechanism for delivery of iron to the brain and other organs. *Am J Physiol Cell Physiol*. 2007;293(2):C641-9.
3. Mintz, A, Gibo DM, Madhankumar AB, Cladel NM, Christensen ND, Debinski W. Protein and DNA-based active immunotherapy targeting interleukin-13 receptor alpha2. *Cancer Biother Radiopharm*. 2008;23(5):581-9. PMCID: PMC2936944
4. Madhankumar AB,Slagle-Webb B, Wang X, Yang QX, Antonetti D, Miller PA, Sheehan JM, Connor JR. Efficacy of interleukin-13 receptor-targeted liposomal doxorubicin in the intracranial brain tumor model. *Mol Cancer Ther*. 2009;8:648-654.
5. Liu, X., Madhankumar, A.B., Webb, B., Suruguledze, N., Sheehan, J., Connor, J.R. Heavy chain Ferritin siRNA Delivered by Cationic Liposomes Increases Sensitivity of Cancer Cells to Chemotherapeutic Agents, *Cancer Res*., *Cancer Res* 2011;71(6):2240-9.
6. Wiley, N.J., Madhankumar,A.B., Mitchell, R.M., Neely,E.B., Rizk,E., Douds,G.L., Simmons, Z., Connor, J.R. Lipopolysaccharide Modified Liposomes for Amyotropic Lateral Sclerosis Therapy: Efficacy in SOD1 Mouse Model, *Advances in Nanoparticles*, 2012; 1(3); 44-53
7. Dellinger, A., Zhou, Z., Connor, J.,Madhankumar, A. B., Pamujula, S.,Sayes, C. M., Kepley, C. L Application of fullerenes in nanomedicine: An update, Nanomedicine, 2013; 8(7): 1191-208