Biosensors for Real-Time Monitoring of Radiation-Induced Biologic Effects in Space

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I. Executive Summary
This proposal seeks to develop cellular biosensors based on dendritic polymers. Nanoscale polymer structures less than 20 nm in diameter will be used as the basis of the sensor/actuators. The structures will be designed to target into specific cells of an astronaut and be able to monitor health issues such as the exposure to radiation or infectious agents. Multiple components can be assembled on the polymers including target directors, analytical devices (such as molecular probes), magnetic particles and metals, and imaging agents. The design and assembly of these devices has been pioneered at the Center for Biologic Nanotechnology in the University of Michigan. These molecules would also be able to administer therapeutics in response to the needs of the astronaut, and act as actuators to remotely manipulate an astronaut as necessary to ensure their safety. The reporting will be accomplished either through fluorescence signal monitoring, with the use of multispectral analysis for signal interpretation, or through functional MRI. These nanosensors coupled to NEMS devices could facilitate the success and increase the safety of extended space flight.
II. Overall Progress: Organized by technical objective and task group.

Technical Objective I: Development of dendritic polymer-based biosensors capable of monitoring radiation-induced changes in lymphocyte populations.

Synthesis of Biosensor
Balazs Keszler, Ph.D., Istvan Majoros, Ph.D.

We have conjugated the porphyrine-based dyes (Chl-NCS, BChl-NCS) to the dendrimer as tracer dyes. These latter dyes emit in the near infrared region, which will enable us to track the biosensor without interference from the other dyes or from autofluorescence. Both were based on a partially acylated G5 PAMAM dendrimer, where 82 of the 110 primary surface amines were acylated. The dendrimer molecular weight of 29,620 g per mole was determined by GPC.

We have studied the reaction of the primary amino groups of the G5 dendrimer with benzyl chloride, which was selected as a model compound to mimic the chloromethyl moiety of MitoTracker® Red CMXRos and MitoTracker® Deep Red 633 mitochondria dyes (Figure 1).

![Figure 1. Mitochondria dyes with a chloromethyl functional group for use in conjugation reactions.](image)

Chl-NCS Dendrimer Conjugate: 10.09 mg (3.4065 \(10^{-7}\) mol) of the partially acylated dendrimer was allowed to react with 1.15 mg (1.7 \(10^{-6}\) mol) of Chl-NCS porphyrine-based dye in dry DMSO under a nitrogen atmosphere (Scheme 1). The reaction mixture was stirred in the dark for 24 hrs. Enough DI water was added to give a 1:1 ratio of water to DMSO. This solution was purified by exhaustive dialysis against deionized water, using a 3,500 MWCO membrane.
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(Spectrum Medical Industries). The retentate was then lyophilized to give a deep brown-black product, which will be tested \textit{in vitro}.

Scheme 1. Reaction scheme of Chl-NCS dye-dendrimer conjugation.

BChl-NCS Dendrimer Conjugate: 2.93 mg (9.892 \(10^{-8}\) mol) partially acylated dendrimer was allowed to react with 0.27 mg (3.886 \(10^{-7}\) mol) BChl-NCS porphyrine-based dye in dry DMSO under a nitrogen atmosphere (Scheme 2). The reaction mixture was stirred in the dark for 24 hrs. Enough DI water was added to give a 1:1 ratio of water to DMSO. This solution was purified by exhaustive dialysis against deionized water, using a 3,500 MWCO membrane (Spectrum Medical Industries). The retentate was then lyophilized to give a deep brown-black product, which will be tested \textit{in vitro}.

Scheme 2. Reaction scheme of BChl-NCS dye-dendrimer conjugation.

Reaction of G5 dendrimer with benzyl chloride as model compound
5.0 mg of G5-(NH\(_2\))\(_{10}\) (1.92x\(10^{-7}\) mol) was dissolved in 1 mL of 0.1M PBS buffer (pH=8). 0.0024 mL of Et\(_3\)N (1.70x\(10^{-5}\) mol, 1.5x molar excess of benzyl chloride) and 0.00176 mL of
benzyl chloride (2.84x10^{-6} \text{ mol}) in 0.5 \text{ mL DMSO} were added to the solution (Figure 1). The mixture was stirred for 60 hrs. The product was purified by dialysis and ultra filtration.

The product was characterized by \textsuperscript{1}H-NMR (Figure 2). The signal of the aromatic protons of the benzyl group shifted after the reaction indicating a change in the chemical structure. We can also noticed broadening of these signals. This phenomena is observed when the benzyl groups are covalently bonded to a polymer, in our case to dendrimer.

Based on this finding we will attempt to conjugate MitoTracker\textsuperscript{®} Red CMXRos and MitoTracker\textsuperscript{®} Deep Red 633 to G5 dendrimer.

**Activities planned for the next reporting period**

In the next period we will

- characterize both dye conjugates described above,
- design the chemical structure of the fluorescent resonant energy transfer reagent,
- look for alternative mitochondria dyes to conjugate to the dendrimer,
- conjugate tetramethylrhodamine ethyl ester to the dendrimer.

**Analysis of Biosensor**

Lajos Balogh, Ph.D.

We continued the development of methods for the analysis of the PAMAM dendrimers. One of the problems to solve is the task of finding an appropriate internal standard, which can be run under the same conditions, does not influence the movement of the compound(s) to be analyzed, and is detectable in small amounts, but whose peak comes separately from all the dendrimer products and derivatives.

We are experimenting with different compounds. To date, dimethylaminopyridine (DMAP) was found to be an acceptable compound for acylated PAMAMs (Figure 3A). DMAP can also be
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detected independently at longer wavelengths where dendrimers have a very small absorption coefficient, thus its detection is unperturbed (Figure 3B).

**Figure 3.** Comparison of capillary electropherograms of a fully acylated PAMAM G5-NHCOCH₃ dendrimer (1 mg/ml, second peak) containing traces of DMAP (0.03 mg/ml, first peak), detected at 210 nm (A) and 250 nm (B).

The peak of DMAP partially overlaps with the peak of the unmodified amine surfaced G5 PAMAM (Figure 4). Therefore it is useful only as an external standard for unmodified PAMAM dendrimers.

**Figure 4.** Comparison of capillary electropherograms of PAMAM G5-NH₂ dendrimer (1 mg/ml, second peak) containing traces of DMAP (0.03 mg/ml first peak) detected at 250 nm.

**Activities planned for the next reporting period**
Continue the electrophoresis development work and looking for better candidates for an internal standard with even shorter elution times.
Technical Objective II: The analysis and verification of the radiation sensing capability in lymphocytes \textit{in vitro}.

The goal of this objective is a systematic analysis the efficacy of single or multiple conjugates as biosensors with intracellular spectral probes as signal carriers.

\textbf{Analysis Using Flow Cytometry}

Andrzej Myc, Ph.D., Jolanta Kukowska-Latallo, Ph.D., Alina Kotlyar, M.S., Katarzyna Janczak, M.S., Jeffrey Landers, B.S., David Leavitt

In order to monitor cellular probes based on dendritic polymers \textit{in vivo}, we have been screening several cell line(s) for specific characteristics. The ideal cell line should:

- have low nutrition requirements,
- grow in cell suspension,
- be of human and hematopoietic origin, and
- be specifically labeled through target antigen expressed on the cell surface using dendrimer-based probes.

As an initial proof-of-principle study, we chose the folate receptor as the molecule to target. We examined six cell lines for their growth and viability in tissue culture.

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<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Characteristics</th>
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<tr>
<td>HL-60</td>
<td>Cell line was established from peripheral blood leukocytes obtained from a 36 year old Caucasian female</td>
<td>Cells express CD 14 antigen after stimulation with Vitamin D3</td>
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<tr>
<td>Jurkat E6-1 clone</td>
<td>Acute T cell leukemia cell line was established from peripheral blood of a 14 year old boy</td>
<td>Clone E6-1 cells express CD 3 antigen</td>
</tr>
<tr>
<td>K562</td>
<td>Cell line was established from cells of 53 year old female with chronic myelogenous leukemia</td>
<td>Erythroleukemia cell line which expresses CD 7 antigen</td>
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<td>Raji</td>
<td>Lymphoblast-like cell line was established from a Burkitt's lymphoma 11 year old Black male patient</td>
<td>EBNA positive</td>
</tr>
<tr>
<td>THP-1</td>
<td>Promyelocytic cell line established from peripheral blood leukocytes obtained from one year old male</td>
<td>The cells are phagocytic and monocytic differentiation can be induced with the phorbol ester</td>
</tr>
<tr>
<td>U-937</td>
<td>Histocytic lymphoma cell line from a 37 year old male Caucasian</td>
<td>Cells express FAS antigen and can be induced to terminal monocytic differentiation by vitamin D3</td>
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Each cell line was split into two equal aliquots. One aliquot was cultured in complete medium (2 μM folic acid), while the other aliquot was cultured in folic acid depleted medium (2 nM folic acid). Cell viability was monitored over a period of 12 days, by staining the cells with propidium iodide which stains the nucleic acid only in dead cells, and was analyzed by flow cytometer (Figure 5). Out of six cell lines, only two grew on folic acid depleted medium.

**Figure 5.** Raji cells were cultured in complete medium (left panel) and in folic acid depleted medium (2nM, right panel) for 11 days. Cells were stained with propidium iodide and analyzed with a flow cytometer. Dead cells were losing their shape as detected by side scatter and compromising their membrane integrity as detected by staining with propidium iodide (increasing red fluorescence — FL-3).
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(Figure 6). These two cell lines (HL-60 and THP-1) will be used for further investigation of folate receptor up-regulation, for adaptation, for targeting with specific probes, and for two-photon excitation/emission studies in vivo.

Figure 6. Cell aliquots were cultured either in complete medium (2μM folic acid; pink square) or in folic acid depleted medium (2nM folic acid; blue diamonds). The cell viability was monitored up to 11 days.

Activities planned for the next reporting period
We will continue studying various cell lines.
Testing Using Microscopic Multispectral Analysis
Felix de la Iglesia, MD, and Timothy Sassanella, M.S.

Targeted G5-(6-TAMRA)-folic acid and non-targeted G5-(6-TAMRA) dendrimer conjugates were tested in KB cells. 6-TAMRA alone and G5-(6-TAMRA) is not detectable in KB cells under the conditions tested. G5-(6-TAMRA)-FA conjugates show faint fluorescence in folate starved KB cells, but the signal is weak and appears to be non-specific, though the cell association appears to be folate specific (Figure 7). The faint fluorescence appears to locate on the plasma membrane.

Two dyes, MitoTracker® Red CMXRos and MitoTracker® Deep Red CMXRos, appear to have good spectral characteristics and have a potentially acceptable reactive group for conjugation. These dyes were obtained and submitted to both the chemistry group for conjugation, and the flow cytometry group for analysis.

Preparation, function testing, and calibration testing of the robotic microscope and individual components continued in July. Technician training continued. The system for image processing and image analysis continued in development. Plans to acquire a workstation for deconvolution and digital spectral analysis are in development, subject to sponsor approval.

Since this work requires lymphocytes to be used as a final test system, and these cells will be needed on an on-going basis, regulatory requirements were investigated, and IRB approval will be obtained. Documentation has been prepared for submission to the Medical School IRB. The KB cell line will be employed as a model system until IRB approval is obtained. Protocols for the preparation lymphocytes were obtained and the required materials were ordered.

Activities planned for the next reporting period:
1) Continue to develop the image processing/fluorescence quantitation system.
2) Submit for project IRB approval for the use of blood.
3) Microscopic testing and analysis of MitoTracker® Red CMXRos and MitoTracker® Deep Red CMXRos will be attempted. Acquisition of a workstation for deconvolution and spectral analysis.
4) Work with the Hoechst 33342 dye was temporarily set aside so as to concentrate on the red and far red dyes that have a direct sensing function and require a more narrow excitation range than the Hoechst 33342 far red dye pair would require. The Hoechst dye work will be resumed when a conjugate is prepared.
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Figure 7. Phase contrast, green and red fluorescent microscopy of folate starved KB cells with and without treatment with 30 nM G5 dendrimer conjugated with 6-TAMRA and 30 nM G5 dendrimer conjugated with 6-TAMRA and folic acid.
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Technical Objective III: The development of a noninvasive laser analysis system to monitor biosensor signals from lymphocytes with radiation-induced damage

The primary goal of this component of the research program is to develop ultrasensitive in vivo fluorescence detection technologies and methodologies for real-time monitoring of radiation-induced biologic effects in space.

Flow Cytometer-Laser System Development
Theodore Norris, Ph.D., Jingyong Ye, Ph.D., Cheng Frank Zhong, B.S.

This month we have worked on three components of the non-invasive readout system.

INTEGRATED MICROFLUIDICS SYSTEM: The fluid pumping system has been built. It consists of a syringe pump, various size syringes, micro-tubes, and connectors. We also acquired a microfluidic chip with integrated pneumatic valves from Prof. Chris Meiners’ group. With the pumping system, we can control the flow rate through the main valve on the chip to as low as 3 ml/s. Several sizes of syringe will be purchased so that the pumping system will be able to provide an even lower flow rate for the microchip than the flow rate we have right now. The main valve on the chip is 10 by 100 micrometers in cross section, the width of which is a little smaller than the size of a blood cell. A specific microfluidics chip will be designed to let blood sample go through.

6-TAMRA DYE DETECTION: We measured the auto-fluorescence spectrum of blood using the optical fiber based detection system (Figure 8). The signal peaks at 515nm with magnitude around 90. (All the numbers reported for the two photon fluorescence are in arbitrary units. They are the photon count rate from the detector used.) To distinguish between the auto-fluorescence signal and the dye signal, we need to choose from those dyes which have emission peaks that do not overlap with the auto-fluorescence signal from blood. We did experiments to compare the signal spectrum and intensity between free 6-TAMRA, G5-(Ac)_{103}-(6-TAMRA)_{3}, and G5-(Ac)_{103}-(6-TAMRA)_{3}-FA_{4} solutions (Figure 9, next page). Each of the dendrimer conjugated dyes contain three 6-TAMRA moieties, which means their fluorescence signals should be three times that of free dye, if measured at the same concentration. However, the signal intensity of the free 6-TAMRA solution is around 1900, which is higher than both of the dendrimer conjugates. Also the peak of G5-(Ac)_{103}-(6-TAMRA)_{3}-FA_{4} is around 800, which is only half as big as the peak of G5-(Ac)_{103}-(6-TAMRA)_{3}. One reason might be that the absorbance cross-section changes after the dye is conjugated with

![Figure 8. Visible wavelength spectrum of blood autofluorescence.](image-url)
dendrimer and FA. Therefore we measured their absorption spectra (Figure 10). The absorption spectra of all three peaked at almost the same height, indicating that their absorbance cross-sections are almost identical. This explains why the signals from 6-TAMRA and G5-(Ac)103-(6-TAMRA)3 are roughly the same. However the different signal levels between G5-(Ac)103-(6-TAMRA)3-FA4 and G5-(Ac)103-(6-TAMRA)3 should come from some other effect, i.e., quenching of the 6-TAMRA by FA.

Figure 9. Fluorescence signal of TAMRA and dendrimer conjugated TAMRA.

Figure 10. Absorption spectrum of TAMRA and dendrimer conjugated TAMRA.

Using the optical fiber based detection system, we measured the fluorescence spectrum of the free 6-TAMRA, the G5-(Ac)103-(6TAMRA)3, and the G5-(Ac)103-(6TAMRA)3-FA4 mixed with water and then with blood (Figures 9-11). The signal level of the mixtures of dye and blood is only $\frac{1}{2}-\frac{1}{2}$ of the signal level of the mixtures of dye and water (all mixtures have the same dye concentration). This is due to the absorption of blood. The blood absorption spectrum has two

Figure 11. Fluorescence signal of mixture of TAMRA and water and mixture of TAMRA and blood.

Figure 12. Fluorescence signal of mixture of G5-(Ac)103-(6TAMRA)3 and water and mixture of G5-(Ac)103-(6TAMRA)3 and blood.

Figure 13. Fluorescence signal of mixture of G5-(Ac)103-(6TAMRA)3-FA4 and water and mixture of G5-(Ac)103-(6TAMRA)3-FA4 and blood.
peaks around 550 nm (Figure 13), which is the emission wavelength of the dyes we used. We concluded that part of the fluorescence signal of the dyes was absorbed by the blood.

![Blood Absorption Spectrum](image)

**Figure 14.** Blood absorption spectrum.

Finally, we repeated some of these experiments in the quartz capillary based system (Figures 15-17), using the same concentrations of dyes in both the quartz capillary system and the optical fiber system. The signal level from the capillary based system is two times higher than that from the optical fiber based system, which means that the capillary based system will be able to provide a higher signal to background noise ratio.

![Auto-fluorescence Spectrum](image)

**Figure 15.** Blood auto-fluorescence from a capillary.

![Fluorescence Signals](image)

**Figure 16.** Fluorescence signal of a mixture of TAMRA and water, and of a mixture of TAMRA and blood in a capillary.

**Figure 17.** Fluorescence signal of mixture of G5-(Ac)_{103}-(6TAMRA)_{3}-FA_{4} and water and a mixture of G5-(Ac)_{103}-(6TAMRA)_{3}-FA_{4} and blood in a capillary.

(3) Monte Carlo Simulation of Bio-Flow Cytometer: The framework of the simulation has been established and tested with Simulink (Figure 16, next page). We used stochastic algorithms to generate initial positions for lymphocytes. Then the flow and excitation processes of the lymphocytes were calculated. In the simulation, each pulse represents a cell (with dyes on it) passing through the excitation volume of the laser beam. The pulse intensities of the result vary to a great extent. This initial simulation was based on a simplified model.
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Figure 18. Bio-flow cytometer simulation.

Activities planned for the next reporting period
Integrated Microfluidics System: A specific microfluidics chip will be designed to let blood sample go through.

Monte Carlo Simulation of Bio-Flow Cytometer: The next step to improve the simulation would be adding in more parameters to control the motion of cells and the process of two photon excitation.

III. Problems encountered and their resolution.
In this period we have tried to conjugate tetramethylrhodamine methyl ester or tetramethylrhodamine ethyl ester to the dendrimer as alternative mitochondria dyes. Several reactions were done, with little success.

We have two explanations for the unsuccessful experiments. One explanation is that the conjugation of the dendrimer to the dye molecule is sterically hindered by the bulkiness of the dendrimer. The other explanation is that after the hydrolysis of the ester group, the newly created carboxyl group may immediately form an inner salt preventing further reaction.

Work on these reactions will continue.

IV. Copies of manuscripts (published or unpublished) derived from the research and copies of all abstracts, manuscripts, preprints and publications.
None.
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| 13. ABSTRACT (Maximum 200 words) | The synthesis of a biosensor with 6-carboxy-tetramethylrhodamine (6-TAMRA) and Folic Acid has been completed, and the material has been characterized. This material will be used in in vitro testing. Method development for the capillary electrophoresis analysis of PAMAM derivatives has been largely completed, while method development for the capillary gel electrophoresis analysis has been initiated and continues. Dyes to stain the cells have been assessed for their ability to be used with the flow cytometer. To overcome cell autofluorescence, we have tested the feasibility of using the 6-TAMRA dye. Cell staining optimization studies were performed. We have set up the robotic microscope and have started to study 6-TAMRA, and tetramethylrhodamine methyl ester in KB cells. Initial tests showed that the microscope is functioning correctly and that these dyes are detectable. The experimental parameters are still being worked out. We have a design for a model system that will simulate a retinal capillary for initial proof-of-principle experiments. We are developing both a very simple system based on a peristaltic pump and a glass capillary tube, and a second, integrated system based on a microfluidics chip, which will enable a much higher degree of control of the flow. Components have been received. |

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