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13. ABSTRACT ***(Maximum 200 Words)***
Our hypothesis is that novel paramagnetic oligonucleotide analogs will be useful MR contrast agents for breast cancer imaging. This novel approach of targeting MR contrast agents will ultimately aid in the early detection of metastatic breast cancer. The technical objectives are as follows along with progress to date.

1: Optimization of Paramagnetic Oligonucleotide Chelates: To prepare a series of paramagnetic chelate modified oligonucleotides to assess maximal proton relaxation enhancement upon binding to a macromolecular target. The oligonucleotide conjugates have been prepared and are ready to assay.
2: Selection of RNA Aptamer for the Tn-antigen: To use the SELEX technology to find a small RNA sequence which binds specifically to the tumor associated Tn-antigen. The SELEX process has been initiated.
3: Evaluation of Paramagnetic Oligonucleotides in Breast Cancer Cell Model System: To use a cell culture model system for evaluating the proton relaxation enhancement of paramagnetic oligonucleotide analogs which have been targeted to breast cancer cells. A significant amount of work has been done to develop and evaluate the novel monolayer assay system. We are now ready to examine our agents using this system.

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Annual Report for Award Number DAMD17-99-1-9339
Paramagnetic Oligonucleotides for Breast Cancer Imaging

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INTRODUCTION

For effective cancer prevention and control it is imperative to have effective imaging diagnostics to accurately asses the benign or malignant nature of a growth as well as to identify metastatic tendencies in a tumor as early as possible. In magnetic resonance imaging (MR imaging), the current approved contrast agents make it difficult to differentiate between these various states and the need exists for developing tumor-specific contrast imaging agents. [1] There are biochemical differences between these states which can be utilized to provide the specificity needed for the imaging agents. Our hypothesis is that paramagnetic oligonucleotide analogs will be novel and useful MR contrast agents for breast cancer imaging. Using either an antisense or aptamer-based targeting strategy, biostable versions of these agents will provide enhanced contrast due to the combined effects of localization and proton relaxation enhancement (PRE). This novel approach of targeting MR contrast agents will ultimately aid in the early detection of metastatic breast cancer.

BODY

B1. Optimization of Paramagnetic Oligonucleotide Chelates

The overall goal of this technical objective was to prepare a series of paramagnetic chelate modified oligonucleotides to assess the optimal chelator, tether length and position for maximal proton relaxation enhancement upon binding to a macromolecular target. The following tasks are covered in this objective.

B1.1 Task 1

> *Construct MR compatible temperature control, prepare oligonucleotide chelates for study with 5S rRNA, analyze R1 on 6.3T spectrometer.*

The MR sample chamber for the monolayer studies needs to hold the tissue culture plate level be readily adapted for temperature control. We have initially opted to use a container of sand and settle the culture plate in it using bubble levels to insure the plate is level. If we decide to continue analyzing these samples using the monolayer system, a temperature control device

can be readily constructed by having a tube of recirculating warm water or air embedded in the sand.

To prepare oligonucleotide chelates we needed to first prepare ITCDTPA (DTPA isothiocyanate). The initial procedure chosen was fraught with difficulties even with our modifications to improve handling characteristics.[2] The challenge was that all the intermediates were gums that were difficult to solubilize in either aqueous or organic solvents. We added benzyl ester protecting groups in an effort to improve the synthesis. While it did improve the handling somewhat, it was still not sufficient for reliable total synthesis. Instead we turned to an alternate synthesis of an aniline derivatized DTPA analog[3] where each intermediate was a solid and had been fully characterized. From this we were then able to make the isothiocyanate after first using dowex resin to prepare the neutral form of the DTPA analog. The overall successful synthetic scheme along with our best yields are shown in scheme 1. We have also optimized conditions for conjugating the ITCDTPA and are ready for R1 studies.

Due to cost, supply and reproducibility issues, we decided to prepare our own 5S rRNA. Following a published procedure[4], we used a commercially available plasmid that contains the *E. coli* 5S rDNA sequence. This sequence (with slight modifications to accommodate primer and restriction enzyme sites) was amplified by PCR. The double stranded DNA was then digested to release the fragment containing just the T7 promotor region and the 5S rDNA. The resulting DNA was then used directly in a T7 RNA polymerase reaction to prepare 5S rRNA. Prepared RNA was identical to commercial 5S rRNA as indicated by denaturing polyacrylamide gel electrophoresis. The recombinant 5S rRNA differs from wild-type only in that the last nucleotide at the 3' end is deleted and the second to last nucleotide is changed to a C. These two changes are necessary to accommodate restriction enzyme cleavage sites on the DNA to ensure an accurate end to the template making it ready for transcription reactions. Previous work[4] indicates that this slight change does not affect the three-dimensional folding or activity of 5S rRNA. Based on these studies, we believe that this will not affect the 5S rRNA in our model binding studies. However, we are planning on running control experiments to verify this.

Due to moving to Ohio University in late June-early July, research was delayed for a month. Consequently, our projected time for analyzing the R1 on the 6.3T spectrometer is this coming month (August). At this point we are scaling up the 5S rRNA preparation and oligonucleotide conjugation/chelation reactions such that we will be ready for all the model studies in August.

B1.2 Task 2

R1 studies of oligonucleotide chelates with 5S RNA at 1.5T, data analysis, UV melting studies of oligonucleotide chelates with 5S rRNA.

As indicated above, research progress was delayed by a month due to our move to Ohio University. We expect that the R1 measurements at 1.5T will be done in August. By that time we will have sufficient quantities of 5S rRNA and chelated oligonucleotides.

Initial UV experiments indicate that it may be difficult to see the very slight changes due to the binding of ON-1 to 5S rRNA. However, as part of the start-up package for the move to Ohio University, a better, more sensitive melt apparatus is being purchased. Consequently, future melt studies with this better instrument may show the desired changes. If not, then evidence for binding of the chelated conjugated oligonucleotide can be obtained from fluorescence studies. Europium chelate anisotropy fluorescence studies could be done in the presence and absence of 5S rRNA. A decrease in the anisotropy of ON-1-ITCDTPA-Eu in the presence of 5S rRNA would indicate that binding had occurred. The usual dextran control would also be run in order to verify that the change was not due to viscosity differences. These studies have an additional advantage in that they could be used to directly measure the correlation time of the chelate complex. This could then be correlated with our observed PRE measurements.

B2. Selection of RNA Aptamer for the Tn-antigen

The overall goal of this technical objective was to use the SELEX technology to find a relatively small RNA sequence which binds specifically to the tumor associated Tn-antigen. The following tasks are covered in this objective.

B2.1 Task 1

Prepare column for SELEX, SELEX selection of RNA aptamer, consensus sequence determination.

We chose a simpler resin bound carbohydrate model system (GalNAcα1-O-PAP) to prototype the SELEX procedure as well as to find an aptamer that might be sufficient for initial studies while the development of GalNAcα1-O-ser is optimized. We have prepared the DNA library, PCR amplified, transcribed and started the SELEX selection and amplification rounds.

B2.2 Task 2

Prepare paramagnetic aptamers, UV melting studies of model system, R1 of model system at 6.3 T, data analysis.

B2.3 Task 3

R1 of model system at 1.5 T, data analysis, optimization of aptamers.

Progress on these two tasks will occur once the SELEX has been completed. We expect to have an aptamer in the next few months. Once an aptamer has been identified, carrying out this task will be quite straightforward since all the protocols will have been worked out for the antisense model systems and can be directly applied to the study of the aptamers.

B3. Evaluation of Paramagnetic Oligonucleotides in Breast Cancer Cell Model System

The overall goal of this technical objective was to use a cell culture model system for evaluating the proton relaxation enhancement of paramagnetic oligonucleotide analogs which have been targeted to breast cancer cells via an antisense complementary to an overexpressed mRNA and via aptamer recognition of the tumor associated Tn-antigen. The following tasks are covered in this objective.

B3.1 Task 1

Assessment of spatial resolution limit of MR, preparation of biostable paramagnetic antisense agent.

B3.2 Task 2

Development of modified MR acquisition & image analysis, R1 measurements in cell layers for antisense agents at 1.5T.

Tasks 1 and 2 are integrally linked and will be discussed together. Our overall assessment of MR studies of monolayers is that they have low signal/noise at high resolution. This will be a problem with very low relaxivity agents. Also, even with the highest possible resolution, one still needs to consider voxel size vs. size of cell since it involves a mixing of cell R1 and buffer R1. Data to date indicates that we can most definitely look at trends, but absolute measurement of R1s may be limited. All initial assessment studies were done using Gd-DTPA. These studies are discussed in detail below.

We have prepared a phosphorothioate antisense agent as well as DNA agents and 2'OMe/RNA chimera antisense agents. All the oligonucleotides were synthesized commercially with a 5' aminohexyl linker. The phosphorothioate analog is the only one that has been studied in a detailed monolayer experiment. The DTPA-phosphorothioate conjugates were made by reaction the oligonucleotide analog with DTPA-dianhydride in DMSO/H_2O pH 8.0 and then purifying with a spin column. Cells grown in the monolayer wells were then treated with the phosphorothioate for 12 hrs. The wells were then photographed, imaged by MRI and the cells removed for Gd determination by ICP-MS. This initial study was somewhat discouraging because there was essentially no correlation between Gd and observed differences in the MRI. However, this was one of the first studies done with the monolayer experiment and since this experiment we have extensively obtimized the assay (see below). Consequently, we are hopeful

that future monolayer experiments will be more helpful. Another reason why the phosphorothioate experiment may have not worked is that phosphorothioates are known to nonspecifically bind proteins. This may have been interfering with our MRI observations. Consequently, we are planning future antisense experiments to use either DNA or chimeric analogs. Previous antisense studies with DNA indicate that the antisense agent is stable and effective in the serum-free media used for the experiments.[5]

An alternate assessment method of cell pellets was examined. Cell pellets are advantageous in that they are larger in size. However, depending on the coil that can be used there is still a problem of signal/noise and resolution. We experimented with preparation of the cell pellets since the literature precedence[6] was difficult to reproduce reliably due to the way the cell lines we use pelleted. Instead, we came up with a new scheme of pelleting cells ultimately in a microcentrifuge tube, overlaying with agarose gel and then securing tubes in a tray where the tubes are immersed in water (in order to provide temperature stability as well as decrease magnetic susceptibility).

The cell pellet volume was determined in two ways. An initial estimate was made at the time of preparation using volumetric pipettes. A more accurate determination was then made using the MRI image of the cell pellet and knowing the pixel size. After imaging, the cell/agarose samples were melted and prepared for Gd determination by ICP-MS.

Monolayers were imaged by MRI as shown in Figure 1. For the monolayers, cell coverage was estimated based on photographs of the inserts prior to imaging by MRI. The cell coverage data were then used to estimate the total number of cells N_{cells}, the total cell volume V_{cells} and (by combination with the IPC-MS data) the Gd-DTPA molarity of the cells. Assuming a cell diameter of 30μm consistent with literature reports[5] and our photography (A_{cell}=7.07 $10^{-4}mm^2$), and given the area of the 6.2mm diameter insert (A_{insert}=30.19mm^2), the number of cells on the insert can be calculated as

$$N_{cell} = \frac{A_{insert}}{A_{cell}} f_{cover}$$

where f_{cover} is the average cell coverage of the membrane estimated from micro-photographs. Assuming spherical shape for the cells, the cell volume can then be calculated as

$$V_{cell} = N_{cell} \frac{4}{3} \pi \, r_{cell}^3$$

This total estimated cell volume and the Gd-DTPA concentration measured by ICP-MS were used to compute the Gd molarity in the cells. There is a linear relationship between the treatment concentration and the estimated final cell molarity (Figure 2). Linear regression fitting of the data indicates that approximately 65% of the treatment concentration is taken up and

retained by the cells. This uptake fraction is somewhat higher than with the cell pellets (data not shown), and may suggest more efficient uptake of agent with the mono-layer cell culture system. However, it has to be stressed that there is significant uncertainty in the volume estimate for the cell on the membrane leading to significant uncertainty for the cell molarity estimate. It is however very encouraging, that cell pellet and mono-layer studies show comparable uptake of Gd-DTPA agent.

The 2D-IR-FSE and 3D-SPGR and 3D-IR-FSE methods were examined for determination of T1s by MRI. The relaxivity graph for all the cell pellet studies is shown in Figure 3 and the graph for the monolayer studies is shown in Figure 4. From these graphs the relaxivity for the cell pellets was 1.6 $mM^{-1}s^{-1}$ (R^2=0.87) and that for the monolayers was 0.083 $mM^{-1}s^{-1}$ (R^2= 0.89). This different relaxivity for the monolayers is expected for a region with "mixed" relaxation properties. This "mixed" relaxation property may result simply from partial volume averaging of signals in the voxels containing cells and media solution. Alternatively, it may be the result of fast exchange of water protons moving in and out of the cells. The simple single exponential analysis used here cannot distinguish the nature of the relaxation mechanism any further. We are exploring details regarding fast exchange and partial volume in the monolayer studies. Despite the differences in relaxivity, we are encouraged by the fact that a relaxivity trend can be measured in the monolayer experiments.

While the monolayer assay method continues to pose some technical challenges and limitations, there is a significant cost advantage for the monolayers over the cell pellet model system. In the initial prototyping and optimization stage of developing the oligonucleotide agents for MRI it is essential to rapidly and cost effectively screen analogs. The monolayer sytem can be prepared in a few days whereas the cell pellet system requires a week or more lead time in order to get enough cells. In addition, to treat the cells with the same amount of oligonucleotide costs 53 times more in the cell pellet model system as opposed to the cell monolayer system. This would result in a cost of $150,000 for one complete study with the cell pellets as compared to $3000 for one complete study with the monolayers. Once an optimized agent has been developed there are various ways to modify & optimize preparation of the agent in order to drastically reduce the cost (e.g. use of biosynthetic techniques). These avenues ultimately could be pursued in order to make future studies (such as animal and clinical studies) economically feasible. At this point, however, our goal is to assay the agents and optimize their properties rather than optimizing their preparation which is a whole research endeavor in itself.

B3.3 Task 3

Prepare biostable paramagnetic aptamers, R1 measurements of cell layers with aptamer agents at 1.5T, data analysis.

These studies will be done once the aptamers are identified, however, by then all the conditions will have been optimized and experimental details worked out such that obtaining the data should be straightforward.

KEY RESEARCH ACCOMPLISHMENTS

✳A novel cell monolayer system has been developed for assessing MRI contrast agents.

✳Novel paramagnetic oligonucleotide contrast agents have been developed.

REPORTABLE OUTCOMES

Currently, one paper is in preparation on the monolayer model system.

One poster presentation is being made at MR Gordon Conference in August. "T1 Measurements In Cell Cultures for Contrast Agent Characterization at 1.5 T" P. Schmalbrock, S.-M. Lee, G. M. Ammar, J. V. Hines

CONCLUSIONS

To date we have completed the initial optimization and feasibility experiments for assaying contrast agents using a cell monolayer system. This assay system will be vital for the efficient analysis of oligonucleotide contrast agents. We have also completed the synthesis of oligonucleotides conjugated to an isothiocyanate modified DTPA as well as to unmodified DTPA. We have done initial studies of DTPA-oligonucleotide-Gd antisense agents in a monolayer system. However, these studies were done before all the optimizations and we believe we are now ready to obtain more informative data. In summary, we have laid the groundwork that is essential for the efficient assessment of our oligonucleotide contrast agents in both the 5S rRNA model antisense system as well as the cell culture antisense system.

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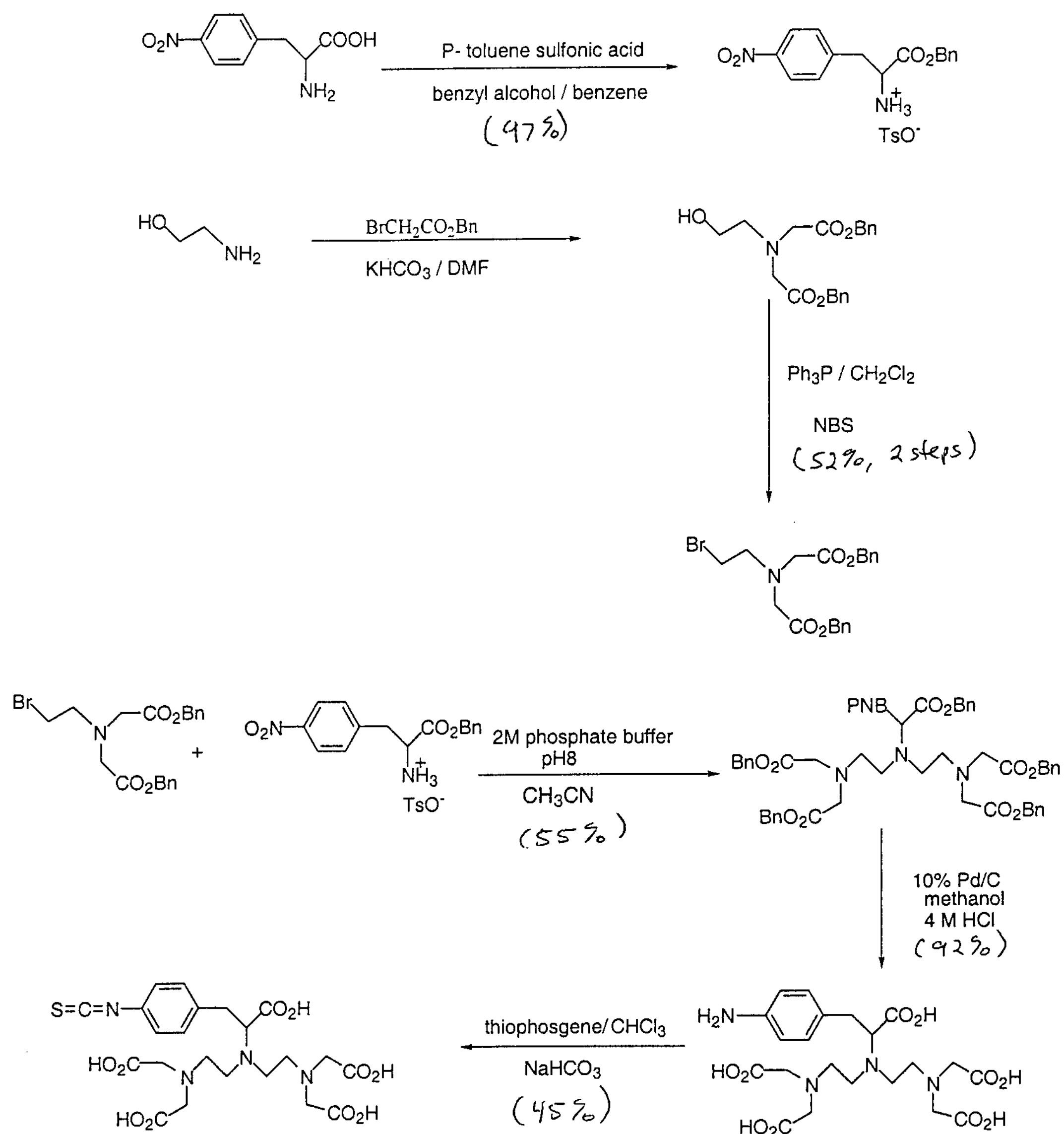
Scheme 1: Synthesis of ITCDTPA



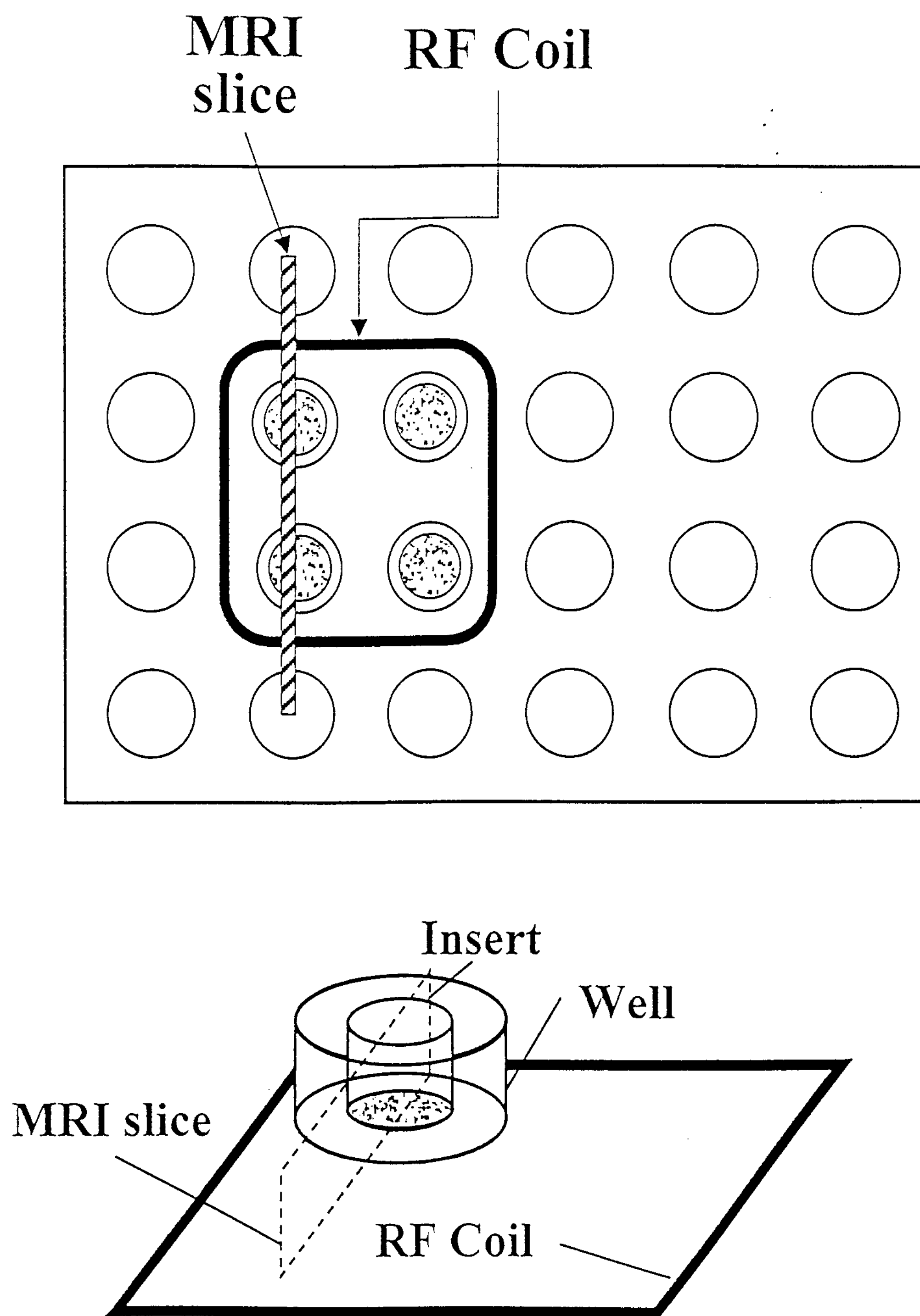


Figure 1: Schematic diagram of transwell monolayer cell culture arrangement in MRI

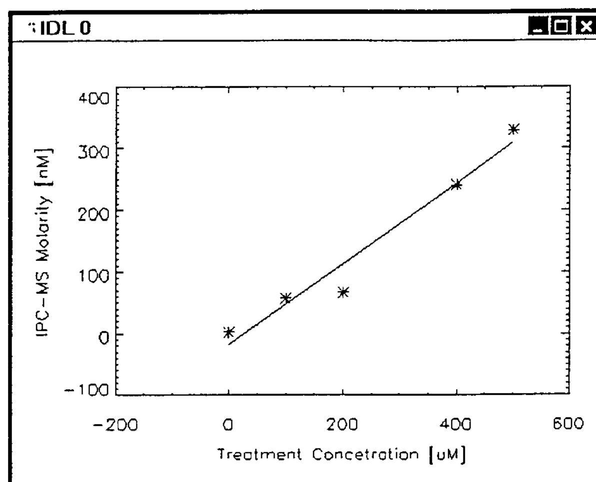


Figure 2: Cell loading in the monolayer cell cultures. Slope is 0.655, R^2=0.98

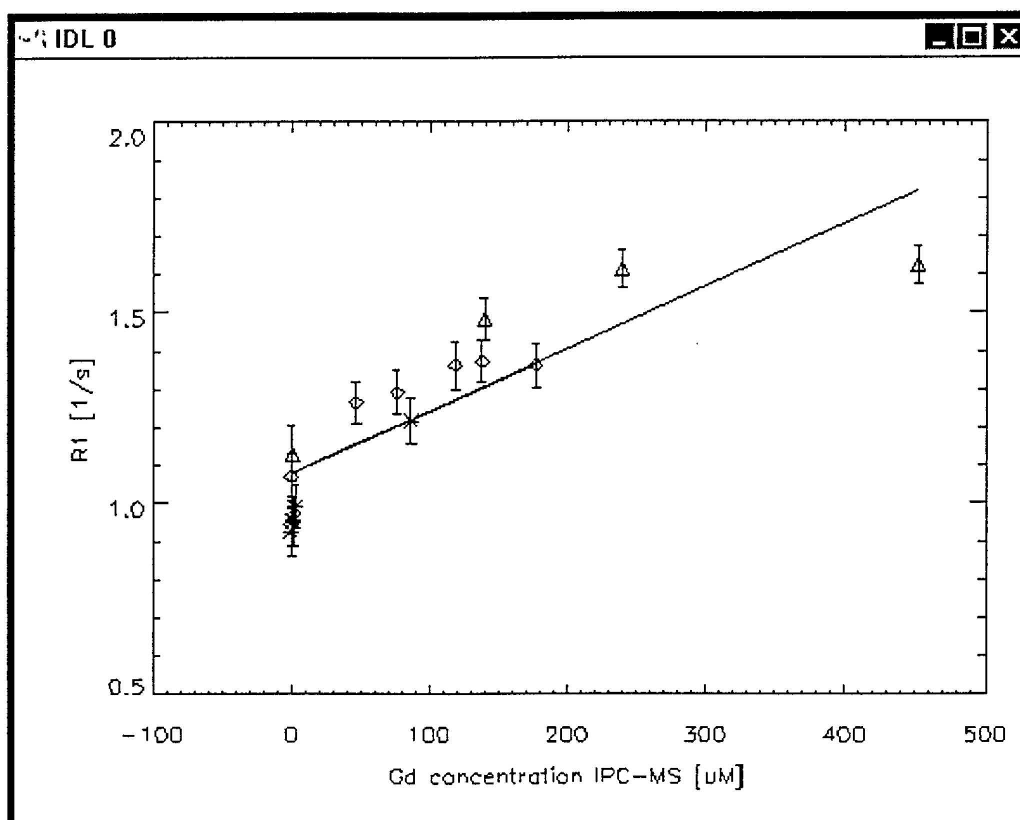


Figure 3: (1/T1) or relaxation rate versus Gd concentration in cell pellets.
Slope = relaxivity = 1.6 $mM^{-1}s^{-1}$ R^2= 0.866

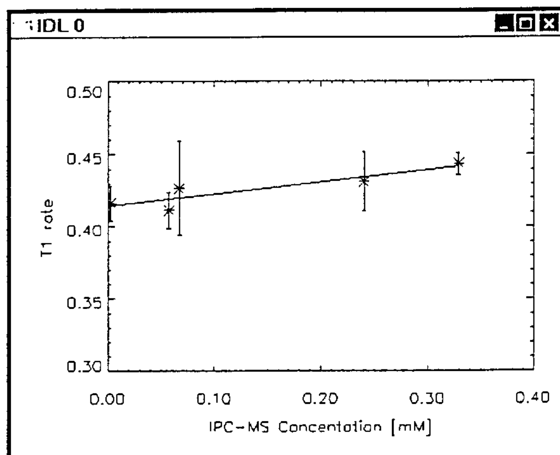


Figure 4: (1/T1) or relaxation rate versus Gd concentration in cell monolayers
Slope = relaxivity = $0.83mM^{-1}s^{-1}$ R^2= 0.89