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Developing Novel Nanoparticulated Imaging System using Luminescence Enhancement of Eu(III) and TB(III) by Single-Strand DNA Encapsulation

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A Graduate Thesis submitted

To Governors State University

By

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MMXI

Dedicated to

My Mom,

My Children: Victoria & Thomas,

and

My friend Diana

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Two years ago, I asked Diana if she would like to collaborate in a joint thesis. If she had known at the time it would take two years to finish, she would probably have said no thank you, but I am most appreciative she said yes to the thesis. I am deeply in debt to her for her time, hard work and assistance, and extremely grateful in keeping me focused in attaining our goal. She was an invaluable partner in this research, her dedication, commitment, and perseverance is what got this thesis to its conclusion. I find myself to be extremely fortunate to have worked with Diana on this thesis.

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ABSTRACT

Non-toxic biosensors are encountering an increase in attention for use in understanding the fate of cells and as a diagnostic tool. Development and incorporation of suitable fluorophores into biological molecules is the key for monitoring proteins *in vivo* research. This study investigated the enhanced emission of Eu (III) and Tb (III) upon binding to the four DNA bases and their respective nucleotides, found the best ratio for effective energy transfer, and developing nanoparticles to deliver the biosensor into the cells.

It is well known that Eu (III) and Tb (III) exhibit very distinctive photo-characteristics. The luminescence of these two lanthanides is weak due to low absorption cross sections. Conversely, the emission of both trivalent ions, upon irradiation, in aqueous solution, is strong when bound to complex ligand systems. The luminescent enhancement is the result of energy transfer (EnT) and the binding with single-stranded DNA, making these ions perfect candidates for luminescent probes [(1)]. The emission lanthanides theory by G.A. Crosby establishes that the intramolecular energy transfer in a lanthanide complex is when the lowest triplet state energy level of the complex equals or lies above the resonance level of the lanthanide [(2)]

To overcome the inherently low absorption of lanthanide ions, researchers have developed sensitizing fluorophores that upon excitation, transfer energy to the lanthanide [3] [4]. One problem with luminescence in an aqueous solution is that another pathway is available for deactivation of the excited state of the lanthanide, in the form of vibrational energy transfer to water molecules [1]. Early research shows that quenching of luminescence is minimized by using ligands which tended to encapsulate the lanthanide ion [1]. Longer emission lifetimes and greater quantum yield intensities can be accomplished by either chelation by ligands [5] or encapsulation of the lanthanides. We ascertained the maximum enhancement for the lanthanide ions occurred through the interaction with the base guanine or its nucleotide guanosine 5'-monophosphate disodium salt.

The research initially pursued the encapsulation of the lanthanide ions by single-strand oligonucleotides as a biosensor. However, an alternative delivery method based on inverse micelles and liposomes was developed and it proved to be economical and simple to encapsulate and deliver the biosensor into the cells. The creation of a double emulsion, or water-oil-water system, and the encapsulation (using palmitic acid as surfactant) of the water soluble biosensors were successful. This thesis determined the particle size achieved of 75nm, for both lanthanides had fallen into the nanoemulsions range. Their small size permits the nanoparticles to be injected intravenously [6].

The in vitro toxicity of the nanoparticles, with both luminescence biosensors, was assessed by BCA assay. Results supported both luminescence nanoparticles biosensors were non toxic to human cells. Therefore, these NP's have a potential to provide a unique detection signature as a contrast agent suitable for medical applications [(7)].

It has been published that nanoparticles (NPs) can rapidly be transported to the liver (about 90%), then kidneys and other organs [(8)]. After a period of time, the NPs are expelled from the human body through feces and urine, unless the size of the NPs is larger than 200 nm, in which case the NPs are retained / trapped by the liver. The particle size obtained in this research, 75nm, is a good indication that the biosensor will have a safe disposal from the body.

1. INTRODUCTION

Non-toxic biosensors are experiencing an increase interest due to the need to understand the fate of cells and as a diagnostic tool. Development and incorporation of appropriate fluorophores into biological molecules is the key for proteins *in vivo* research. It is well known that Eu (III) and Tb (III) exhibit very unique photo-characteristics upon irradiation.

It has been shown that the emissive lanthanide properties of Eu (III) and Tb (III), including their luminescence enhancement through energy transfer, and their ability to bind single-stranded regions of DNA make these ions the perfect candidates for life-cell imaging system. Additionally, single-stranded oligonucleotides are known to enhance the emission of Eu (III) and Tb (III) ions in solution [(1)]. Previous studies have confirmed that lanthanide ions also bind oligonucleotides [(9) (10)] , and the resulting bioconjugates provide in the monitoring of hybridization reactions and phosphodiesterase activity by FRET, Föster resonant energy transfer, technology [(11) (12)].

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As stated earlier, it is well known that Eu (III) and Tb (III) exhibit very unique photo-characteristics upon irradiation. To overcome the inherently low absorption of lanthanide ions, researchers have developed sensitizing fluorophores that upon excitation, transfer energy to the lanthanide [(13) (4)]. There is a major problem with luminescence in an aqueous solution that another pathway is available for deactivation of the excited state of the lanthanide, in the form of vibrational

energy transfer to water molecules in particular. This quenching of luminescence can be minimized by using ligands which tend to encapsulate the lanthanide ion [(1)]. To overcome the weak luminescence in aqueous solution of Eu (III) and Tb (III), addition of chelating agents or encapsulation of the lanthanides leads to longer emission lifetimes and quantum yields.

Our research pursues the encapsulation of the lanthanides. Encapsulation is to protect the enclosed lanthanides from substances or processes in the vicinity of these capsules. The unique optical property of Eu(III) and Tb(III) Nanoparticles(NP) will provide us a new live-cell imaging system which is safe, photostable, and photosensitive. The stability both in the excited state and in oxidation state makes these lanthanides good imaging molecules to monitor cellular activities such as enzymatic reactivity, DNA hybridization, drug binding, electron transfer, and nucleic acid solvation environment.

The ultimate goal of this thesis study is to develop a delivery mechanism that packages theses two lanthanides into a biosensor, insert the biosensor into the bloodstream and release it at the specific point of interest [(14)].

We discovered that the maximum enhancement for the lanthanide ions occurred through the interaction with the base guanine (G) and the phosphate groups of its nucleotide guanosine 5'-monophosphate disodium salt (GMP), (Figure 1 and 2). It was found that guanine enhances both of the trivalent ions emission with Eu

(III) being more emissive. In accordance with literature research, energy transfer from intrinsic fluorophores, such as nucleic acids, generally gave rise to enhanced Eu (III) and Tb (III) emission [15]. However, the results which are to be discussed later, shows Eu (III) opposed this statement.

Although previous studies reported energy transfer from the bases (C and G) and nucleotide 5'-deoxymonophosphate (dGMP), unexpected behavior of the 2' deoxyguanosine 5'-triphosphate disodium salt (dGTP) nucleotide was found. Actually, none of the triphosphate nucleotides enhanced the fluorescence of the trivalent ions. Phosphate interference was determined as the main cause for unsuccessful energy transfer. Research has shown that in the cases of G and dGMP, the phosphate group appears to aid in the biding of the donor and acceptor. A comparison of the enhanced luminescence among GMP with its triphosphate and diphosphate analogues, GTP and GDP, respectively, has shown that GMP is a better energy transfer donor in the order GMP> GDP > GTP [16] [17]. The results indicated that the lanthanide bounded to the monophosphate group (GMP) was closer for effective energy transfer than the triphosphate groups (dGTP). The degree of enhancement of GMP bound to Tb (III) was greater than for Eu (III), respectively. The enhancement of the trivalent ions by GMP was much greater than the base G.

Toxicity studies through BCA assay, (bicinchoninic acid), determined that our nanosensors were non-cytotoxic. The w/o/w double emulsion technique

successfully created nanoparticles which encapsulate the water soluble biosensor. A TEM image showed that the size of the particle was 75 nm, which fell into the nanoemulsions range (Figure 29).

The choice of the rare earths or lanthanides was not arbitrarily chosen. The rare earths (RE) form a group of chemically similar elements which have in common an open 4*f* shell. They are chiefly trivalent and it is principally the properties of the trivalent ions which are important rather than those of the neutral atoms. They are strongly paramagnetic, in some cases ferromagnetic or anti-ferromagnetic at low temperatures. Their trivalent salts have absorption spectra and in some cases fluorescence spectra with sharp lines in the visible or neighboring spectral regions [(27)]. Some trivalent lanthanide ions exhibit excellent luminescence characteristics when the native luminescence is enhanced by coordination with suitable organic ligands.

Having chosen the lanthanides Eu (III) and Tb (III), the design of the biosensor was the next crucial step and was based on chemical, photo-physical and biochemical guidelines published from literature research. For in vivo experiments, the matter of toxicity is highly critical, as well as the ability of the probe to be excreted in a reasonable span of time (typically 12-48 h). Lanthanides are relatively innocuous due to poor absorption in the gastrointestinal track and, even when injected, normally cannot penetrate living cells [(19; 20)]. According to the systematic studies published to date, extraordinary rules have been established

based on a simplistic model which the main energy transfer path implies the ligand triplet state and that the only parameter of importance is the energy gap between this state and the emitting Ln(III) level [21] [22] [23].

Figure 1. Structure of the four DNA bases

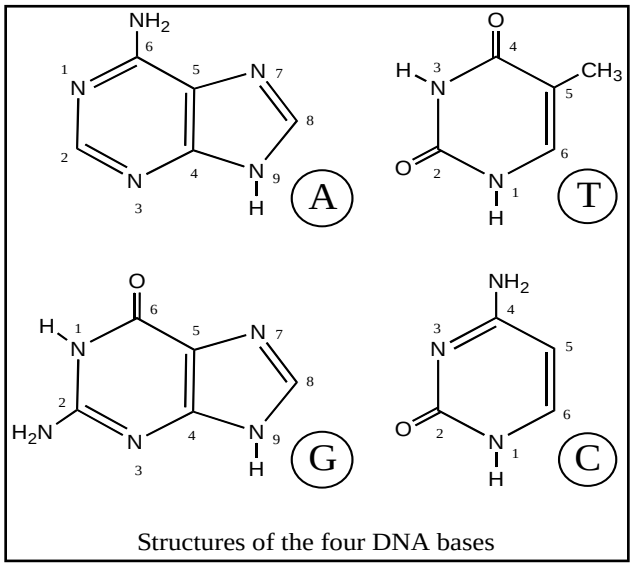
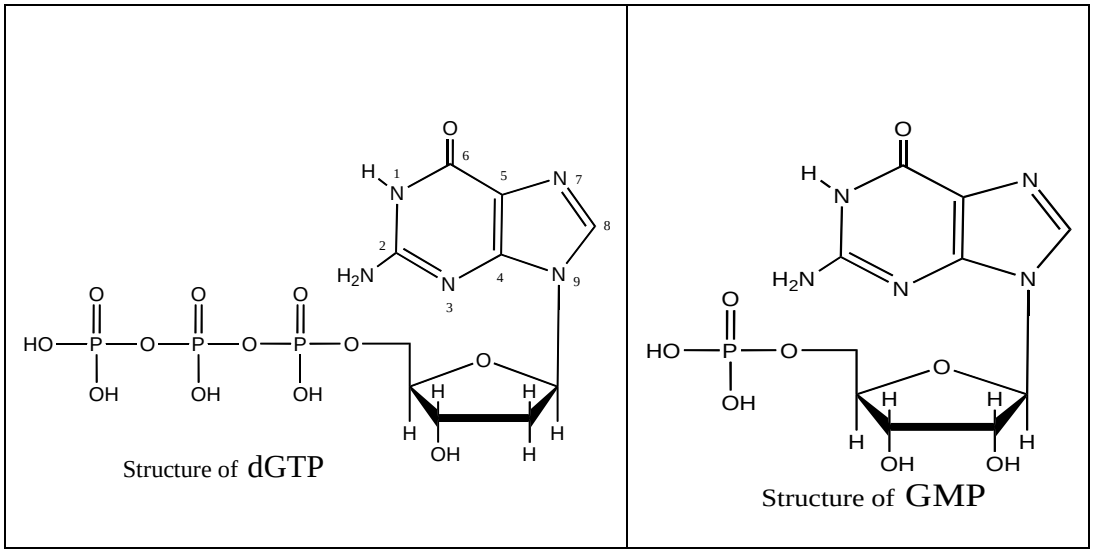


Figure 2. Structure of dGTP & GMP Nucleotides



1.a. Spectroscopic characteristics of Europium and Terbium

The trivalent ions of Eu (III) and Tb (III) show excellent luminescence characteristics when enhanced by coordination with suitable ligands. The enhancement of luminescence intensity by complexation of the tri-positive luminescent lanthanide, Ln (III) ions, has been explained on the basis of a ligand-to-metal energy transfer mechanism [(24).] The mechanism for energy transfer was derived from research by Kasha, Crosby, and their co-workers [(30)] . (See Figure 3.) When an excited triplet state of the coordinating ligand overlaps a lanthanide excited electronic level, the lanthanide luminescence is pumped by a large cross section molecular absorbance, rather than by its own weak absorbance. To understand the photo-physics and photochemistry of the lanthanides see energy level diagram, Figure 4. [(3)] This diagram illustrates the largest gaps in energy level bands for Eu (III) and Tb (III). Eu (III) has a regular ^7F multiplet as ground multiplet followed by a ^5D multiplet. At low temperatures, the absorption spectrum in the visible is due to the transitions from the ground state $^7F_0$ to $^5D_0$, $^5D_1$, $^5D_2$, $^5D_3$. Shortly above the level $^5D_3$, a whole series of levels is found and the situation is that the expected levels are so crowded that a detailed analysis is difficult [(27).]

Figure 3. Schematic diagram of the ligand-to-lanthanide energy transfer [28].

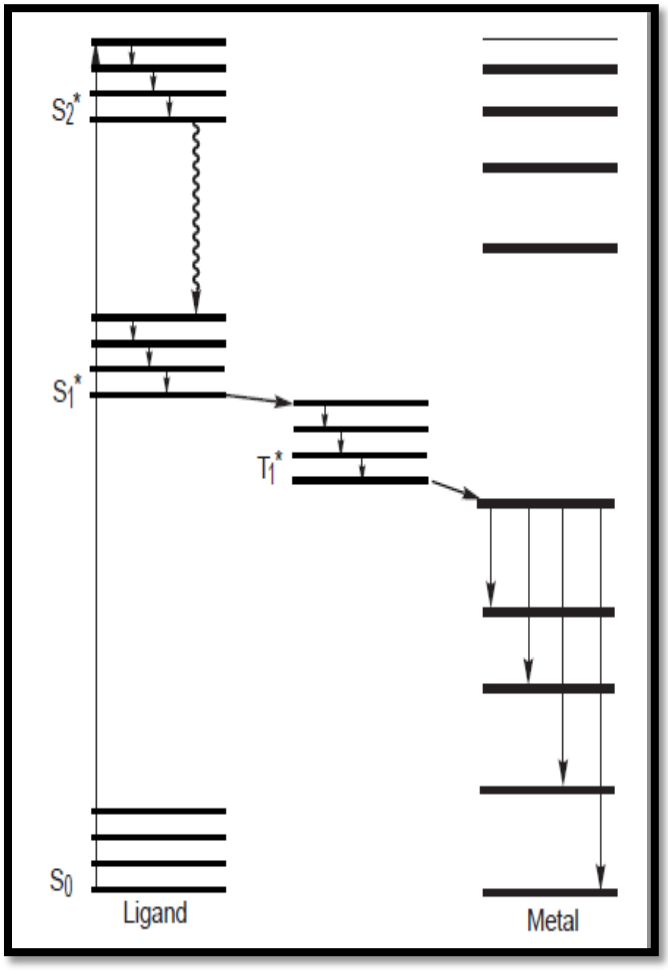
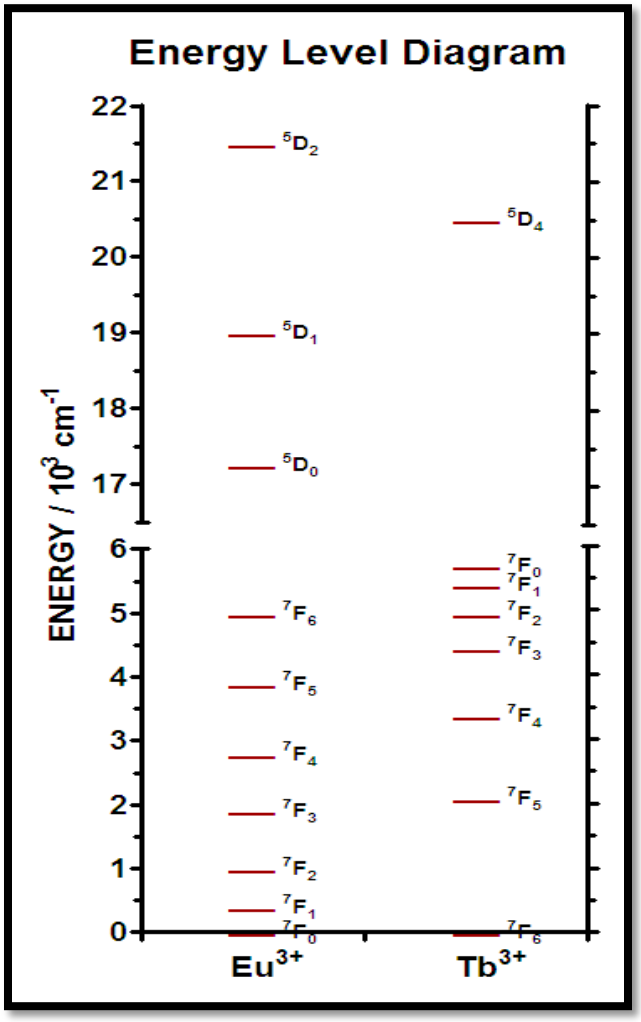


Figure 4. Energy Level Diagram



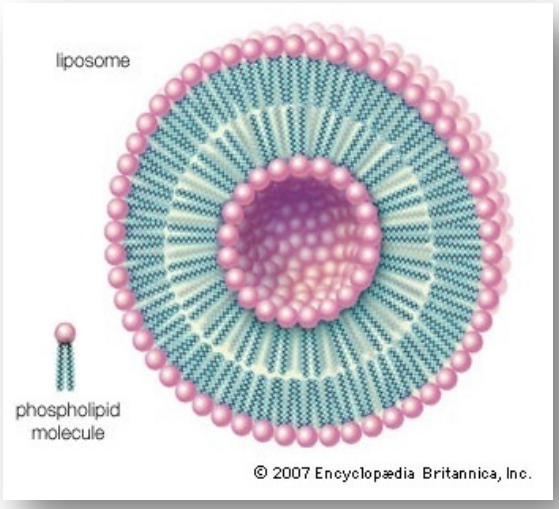
1.b. Nanoparticles Design for biosensor delivery

Nanoparticles were designed by utilizing the characteristic behavior of dual structure, hydrophilic and hydrophobic region in a single molecule. The delivery system must have the property of solubilizing the aqueous biosensor in a hydrophilic (polar) micelle core. Using amphipathic molecules which are everywhere in biological systems. The nanoparticles were designed by using

inverse micelles and double emulsions (Figures 5, 6 and 7). Two particular amphiphilic molecules were chosen. One was a synthetic surfactant: palmitic acid which is anionic, has low toxicity and forms charged micelles that can bind to oppositely charged particles. Second one was a membrane lipid: phospholipids, particularly phosphatidylcholine, which is used for preparation of vesicle suspensions commonly called liposomes or as monolayers [34] (Figure 5). Effective delivery and release of the biosensor were the main purpose of the nanoparticles design.

Palmitic acid or hexadecanoic acid was selected because it is one of the most common saturated fatty acids found in animals and plants. Phospholipid micelles were chosen due to the fact they are an ideal drug and biosensor carrier.

Figure 5. Liposome [35]



Phospholipid micelles are ideal drug carrier systems for multiple reasons. First, the phospholipid nanoparticles are biocompatible and biodegradable. Second, preparation is simple and reproducible [33]. Finally, phospholipids improved in vitro and in vivo stability, bioactivity, targetability and reduced toxicity [33].

Disadvantages are the body can identify the liposomes as foreign intruders and destroy the delivery vehicle. Also, liposomes are very sensitive to temperature, surfactant concentration, and moisture

The nanoparticles were formed by creating inverse micelles and then invert the emulsion. The supramolecular living aggregates can be formed by dissolving surfactants in strongly polar or totally apolar solvents (oils). A schematic representation of reversed micelle is shown in Figure 6.

As stated before the building blocks to our delivery mechanism are molecules called amphiphiles. The strategy devised the use of amphiphiles introduced into an oily liquid (forming reverse micelles) and then obtained a double emulsion.

Figure 6. Schematic Representation of a Reverse Micelle [35].

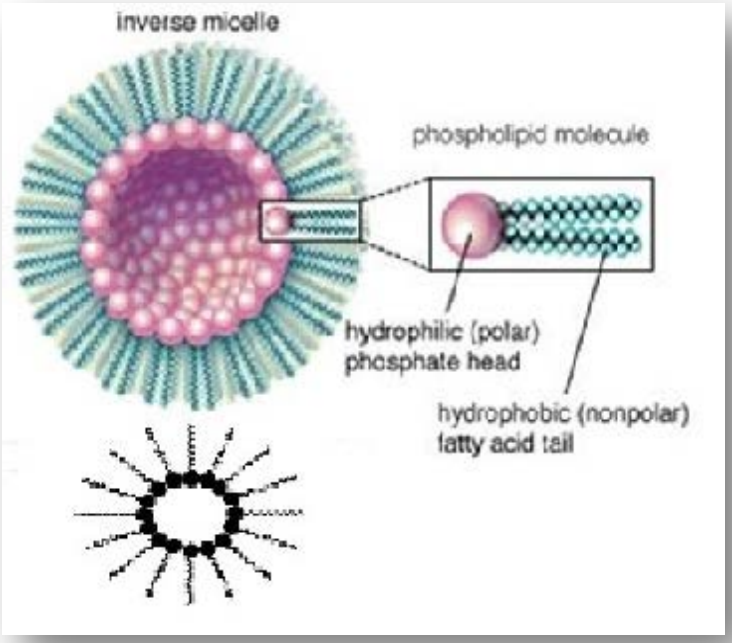
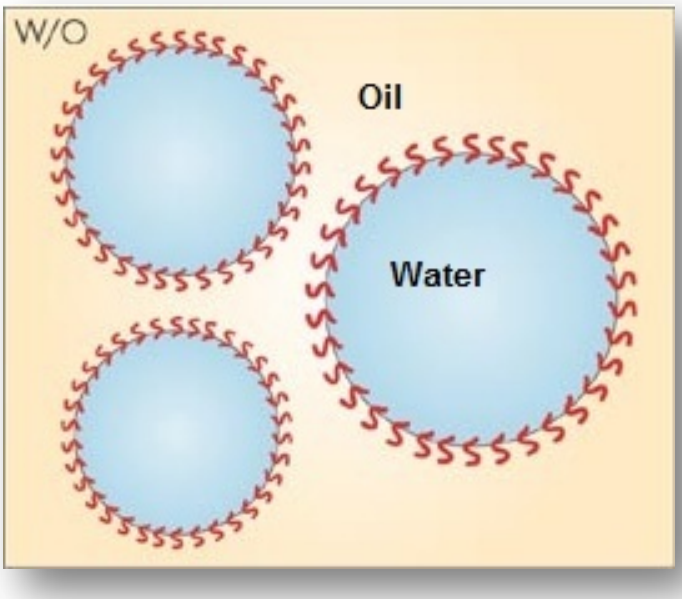


Figure 7. Schematic Representation of Water in Oil emulsion [39].



The difference between reverse micelles and w/o microemulsions is that in the micelles the emphasis is on the surfactant forming the aggregate, while in microemulsions the amount of solubilizate compartmentalize in the micellar core constitutes a relevant part of the entire aggregate.

It is one thing to produce nanoemulsions, it is another thing to determine if they were formed. Different methods such as NMR self-diffusion, transmission electron microscopy (TEM) and scattering techniques (small angle X-ray scattering (SAXS)) and small angle neutron scattering (SANS) provide information of the structure and dynamics of microemulsions and nanoemulsions.

Size and shape of a droplet microemulsions and nanoemulsions can be investigated using a combination of different scattering techniques. Small angle neutron scattering (SANS) is an excellent tool to study the shape, size and polydispersity of the microemulsion droplets. The translational diffusion of the droplets can be studied using dynamic light scattering (DLS). Finally, neutron spin-echo spectroscopy (NSE) gives direct access to the shape fluctuations of the droplets [(40).]

2. MATERIALS AND METHODS

2. a. Materials

Materials were obtained from commercial suppliers and used without further purification. Analytical reagent grade chemicals were used along with de-ionized water to prepare solutions. Lanthanides chlorides were obtained in purities of 99%. Stock solutions of different concentrations of the lanthanides were prepared by dissolving a known amount of trivalent ions in PBS or water (refers to table 1 and table 2). A series of serial dilutions were made with PBS at first for the triphosphate nucleotides and later with de-ionized water, for both the triphosphate and monophosphate nucleotides.

Lanthanides

- Europium$^{(III)}$ chloride powder, $EuCl_3$,

  Purity: 99.9%; was obtained from Aldrich.

  F.W=258.3, Lot number: 05585JJ,

  Appearance: pale yellow powder. CAS 10025-76-0, mp 850 ^{O}C
- Terbium$^{(III)}$ chloride anhydrous powder, $TbCl_3$,

  Purity: 99.9% was obtained from Aldrich.

  F.W=265.28, Lot number: MKAA3919,

  Appearance: white powder. CAS 10042-88-3 mp 588 ^{O}C.

Nucleotides:

- 2’ Deoxyadenosine 5’-triphosphate disodium salt (dATP), 97%, F.W= 535.15 Sigma Aldrich D6500-10MG
- 2’ Deoxycytidine 5’-triphosphate disodium salt (dCTP), 95%, F.W= 511.1 Sigma Aldrich D4635-10MG 98% Purity (HPLC); 4 % Solvent;
- 2’ Deoxyguanosine 5’-triphosphate sodium salt (dGTP), 96%, F.W=507.18 Sigma Aldrich D4010-10MG
- 2’ Deoxythymidine 5’-triphosphate sodium salt (dTTP), 96%, F.W=482.2 Sigma Aldrich T0251-10MG
- Adenosine 5’-monophosphate disodium salt (AMP), 99%, F.W=391.18, from Biochemika-Fluka Analytical 01930-5G $C_{10}H_{12}N_5Na_2O_7P$, Lot 0001443266
- Cytidine 5’-monophosphate disodium salt (CMP), 99%, F.W=367.16, from Sigma C1006-1G $C_9H_{12}N_3Na_2O_8P$, Lot 109K1631
- Guanosine 5’-monophosphate disodium salt (GMP), 99%, F.W=407.18, from Sigma G8377-5G $C_{10}H_{12}N_5Na_2O_8P \times H_2O$, Lot BCBB5515
- Thymidine 5’-monophosphate disodium salt (TMP), 99%, F.W=366.17 from Sigma T7004-250MG $C_{10}H_{13}N_2Na_2O_8P$, Lot 129K1338V

Nucleobases

All were obtained from Sigma Aldrich

- Adenine A8628-5G, $C_5H_5N_5$, 99%: , F.W=135.13, Lot 108K0136
- Cytosine C3506-5G, $C_4H_5N_3O$, 99%:, F.W= 111.1, Lot 059K1010
- Guanine G11950-25G, $C_5H_5N_5O$, 98%, F.W= 151.13, Lot 03420LH
- Thymine T0895-5G, $C_5H_6N_20_2$, 99%, F.W=126.11, Lot 0001435947

PBS

PBS 1X Phosphate Buffered Saline solution without calcium & magnesium, pH: 7.36, obtained from Cellgro Mediatech Inc. CAT No. 21-040-CV

From scratch take the following to make 500 mL solution:

10.9g Na_2HPO_4

3.2g NaH_2PO_4

90g NaCl

500mL distilled H_2O

To adjust the pH add NaOH until final pH = 7.36

Nanoparticles

- α-Tocopherol, F.W=472.74, density=0.953 g/ml, Lot No. 1320538 was obtained from Fluka
- L-A Phosphaditylcholine, obtained from Sigma Aldrich, F.W=776 g/mol, CAS No. 8002-43-5, Lot No. 119K5200, (See attachment 1 for MSDS)

- Palmitic Acid (Hexadecanoic Acid), obtained from Sigma appox 99%, No P-0500 chemical formula is $CH_3(CH_2)_{14}COOH$ Anhyd mol wt 256.4 Lot 42F-0615, (See attachment 1 for MSDS
- Adherent cell Lysis solution was obtained from Origene, hypotonic lysis buffer lot No. 1010
- N-Lauroylsarcosine sodium salt for Molecular Biology (Warning use Mask) from Sigma life sciences Pcode 1000678233, CAS-137-16-6, $C_{15}H_{28}NNaO_3$, MW=293.38g Assay Spec >94% (used 2g in 10mL H_2O)
- BCA™ Protein Assay Kit (bicinchoninic acid) obtained from Thermo Scientific product # 23225 Lot KL136078. BCA™ Protein Assay is shown in Figure 8

  Contains:

  Product #23228 BCA Protein Assay Reagent A

  Product # 1859078 BCA Protein Assay Reagent B

  Prepared the working reagent by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B.

- Hs27 Foreskin Human (Homo sapiens) from ATCC product code CRL-1634 lot number 4012887 1mL volume ampoule containing 8.4 x 10^5 cells. Tissue: Normal; foreskin. Age: Newborn. Gender: male. Morphology: fibroblast. Date frozen: 02/25/05. Expected Viability: 95% to 100%.

- Thermo Scientific Hyclone Classical Liquid Media Dulbeccos Modified Eagles Medium (DMEM) / High Glucose; without L-Glutamine and Sodium Pyruvate. Media; Cell culture; Liquid; Phenol Red. Catalog number SH30081.FS
- Standard Fetal Bovine Serum Collected and processed in USA by HyClone. Triple 0.1 um Sterile Filtered Cat # SH30088.03
- Amphotericin B Solution obtained from Sigma A2942 100mL 250 μg/mL, Lot 0109M00052
- Gentamycin solution from Sigma 10mg/mL in deionized water, liquid, sterile-filtered, BioReagent, suitable for cell culture. Prod # G1272
- Trypsin 10x solution from Sigma T4549 Sterile filtered
- PLGA – Poly(DL-Lactide-Co-glycolide)(50:50), inherit viscosity 0.15 – 0.25 Aldrich 531154-16 mk BB7347
- PEG – Polyethylene glycol from Sigma-Aldrich P3015-500g Batch # 029K0174, CAS 25322-68-3
- PVA – Polyvinyl Alcohol 98% hydrolyzed, from Aldrich 348406-25G Lot 04904DJ CAS# 9002-89-5

Figure 8. BCA Reagent

- http://www.piercenet.com/

INSTRUMENTATION.

All UV-Vis absorption intensity measurements were measured on Ocean Optics USB4000-UV-VIS (USB4F07697) and USB-ISS-UV/VIS (UUSC1562). Software to plot excitation and emission spectra was Logger Pro 3.8.2 Ver. Dtd. Feb 11, 2010 ISBN- 1-929075-24-3.

Figure 9. Ocean Optics UV-Vis Spectrophotometer

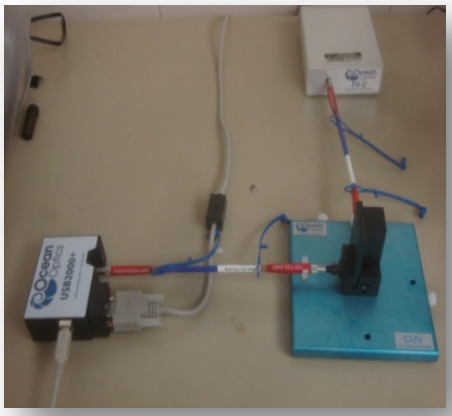
All luminescence intensity measurements were made on a Ocean Optics USB 2000+, CuV, PX-2 spectrophotometer equipped with Spectra Suite Spectroscopy Software 2008 for o/s Win XP ver. 5.1 running X86. The USB2000+ Miniature Fiber Optic Spectrometer is a powerful 2-MHz analog-to-digital (A/D) converter,

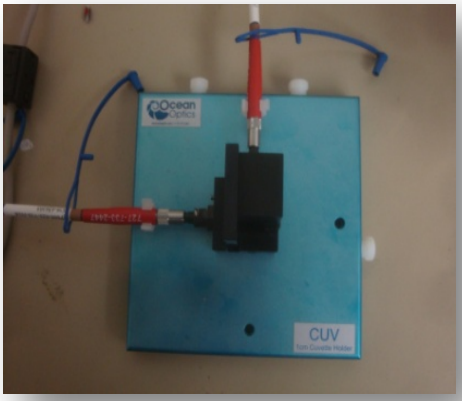
programmable electronics, a 2048-element CCD-array detector, and a high-speed USB 2.0 port.

The PX-2 Pulsed Xenon Light Source was a high flash rate, short-arc xenon lamp for applications involving absorbance, reflection, fluorescence and phosphorescence measurements. Fluoremeter, integration time 2 sec, Scans to average 10, Boxcar width 10.

Both sets of measurements used 1.0 cm quartz cuvettes.

Figure 10. Ocean Optics USB2000 spectrophotometer





To obtain data of emission of the nanoparticles in the cells, the BioTek El x800 with Gen 5 1.09 software to read @ 570 & 630, two times 24 well plate was used.

Figure 11. Bio Tek Fluorometer

http://www.biotek.com/

Finally to obtain the picture of the fluorescence nanoparticles inside the cells, the Olympus BX51 quality research microscope was used. This instrument is capable of bright-field, dark-field, fluorescence, phase contrast and differential interference contrast (Normarski DIC) viewing of samples. The DP 70 Digital Camera was used in conjunction with Olympus DP manager and DP Controller Software.

Figure 12. Olympus BX51 Microscope

2.B. Methods

To find out which nuclei base and nucleotide absorbs and/or emits the best for effective energy transfer; intensity of absorption was measured. Then, emissions were measured after titration of lanthanides with each one of the nucleobases and nucleotides, respectively. The absorption was performed using the Ocean Optics USB 4000-UV-VIS. Solutions were prepared in the dark, due to materials photosensitivity. Logger Pro 3.8.2 software was used to collect the data. Once in Logger Pro, experiment was picked, calibration was performed with a blank and once calibrated; data was collected, and exported as text to graph later. In the same way emission was performed using Ocean Optics USB 2000+, spectrophotometer equipped with Spectra Suite Spectroscopy Software 2008

Originally, the Stock solutions were prepared in PBS shown in Table 1. Performed the UV/Vis measurements, but realized when executing the procedure on the spectrophotometer, the phosphate in the PBS would interfere with the luminescence. Stock solutions were redone in de-ionized water for the nucleotides at 5mM and nucleobases at 10mM, Table 2 shows the real concentrations obtained. Afterwards, diluted the Bases to 100µM, 200µM, 500µM and 1000µM, in addition, the nucleotides stock solutions were diluted to 50 µM and 100µM, and then measured UV-Vis for all of them. In Table 3, the monophosphates nucleotides Stock solutions were prepared using de-ionized water. These nucleotides were used at a diluted concentration of 1mM when combined with the respective lanthanides.

The process called “titration of the lanthanide” was performed as follows: prepared stock solutions in de-ionized water for $EuCl_3$ at 9.80mM, then diluted them to 100µM , 200µM, 500µM, 1,000 µM; likewise $TbCl_3$ at 10.05 mM was diluted to 100µM, 200µM. The two lanthanides were diluted to a concentration of 40 µM.

Table 1. Stock Solutions Bases and Triphosphates in PBS

Nucleotide/Nucleobase	***Stock Solution (concentration mM)***
DATP	4.95
DCTP	5.02
DGTP	5.05
DTTP	5.11
Adenine	10.01
Cytosine	11.58
Guanine	10.59
Thymine	9.68

Placed 2mL of $EuCl_3$ or then $TbCl_3$ of the specified 40 µM concentration into a cuvette and titrated with each one of the nucleobases respectively, in increments

of 10 µL until the peak point of the curve had been reached, or stopped if the absorption/emission readings followed cycles without reaching a peak.

Table 2. Stock Solutions Bases and Triphosphates

Nucleotide/Nucleobase	***Stock Solution (mM)***	***Dilution mM***	***Wavelenght ʎ***
DATP	8.69	1	375.81
DCTP	5.39	1	375.81
DGTP	3.21	1	486.82, 529.32 & 542.54
DTTP	3.38	1	375.81
Adenine	9.88	1	375.81
Cytosine	10.03	1	375.81
Guanine	10.00	1	486.82, 529.32 & 542.54
Thymine	10.05	1	375.81

Placed 2ml of $EuCl_3$, then $TbCl_3$ of the specified 40 µM concentration in a cuvette and titrated with each one nucleotides respectively, in increments of 10 µL until

the peak point of the curve had been reached, or stopped if the absorption/emission readings followed cycles without reaching a peak .The dilution concentrations of the triphosphates and bases were at 1 mM.

Table 3. Stock Solutions Monophosphates

Nucleotide/Nucleobase	***Stock Solution (mM)***	***Dilution mM***	***Wavelenght ʎ***
AMP	9.97	1	375.81
CMP	10.07	1	375.81
GMP	10.00	1	486.82, 529.32 & 542.54
TMP	10.01	1	375.81

Stock solutions were prepared in de-ionized water for $EuCl_3$ at 8.17mM and $TbCl_3$ at 8.04mM. The two lanthanides were diluted to a concentration of 40 µM when placed in a cuvette for measurements.

Placed 2mL of $EuCl_3$ of the specified 40 µM concentration into a cuvette and titrated with each one of the mono nucleotides in increments of 10 µL until the peak point of the curve had been reached, or stopped if the absorption/emission readings followed cycles without reaching a peak.

Preparation of Nanoparticles

During our research two different kinds of Nanoparticles were prepared. The first one was based on Governors State University thesis research by authors Bandaru and Fu. The researchers developed nanoparticles with PLGA-PEG polymer combination to increase the hydrophilicity of Rifampicin (RIF), a hydrophobic drug [26]. Their work, "Photo Physical Properties of Non- Encapsulated and Encapsulated Rifampicin: A comparative study", was the base for becoming familiar with the methods and measurement of fluorescence nanoparticles. The hydrophobic nanoparticle was not a good fit for this research but helped to understand the delivery mechanism into the cells. The second type of nanoparticles developed was based on nanoemulsion technology using two different types of surfactants. Following is the description of the two methods pursued to develop hydrophobic and hydrophilic nanoparticles.

Preparation of Nanoparticles with luminescence marker (Rivoflavin+Rifampicin) [26]

- A calculated amount of Poly (DL-Lactide-Co-glycolide (PLGA) and 5% (w/v) polyethylene glycol (PEG) were dissolved in 2 mL of dichloromethane (DCM) in separate tubes. Then mixed for 30 minutes.
- Prepared luminescence marker Rifampicin (RIF)
- Prepared PVA solution.
- The luminescence marker and the polymer solution PLGA and PEG were mixed and vortexed until emulsified.

- The emulsion was poured into PVA solution which led to the double emulsification of the particles.
- Mixture from previous step was sonicated for 30 minutes then centrifuged for 15 minutes at 13000 rpm, washed with de-ionized water twice. Repeated centrifugation step 4 times, then resuspended in water and placed in the refrigerator for storage.

Preparation of W/O/W Nanoparticles with luminescence Lanthanide marker

The following drug delivery system allows the encapsulation of hydrophilic species into nanoemulsions. The reverse-micellar system involved the solubilization of hydrophilic molecules (solutions of Eu^{3+} and Tb^{3+} with Guanosine 5'-monophosphate disodium salt hydrate (GMP), respectively, in oil (α-Tocopherol) to form a stable w/o emulsions (encapsulation of the marker). The reverse micelle–loaded oil was injected into an aqueous phospholipid or palmitic acid solution to form a w/o/w emulsion.

The phase-transfer method for preparation of reverse micelles [27] was pursued. In this method the organic phase (oil) loaded with L-α Phosphaditylcholine or palmitic acid (surfactants) was titrated with the luminescence marker. This phase was mixed with an aqueous phase containing its particular surfactant and phase separation occurred after equilibrium was reached. The method follows:

1. Prepared the w/o emulsion: oil plus particular surfactant was titrated with aqueous solution of Eu^{3+} and Tb^{3+} with GMP respectively. Ensuring that the luminescent marker was added in the same ratio of energy transfer found in the emission experiments, as Eu(III) to GMP, ratio 1:11 with concentrations, Eu(III)at 40µM and GMP at 444 µM. And TB(III) to GMP, ratio 1:10.7 with concentrations, TB(III)at 40µM and GMP at 428 µM. The process of preparation of the water in oil emulsion for the L-α Phosphaditylcholine surfactant began by measuring 5 mL (weight was 4.94g) of α-tocopherol and mixed with 0.0019g of L-α phosphaditylcholine (500 µM). Likewise, measured 5 ml of α-tocopherol (weight was 4.72g) and mixed with 0.006 g of palmitic acid (500 µM).

   After the previous solution was made and in the process to be titrated with luminescence marker, it must be sonicated until no more water solution could be intake by the emulsion. The L-α phosphaditylcholine emulsion titrated by the Eu(III) and GMP solution had a maximum intake 700 µL of the marker. The Palmitic Acid emulsion had a maximum intake of 1200 µL. In the same way, the L-α phosphaditylcholine emulsion titrated by the TB(III) and GMP solution had a maximum intake of 1200 µL and for the palmitic acid emulsion the maximum value of intake was 1500 µL.

2. Sonicated the mixture for 1 hour, then centrifuged for 15 minutes at 13000 rpm. Decanted fluid.

3. Mixed w/o emulsion of step 1 with aqueous solution of surfactant L-α phosphaditylcholine or palmitic acid respectively. The aqueous solution was prepared at a concentration of (250 μM) as follows:

   22 mL of de-ionized water were measured and mixed with 0.0043 g of L-α phosphaditylcholine. The second solution consisted of 22 mL of de-ionized water, mixed with 0.0014 g of Palmitic acid.
4. Formation of double emulsion. The addition of w/o to an aqueous surfactant solution formed a milky inverse emulsion.
5. Sent samples to outside laboratory to find particle size through TEM images.

Cell study

Cell penetration study was performed to assess the diffusion and the releasing of the biosensor into the cells. We obtained a good encapsulation of the hydrophobic luminescence biosensor (rivoflavin + rifampicin). The cell study was performed with this hydrophobic nanoparticle. Cells obtained from ATCC were grown per the prescribed protocol (see below Cell Preparation Procedure); cell plates were washed with PBS two times. Then into a 24 well plate, 1mL of PBS was placed into 17 wells. Placement on the plate were as follows: by columns on the plate (4 wells) from left to right the following concentration of 0, 200, 400, and 800 uL of the nanoparticle and 1 well (5^{th} column) had 1000 uL of the nanoparticle solution set as control. The plate was incubated for one hour. A cell

lysis solution using N-lauroylsarcosine sodium salt was previously made. Discarded the solution from the 17 wells and added 500 µL of the lysis solution in each well, placed on an incubator shaker for overnight shake. After complete breakthrough of cells, took emission spectra at ʎ=530.39. (Figure 30 and 31). The maximum emission was found at ʎ=530.39 with an intensity of 14,298.

Cell Preparation Procedure

1. Prepared modified DMEM Media food starting with one 500mL bottle of Hyclone DMEM/HIGH media. Added 50mL of Standard Fetal Bovine Serum to DMEM/HIGH media. Additional, added 2.5 mL of Gentamycin solution and 5.0 mL of Amphotericin B Solution to the DMEM/HIGH media. The Amphotericin B Solution and Standard Fetal Bovine Serum are stored in freezer. These two components must thaw out before added to DMEM/HIGH solution. Modified DMEM media placed in incubator to warm up to 37^{o} C.
2. Removed 1mL ampoule from cryogenic storage container. Allowed to thaw and placed into 750 mL flask with 50 mL modified DMEM media. Placed cell culture in incubator at 37^{o} C and 5.0% $CO_{2.}$
3. Waited 24 hours, rinsed flasks with sterilized PBS solution twice and added 50 mL modified DMEM media to flasks. Returned to incubator.
4. Waited 48 hours, checked progress of growth of cells. Changed modified DMEM media with fresh modified DMEM media. Repeated this cycle until flask was saturated with cells.

5. Re-plated cells starting with making 9mL de-ionized water to 1 mL 10x Trypsin solution. Drained media from flask and washed with PBS 3 to 4 times till all color was removed from flask. Added the 10mL Trypsin solution to the flask. Spread throughout flask and kept in incubator for two minutes. Took out of incubator and gave gently but stern slaps to the incubator to loosen cells from flask's walls. Checked for any cell attachment using microscope. If still attached continue stern slapping of flasks. Once all cells all cells are floating. Then immediately add 50 mL of modified DMEM media, otherwise Trypsin will inactivate the cells. The modified DMEM media inactivates the Trypsin. Added additional 20 mL of modified DMEM media to flask. Proceeded to transfer 2 mL aliquots to 40 circular Petri dishes. Added additional 1 mL of modified DMEM media for a total of 3 mL of solution in each Petri dish. Placed all Petri dishes in incubator.
6. Returned 24 hours later and rinsed each Petri dish twice with PBS and placed 5 mL of modified DMEM media into Petri dishes and placed back in incubator to be checked at 48 hour intervals.
7. At the 48 hour interval checked progress of cells in Petri dishes on the microscope. If dishes were not full then drained old media off and refilled with 5 mL of fresh modified DMEM media. Placed back into incubator.
8. Once plates are full with cells, then plates can be used for further testing such as toxicity or preliminary uptake of nanoparticles. Precautions such as maintaining sterile conditions are extremely important. Do not touch

tips of containers. Proper pipetting techniques should be used. Never pipette from original bottles. Transfer quantity needed to secondary bottle and pipette from this bottle. Lids of flask should not be too tight. Powder free latex gloves should be worn at all times when handling cell cultures and washed repeatedly with a 70/30 % ethanol to de-ionized water solution.

As previously explained, hydrophilic nanoparticles were developed; these ones encapsulated the luminescent biosensor so cell study and toxicity were performed as follows:

1. Had cells ready and then transferred them into the wells placing the same amount 250µL of cells in each well. Emptied out the media and washed the cell plates with PBS. Put 1 mL of PBS in each one of the 17 Petri dishes.
2. Incubated plate for one hour after placing the following amount of nanoparticles into the wells with cells:

   Control (no nanoparticles), 1ml, 2ml and 3 ml, and incubate for 1 hour. This procedure was followed for the de-ionized water in oil emulsions of palmitic acid and The L-α phosphaditylcholine as a concentration dependant study.

   At the same time placed the biosensor (Lanthanide + GMP) into the wells with the cells:

For Eu (III) at a concentration of 40 µM, 80 µM and 120 µM, GMP added at concentrations of 150 µM, 300 µM and 450 µM,

For Tb (III) at a concentration of 40 µM, 80 µM and 120 µM, GMP added at concentrations of 150 µM, 300 µM and 450 µM.

Plates were incubated for 1 hour.

3. Took away the emulsions and do not wash with PBS.
4. Put media back and incubated for 48 hours
5. Performed toxicity test using BCA Reagent Assay Kit

Toxicity test

The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein

concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction [28].

The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein. However, unlike the Coomassie dye-binding methods, the universal peptide backbone also contributes to color formation, helping to minimize variability caused by protein compositional differences. [28]

If the biosensor is toxic, then those cells killed have detached from the plate and are free floating. These dead cells are then rinsed away when washing the plates with regular PBS. Therefore, the BCA test, as explained in the previous paragraphs, is measuring the protein content from only those cells left alive that undergo the lysis solution step. This step, (lysis) has been determined to be highly critical in the accuracy of the toxicity test. Any large portion of a well's cells which are not lysis would give an undercount of the true value of absorbance compared if all cells were lysis. Using the absorbance results obtained by the Micro plate reader, a ratio of absorbance of each concentration dependant series versus the three control wells' would be determined. This ratio when translated to a percentage would start the foundation of determining the LD50 for the given concentration which were tested and find if the biosensor is toxic. The procedure used is as follows:

1. Outside of the hood, washed plates twice with regular PBS

2. Put 1 mL of lysis solution
3. Shook 15 minutes lightly at 100 rpm
4. Prepared the BCA reagent as 50 parts of reagent A and 1 part of reagent B, a green solution was formed and the solution must be protected from the light. Added 3 mL of working reagent per sample.

   Reagent A: Bicinchonic acid and tartrate in an alkaline carbonate buffer.

   Reagent B: 4% copper sulfate pentahydrate solution
5. Transferred 1.1 mL of sample in a 24 well plate, replicated three times for each sample.
6. Used the BioTek El x800 absorbance microplate reader with Gen 5 1.09 software, and selected protocol1. Read Plates @ 570nm and 630 nm.

Fluorescence Study

1. Prepared 3 flasks of cells over a two week period. Fed cells per lab protocol using Standard Fetal Bovine Serum with Amphotericin B and Gentamycin.
2. Re-plated grown cells using Trypsin to make cells detach from walls of flasks. Placed cells into five 100mm x 100mm square petri dishes this had two 75x25mm (1.0 – 1.2mm thick) pre-cleaned plain microscope slides.
3. Cells grew for week.
4. Prepared emulsions (L-α phosphaditylcholine + de-ionized water + α-tocopherol) and (palmitic acid + de-ionized water + α-tocopherol) and

added Eu(III) + GMP and TB(III) + GMP to come up with four plates plus one control plate.

5. Sonicated the emulsions for one hour, prepared square petri dishes, washed with PBS twice. Placed emulsions on slides in the square petri dishes and incubated for one and half hours.
6. Using the Olympus BX-51 microscope (fFigure 12) filtered by the standard U-RSL 6 filter bar with attached DP 70 camera and using Olympus DP Manager and DP Controller software captured images (See Figure 33).
7. Images captured were not well defined. Those images of (Palmitic Acid + de-ionized water + α-Tocopherol) and added Eu(III) + GMP and TB(III) + GMP were the better of the images.
8. Grew cells for week in flask, re-plated into three square petri dishes and let grow for another week.
9. Sonicated only the emulsions of (palmitic acid + de-ionized water + α-tocopherol) and added Eu(III) + GMP and TB(III) + GMP for two hours. Prepared square petri dishes, washed with PBS three times. Placed emulsions on slides in the square petri dishes and incubated this batch for three hours.
10. Using the Olympus BX-51 microscope filtered by the standard U-RSL 6 filter bar with attached DP 70 camera and using Olympus DP Manager and DP Controller software captured images (See Figure 34).

3. RESULTS AND DISCUSSION

3.a. Basic Spectroscopic Properties of Europium (Eu) and Terbium (Tb)

The absorption spectrum of the lanthanides, bases and nucleotides give us valuable information concerning the initial step involved in any possible photochemical process. A molecule may be excited in several different ways, depending on the frequency of radiation absorbed. It is anticipated that the absorption causes displacement of outer electrons in the molecule because the frequency of radiation is in the visible and ultraviolet region of the spectrum [29].

Europium (Eu) and Terbium (Tb) are part of the lanthanides, fifteen rare earth metal elements in the sixth row of the periodic table. They are often referred to as 4*f*-metals, because each new electron added as one proceeds from Lanthanum (La) to Lutetium (Lu) enters into the 4*f*-shell. Furthermore, since the 4*f*-shell is located inside of the shell of the 5*d*6*s*-conduction state, the nature of the latter changes little as a function of atomic number. The chemical properties of the lanthanide ions are very similar, since the 4*f* electrons are well shielded by the 5*s* and 5*p* electrons [30]. The most impressive feature about the spectra of rare earth (RE) ions in ionic crystals is the sharpness of the many lines in their absorption and emission spectra. These lines can be as narrow as those commonly observed in the spectra of free atoms of free molecules. The narrow optical lines suggest that the interaction or rare earths (RE) ions with the crystalline environment is

relatively weak [31]. Table 4 shows some basic spectroscopic information of Eu (III) and TB (III).

The rare earths when solids are either divalent or trivalent. By far the most common valence state of the RE ions in solids is the trivalent state with electronic configuration $4f^{N-1}5s^25p^6$. The 4*f* electrons are clearly not the outermost electrons. These electrons are shielded from external fields by two electronic shells with a larger radial extension ($5s^25p^6$), this explains the atomic nature of their solid state spectra. Due to this shielding, the 4*f* electrons are only weakly perturbed by the charges of surrounding ligands. This characteristic is why RE ions is such a useful probe in a solid. The crystal environment constitutes only a small perturbation on the atomic energy levels and many of their solid state, hence spectroscopic, properties can be understood from a consideration of the free ions [32].

Eu (III) has been used in luminescence studies due to the unique 4*f*-to-4*f* absorption/emission band near 580 nm which cannot be split by a ligand field. Usually a tunable dye laser is necessary to study the Eu (III)'s 580 nm band because of the resolution and sensitivity needed for analysis of the band shape. Eu (III) is also the least stable lanthanide with respect to redox chemistry. It's reduction potential of about -3.5 V, depending on the coordination environment is within the range of weak biochemical reducing agents. All other trivalent lanthanides have oxidation/reduction potentials unfavorable by 1 V or more.

Consequently, due to either instrumental or chemical reasons, it is not always possible to take advantage of some of the superior probe properties of Eu (III) [15].

Table 4. Basic Spectroscopic Properties of Eu & Tb in the trivalent state (32)

Atomic Number	***Element***	***Electron configuration RE(III)***	***Ground Term RE(III)***
63	Europium (III)	$4f^6 5s^2 5p^6$	$^7f_0$
65	Terbium (III)	$4f^8 5s^2 5p^6$	$^7f_6$

TB (III) has 4*f*-to-5*d* absorption bands which provide an alternative to the demanding instrumental requirements of studying the 580nm band of Eu (III). Since the 5*d* orbitals are immediately exposed to the ligand field, the 4*f*-to-5*d* absorption bands move by thousands of wavelength numbers with changes in coordination, providing a convenient monitor of site multiplicity, and chemical exchange. However, some of the TB (III) absorption bands are too far into the ultraviolet range to be studied with conventional fluorescent equipment and often overlap with ligand absorption bands. Energy transfer from intrinsic fluorophores, such as nucleic acids, generally gives rise to enhanced TB (III) [33] emission. This is often not the case with Eu (III) if charge transfer occurs [15].

Basically, all of the lanthanide absorption bands observed in the near-infrared to near-ultraviolet range of the spectrum are attributed to electric dipole transitions, although these transitions are parity forbidden since they occur between states

within the same configuration. The magnetic properties of lanthanide ions vary appreciable along the series. The ions La (III) and Lu (III) are diamagnetic. Among the paramagnetic ions, Pr (III), Eu (III) and Yb (III) have short electron spin relaxation time. An extremely important application of lanthanides is the ability to get quantitative answers to conformational problems. Enhancement of the luminescence of Eu (III) and TB (III) ions on biding to a ligand are capable of providing a detailed knowledge of the metal ions biding sites [30].

3.b. Absorption of Europium (Eu) and Terbium (Tb)

Lanthanides are extremely weak absorbers with Europium and Terbium having shown weak absorption bands in the UV-Vis spectrum. No sharpness of lines are observed in their absorption spectra. The absorption spectra of aquo-complexes showed values of extinction coefficients (ε) in the literature for TB(III) near 310 $M^{-1}cm^{-1}$ at 220nm corresponding to the fully allowed 4*f*-to-5*d* band [15]. For Eu(III) the $^7F_1$ state was only about 360 cm^{-1} above the $^7F_0$ ground state and excitation from both levels was observed. Unlike TB (III), the Eu (III) 5*d* levels lie well above 50,000 cm^{-1} and no 4*f*-to-5*d* absorption bands are observed above 200 nm. Like TB (III), Eu (III) had many 4*f* levels accessible within the visible and ultraviolet regions yielding a large number of absorption and emission bands (see Figure 4) [15].

For a foundation for the research, the extinction coefficients for Eu (III) and Tb (III) were established as a measure of how strongly these lanthanides absorbed

light at a particular wavelength. Dilutions from a stock solution generated four different solutions with the following concentrations: 100 µM, 200 µM, 500 µM and 1,000 µM of each one of the lanthanide ions, then the absorption of each solution were measured. The excitation wavelength was λ =226.50 nm for Eu (III) and the average absorbance value for all the solutions were 0.21. Likewise, the excitation wavelength for Tb (III) was λ =221.60 nm and the average absorbance value were 0.22.

After finding the linear equation, that represented the relationship between concentration (X-Axis) and absorbance (Y-Axis) for the specific lanthanide ion, the value of the molar extinction coefficient (ε) was calculated (Figures 13 and 14). The calculation based on Beer's law found that Eu (III) molar extinction coefficient was 457 $M^{-1}cm^{-1}$ and the one for Tb (III) was 477 $M^{-1}cm^{-1}$. The experimental value of Tb (III) was close to the reference value of 310 $M^{-1}cm^{-1}$ at 220 nm. Details of the experimental conditions of terbium chloride in water can be found on the cited reference [15]. This wavelength presumed corresponds to the fully allowed transition 4*f*- to-5*d*. An expected value to compare the molar extinction coefficient of Eu (III) was not available.

The absorption spectra of the nucleic bases were analyzed. The base adenine (A) dissolved in water at excitation wavelength λ max=260.5 nm had a molar extinction coefficient ε =7,772 $M^{-1}cm^{-1}$. Literature research shows that adenine dissolved in water, pH 7, has a molar extinction coefficient of 13,400 $M^{-1}cm^{-1}$ at

261 nm [34]. Due to an expected low fluorescence yield, estimated to be $2.6x10^{-4}$ a fluorescence spectrum was not collected [35].

The base cytosine (C) dissolved in water at excitation λ max=220.34 nm had a molar extinction coefficient ε =6,571 $M^{-1}cm^{-1}$. Literature research shows that cytosine dissolved in water, pH 7, has a molar extinction coefficient of 6,100 $M^{-1}cm^{-1}$ at 220 nm [34]. Due to an expected low fluorescence yield, estimated to be $8.2x10^{-5}$ a fluorescence spectrum was not collected [35].

Guanine (G) dissolved in water at excitation λ max=246 nm, had a molar extinction coefficient of ε =390 $M^{-1}cm^{-1}$. Literature research shows that guanine dissolved in water has a molar extinction coefficient of 10,700 $M^{-1}cm^{-1}$ at 243 nm [34]. Due to an expected low fluorescence yield, estimated to be 3.0×10^{-4} a fluorescence spectrum was not collected [35].

Finally, thymine (T) dissolved in water at λ max=263.2 nm, had a molar extinction coefficient of ε =5,075 $M^{-1}cm^{-1}$. Literature research shows that thymine dissolved in water has a molar extinction coefficient of 7,900 $M^{-1}cm^{-1}$ at 263.75 nm [34]. Due to an expected low fluorescence yield, estimated to be $1.02x10^{-4}$ a fluorescence spectrum was not collected [35].

Figure 8. Eu (III) absorption at wavelength max λ =226.5 nm

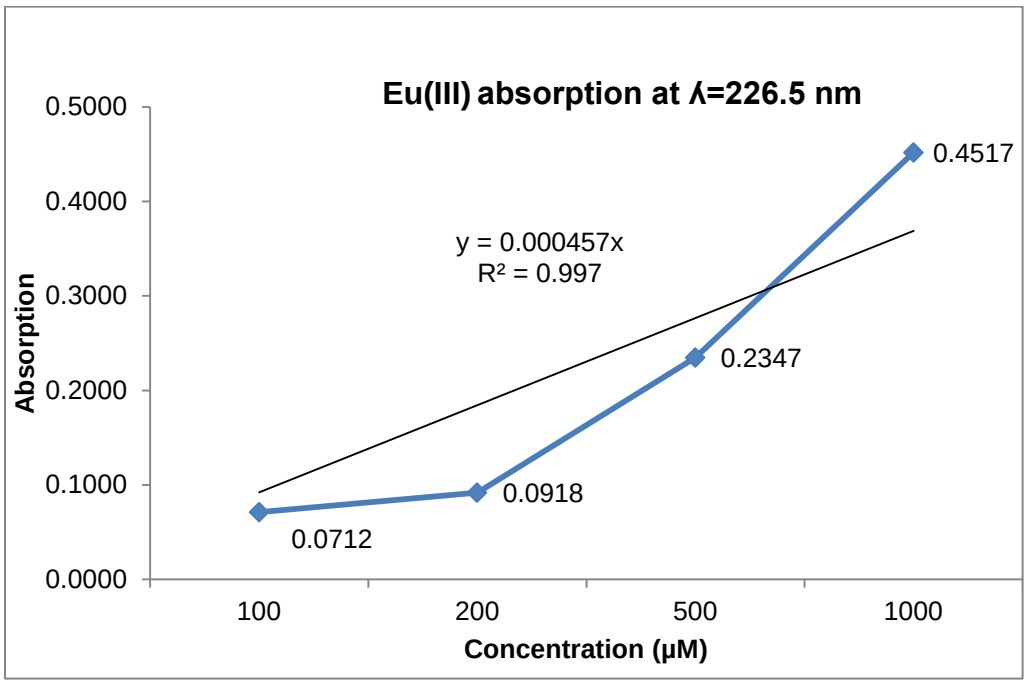
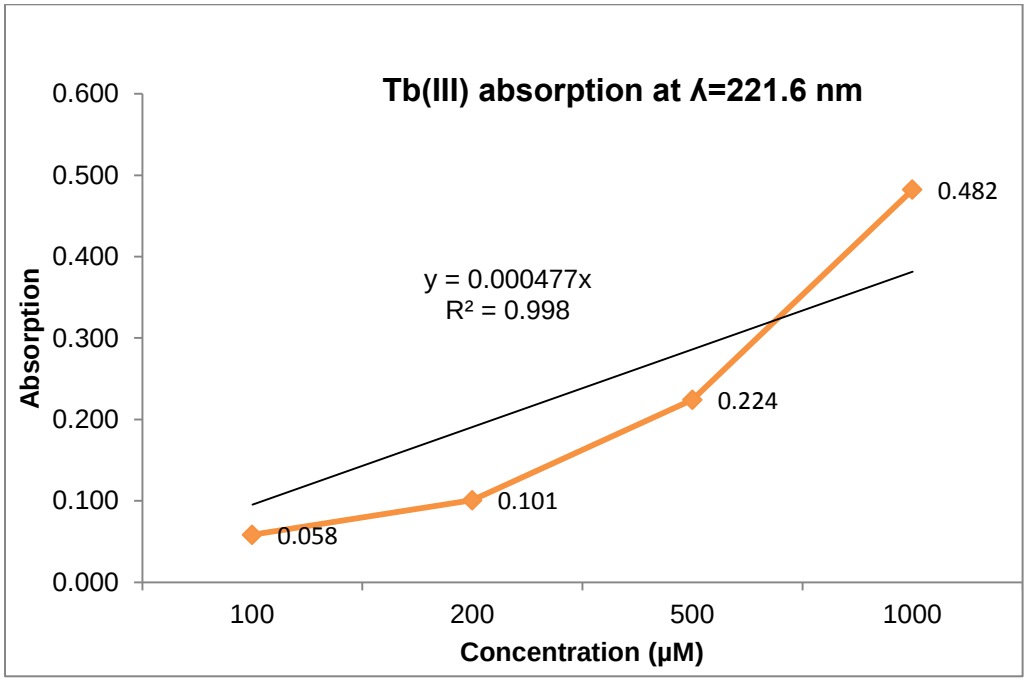


Figure 9. Tb (III) absorption at wavelength max λ = 221.6 nm



The same procedure was followed to measure the absorption of the nucleotides. dATP in aqueous solution had a wavelength maxima of λ =261.55 nm and a molar extinction coefficient of ε=13,189 $M^{-1}cm^{-1}$. Likewise dCTP in aqueous solution had a λ max=220.32 nm and ε =7,896 $M^{-1}cm^{-1}$; dGTP had a λ max=253.28 nm and ε =8,821 $M^{-1}cm^{-1}$; finally dTTP had a λ max=266.84 nm and ε =6,607 $M^{-1}cm^{-1}$. There were not literature values available to compare the accuracy of the results. However, the molar extinction coefficients were similar to the values found for the respective bases, which would be a good indication of the validity of the nucleotides absorption results.

Based on the experimental data for wavelength maxima (λ max) and extinction coefficient (ε), it was clear that the values for adenine and guanine were different than the ones reported in the literature. Cytosine and to a lesser degree thymine extinction coefficients were the only nucleotides comparable with the expected values. The extinction coefficient (ε) is a characteristic of the solute and depending upon the wavelength of light, the solvent and temperature, any change in the experimental conditions can be the cause for inaccuracy. Literature research states that membrane systems are probably the most difficult to study, due to their unavoidable turbidity and tendency to settle [(15)]. The differences in absorption values of the nucleic acids can be attributed to its physical characteristics. Because, centrifugation and microfiltration were not performed on the nucleic bases or its nucleotides in order to avoid turbidity and precipitation.

The homogeneity of the sample might be compromised in the absorption and emission readings.

3.c. Emission of Europium (Eu) and Terbium (Tb)

The purpose of the emission experiments was to determine the best concentration in which effective energy transfer from the donor, nucleic bases and nucleotides, to the acceptor, trivalent ion, would occur. After many trials of adding nucleic bases (donor molecule) to a fluorescent trivalent ion solution (acceptor ion) at different concentrations, the best array of concentrations for efficient energy transfer was finally devised. The initial concentration of the trivalent ions was set at 40 µM. Aliquots were added in increments of 10 µL, of nucleic acids at 10 mM stock solution, to each one of the trivalent ions. Similarly, aliquots were added in increments of 10 µL, of nucleotides at 1 mM stock solution, to the respective aqueous trivalent ions solution. We called this process, titration of the trivalent lanthanide ion by either nucleic bases or nucleotides. Spectra emission was obtained but for analysis focus was on the visible spectrum. Also, notice the excitation readings at a single wavelength were not performed.

A solution containing 40 µM Eu (III) or Tb (III) was weakly emissive upon excitation, due to the low extinction coefficient of the lanthanide ion. Since nucleic acids exhibit large absorption cross sections in the 250-280 nm range [(1)], energy transfer (EnT) from the excited base to the emissive state $^5D_0$ of bound Eu (III) or $^5D_4$ of Tb (III) is possible. Although Tb (III) excitation can involve the 5*d*

levels, there is rapid internal conversion of all excited states to the $4f^5D_4$ level, from which virtually all the emission occurred [15]. The enhanced Eu (III) or Tb (III) luminescence in the presence of each nucleic base was established. The relative emission intensity of 40 µM Eu (III) with increasing concentration of each nucleic base is shown in Figures 15 and 16. Similarly, the relative emission intensity of 40 µM Tb (III) with increasing concentration of each nucleic acid is shown in Figures 17 and 18.

The relative emission intensity of 40 µM Eu (III) spectra at λ=375.81 nm, 486.82 nm and 542.54 nm with increasing concentration of each nucleic bases displayed decreasing behavior for overall luminescence for A (adenine), C (cytosine) and T (thymine). As the aliquots at the following concentration; A (adenine 9.88 mM), C (cytosine 10 mM), and T (thymine 10.05 mM) were increased, there was no enhancement of Eu (III). Significant enhancement of the Eu (III) emission intensity at λ=375.81 nm and 542.54 nm was observed upon addition of G (guanine) which was quite the opposite of A, C, and T. (Figure 15 and 16).

The relative emission intensity of 40 µM Tb (III) spectra at λ =375.81 nm and 542.54 nm with increasing concentration of each nucleobase showed decreasing behavior in the overall luminescence for adenine, cytosine and thymine bases. As the aliquots at the following concentration; A (adenine 9.88 mM), C (cytosine 10 mM), and T (thymine 10.05 mM) were increased, there was no significant enhancement of Tb (III). In contrast, important enhancement of the Tb (III)

emission intensity at λ =375.81 and 542.54 nm was observed upon addition of G (Figure 17 and 18).

From the analysis of the emission results with nucleic bases, it was deduce that only one of the bases showed effective enhancement. Guanine displayed emission for both Eu (III) and Tb (III). It is known that strong chelation of Tb(III) in water is better accomplished by ligands that possess two or more adjacent electron density rich regions, especially when at least one of them is an oxygen atom [(36)]. Inspection of the structures of the nucleic acids (Figure 1) revealed that this is only possible in C (through O2 and N3) and G (through O6 and N7). The difference in enhancement between C and G may be due to differences in quantum yield or triplet state formation or differences in binding stability and kinetics [(1)].

The relative emission intensity of 40 μM Eu(III) and Tb(III), at λ=375.81 nm and 542.54 nm was measured as the concentration of 2' Deoxy 5'-triphosphate disodium salt of each nucleobase, dATP, dCTP, dGTP and dTTP, was increased (Figure 19 through 22). It was expected that dGTP or dCTP would show luminescence enhancement. Surprising results were obtained when none of the nucleotides showed emission.

Figure 10. Emission of Eu (III) with nucleobases at λ=375 nm

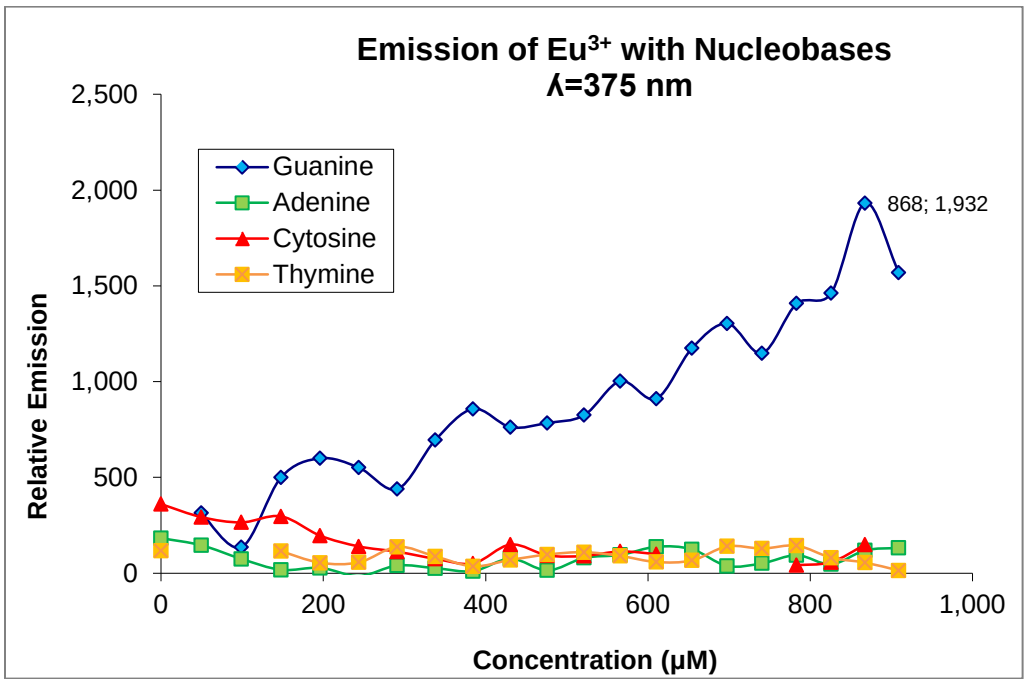


Figure 11. Emission of Eu (III) with nucleobases at λ =542.54 nm

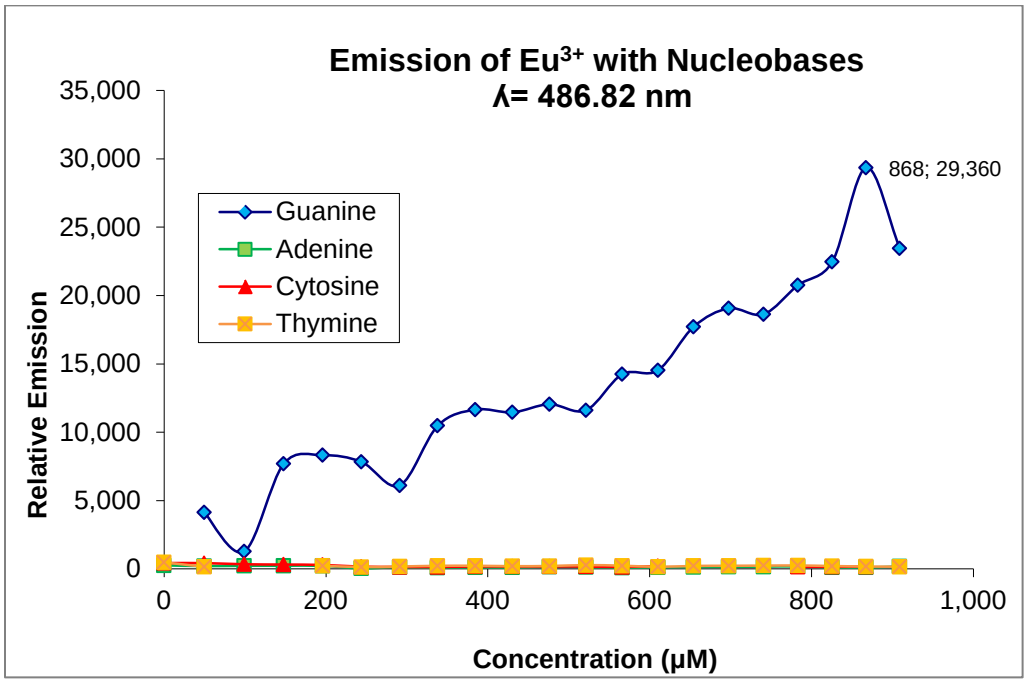


Figure 12. Emission of Tb (III) with nucleobases λ =375 nm

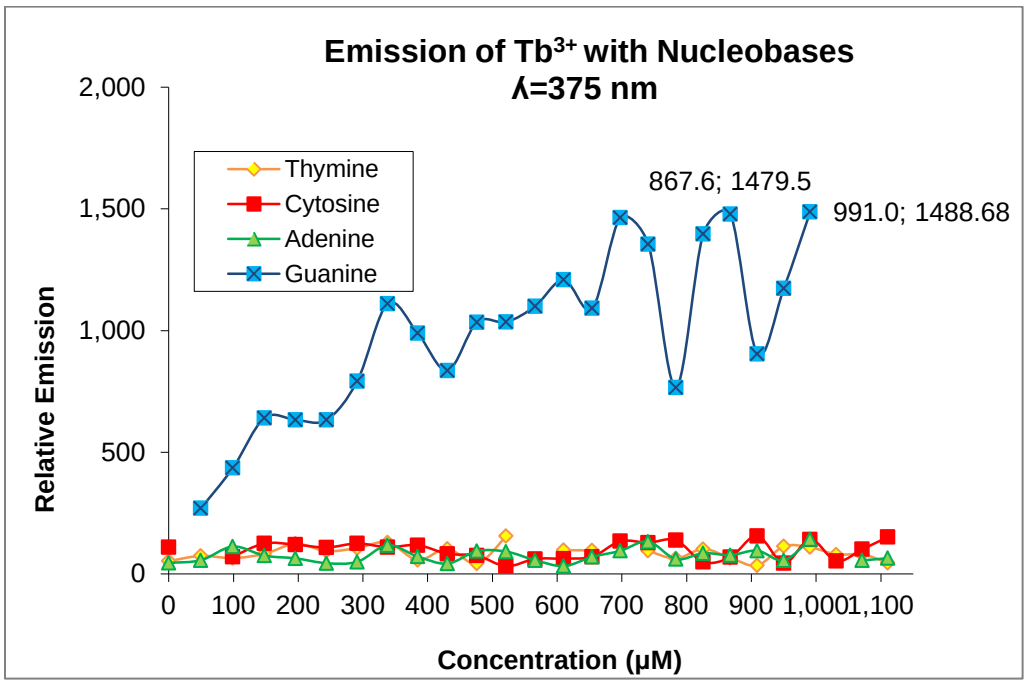
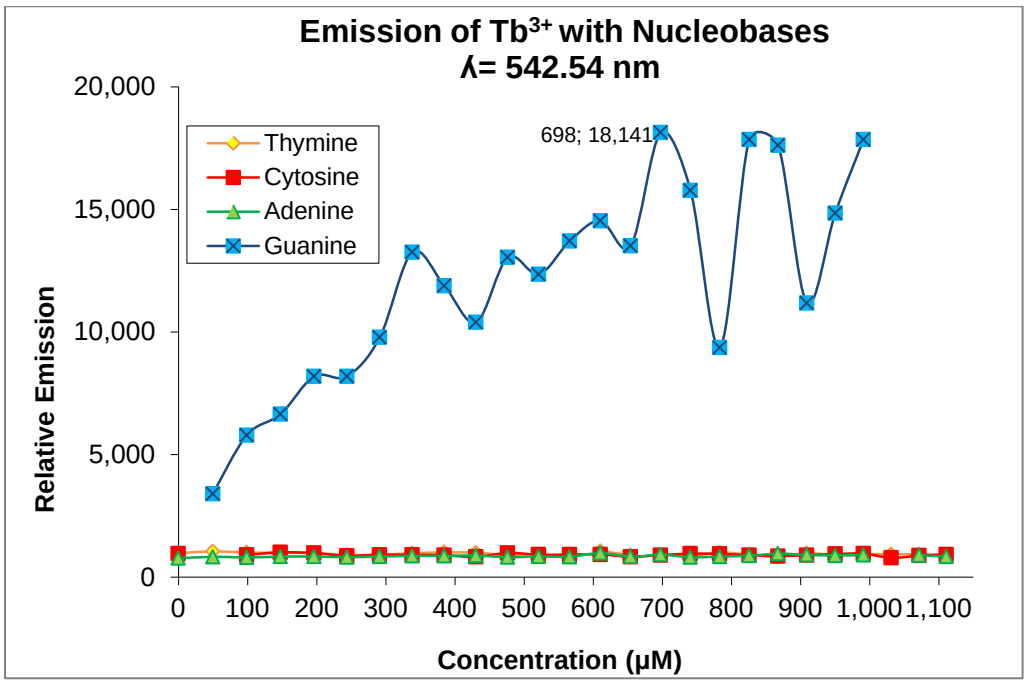


Figure 13. Emission of Tb (III) with nucleobases at λ =542.54 nm



Apparently, there was no energy transfer due to phosphate interference and impractical binding with the three phosphates available for complexation (Figure 2, dGTP). Previous studies measured the emission intensity of 25 μM of Tb (III) as the concentration of that 5' deoxymonophosphate disodium salt of each nucleobase, dAMP, dCMP, dGMP, and dTMP was increased. As previously reported, only dGMP, showed enhancement of Tb (III) emission [(1)]. Therefore, we performed fluorescence experiments using 5'-monophosphate disodium salt of each nucleobase, at concentration of 1 mM respectively (Figure 2, GMP).

The relative emission intensity of Eu(III) and Tb(III) spectra at λ=486.82 nm and 542.54 nm was measured as the concentration of the 5'-monophosphate disodium salt of each nucleobase (1mM), AMP, CMP, GMP and TMP, was increased (Figure 23 through 26).

Only GMP showed a slight enhancement of the Eu (III) emission at 486 nm but no enhancement at 542 nm (Figure 23 and 24). Addition of similar concentrations of AMP, CMP, and TMP to Eu (III) did not appear to enhance the luminescence of the lanthanide ion. Relatively, for Tb (III), GMP showed considerable enhancement of the Tb (III) emission at both wavelengths 486 nm and 542 nm (Figure 25 and 26). A negligible degree of enhancement for Tb (III) was formed by addition of AMP at 486 nm but not at 542 nm. Addition of similar concentrations of CMP, and TMP to Tb (III) did not appear to enhance the luminescence of the lanthanide ion.

Figure 14. Emission of Eu (III) with triphosphate nucleotides at λ=375 nm

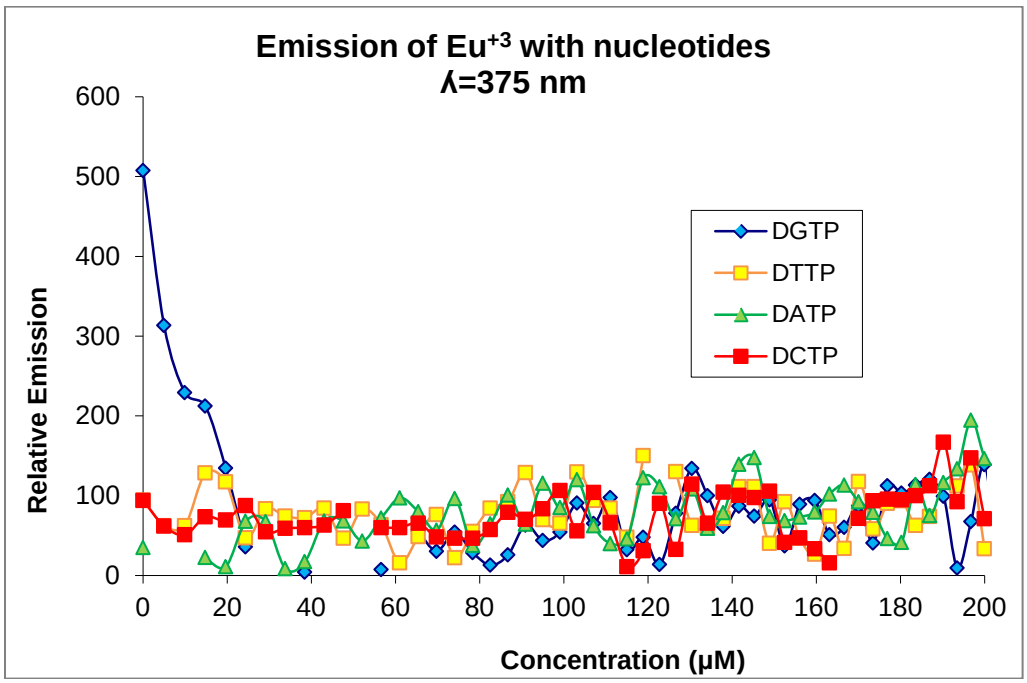


Figure 15. Emission of Eu (III) with triphosphate nucleotides at λ =542.54 nm

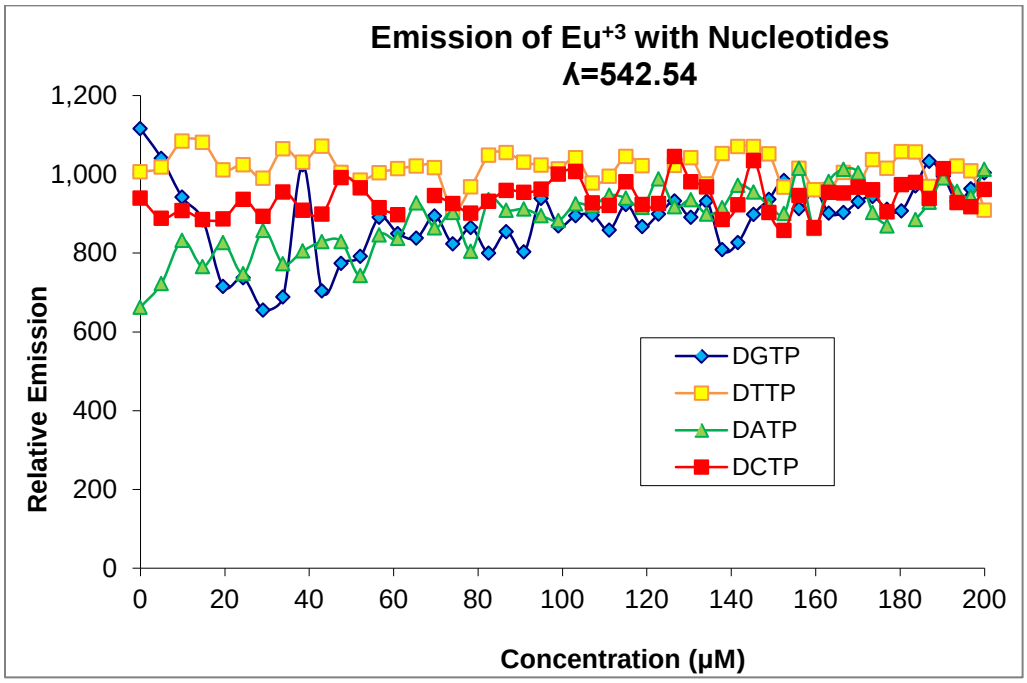


Figure 16. Emission of Tb (III) with triphosphate nucleotides at λ =375 nm

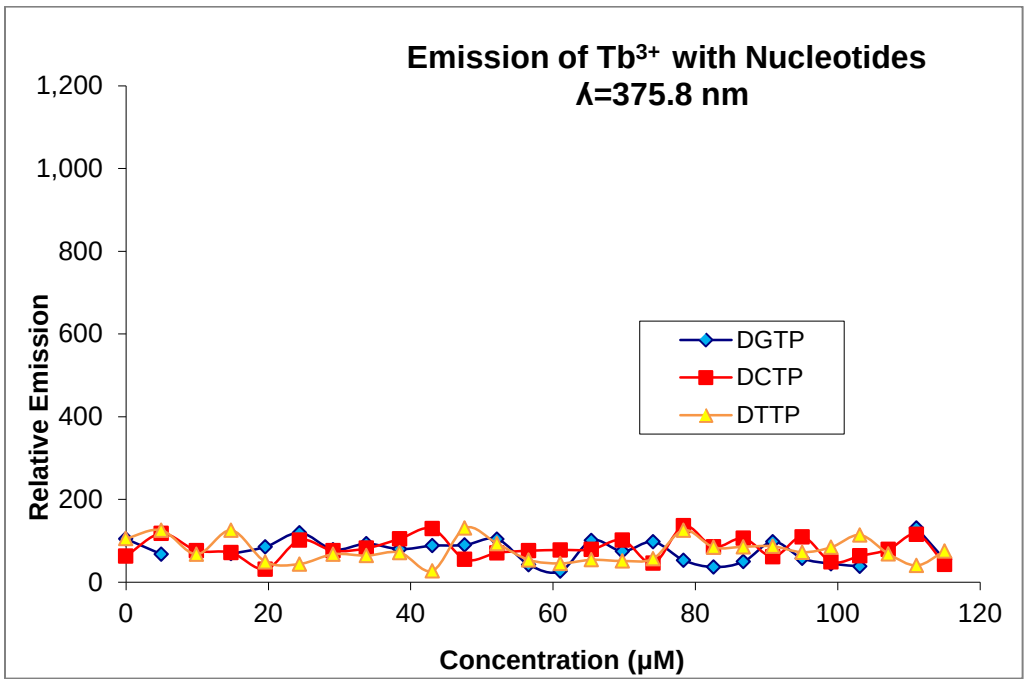
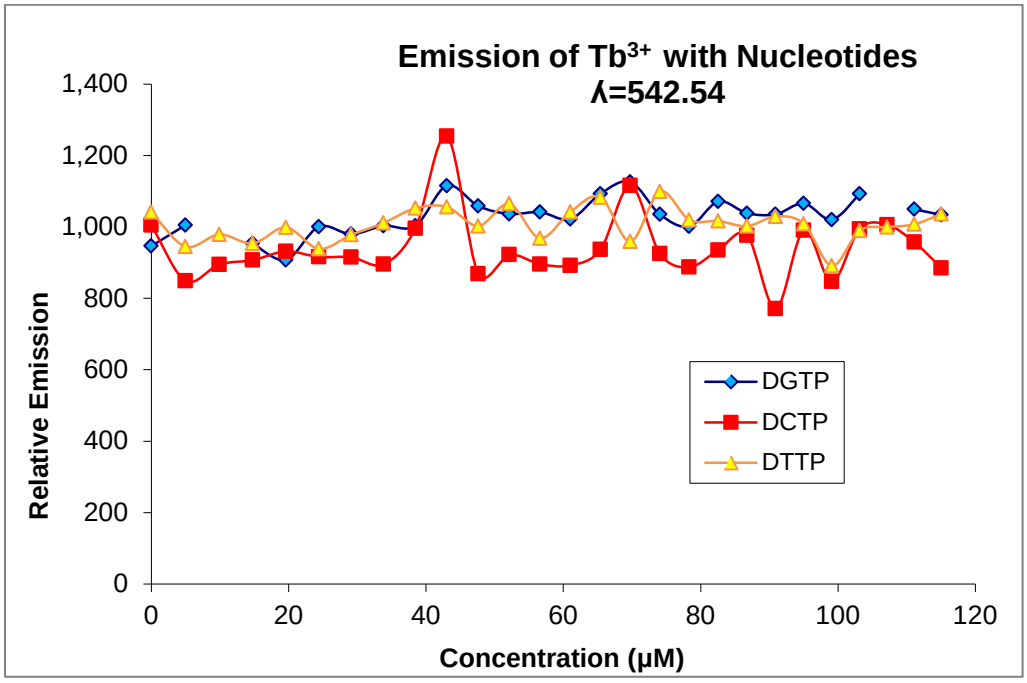


Figure 17. Emission of Tb (III) with triphosphate nucleotides at λ =542.54 nm



As reported previously by various authors, dGMP led to enhancement of the Tb (III) emission, whereas the other nucleotides did not [37] [1]. Comparably, the emission experiments found the base, guanine (G), and its nucleotide, 5'-monophosphate disodium salt (GMP), enhanced the luminescence of both trivalent ions. Among the bases and nucleotides, GMP, was the ligand that enhanced the luminescence of both lanthanide ions to a greater extent. However, GMP bound to Tb (III) appeared to be more emission efficient than bound Eu (III). The difference in emission could be explained based on water interference, deactivation pathway, or/and energy transfer (EnT) efficiency parameters.

First, it is believed that Eu (III) sensitive emission state is weaken by water molecules interference and its deactivation pathway is affected. Upon ligand excitation in the presence of Tb (III) or Eu (III) two mechanisms for the enhancement of the lanthanide emission are possible in water. Energy transfer from the excited ligand to Tb (III) or Eu (III) is expected to provide the largest enhancement, although a small increase in the emission intensity could arise from the replacement of water molecules from the first coordination sphere of the ion by other ligands, resulting in a decrease of the excited state deactivation through the O-H vibrational modes of coordinated water molecules. Whereas the former is dependent on the excitation wavelength (where the ligand absorbs), the latter is not. However the emissive state of Tb (III) is not as sensitive to water vibronic deactivation compare to that of Eu (III) [1]. Since the coordination sphere of the

lanthanide ion is saturated by the ligand, the ability of water to quench the luminescence is small [25].

Second, the difference in emission between, bound Eu (III) and Tb (III) can be justified by EnT parameters. Energy transfer (EnT) takes place from the excited base of a given base (donor) to the emissive state $^5D_0$ of bound Eu (III) or $^5D_4$ of Tb (III). The efficiency of the EnT process depends on the binding of the lanthanide to the base, rate of energy transfer, and quantum yield of formation of the ligand donor excited state. The study of these efficiency parameters is out of the scope of our research but nevertheless helps in the explanation of the lanthanides fluorescence behavior. It is believed that the emission differences between the two lanthanide complexes could be explained by either the quantum yield of formation of the triplet state of the donor or to differences in binding of Eu (III) to each nucleotide.

It has been reported that cytosine (C) and dGMP had the best EnT performance when bounded to Tb (III). That study established excited-state kinetics of the nucleotides is known to be very similar to those of the corresponding bases [1]. So, being cytosine, a great ligand, we expected that dCMP followed the trend. However, CMP bounded to the lanthanide ions was not emissive at all. The observed differences in energy transfer to the lanthanide ion between CMP and GMP could be explained by variations in binding of the trivalent ion in the presence and absence of the anionic phosphate group. It appeared that in CMP

the Tb (III) and Eu (III) bounded to the phosphate group could be too far away from the base for effective energy transfer. The difference between GMP and CMP could be due to the ability of the phosphate to fold over and interact with the lanthanide ions trough O6 and N7 in GMP, whereas an analogous fold over in CMP did not take place. In the cases of G and GMP, the phosphate group appeared to aid in binding of the donor and acceptor. Research had shown that GMP is a better energy transfer donor in the order GMP> GDP > GTP [16] [17]. This fact is supported by the initial experiments where dGTP did not show any enhancement for the trivalent ions. When we replaced the triphosphate by monophosphate, the process of energy transfer happened. The difference in binding between dGTP and GMP can be explained in terms of phosphate interference and molecular geometry for the binding with the trivalent ions.

The emission experiments discovered the best ratio for effective energy transfer (Figure 27). The ratios were: Eu (III) to GMP 1:11 and Tb (III) to GMP 1:10.7. The concentrations at which effective EnT happened were: Eu (III) plus GMP at 444 µM; and Tb (III) plus GMP at 428 µM.

Figure 18. Emission of Eu (III) with monophosphate nucleotides λ =542.54 nm

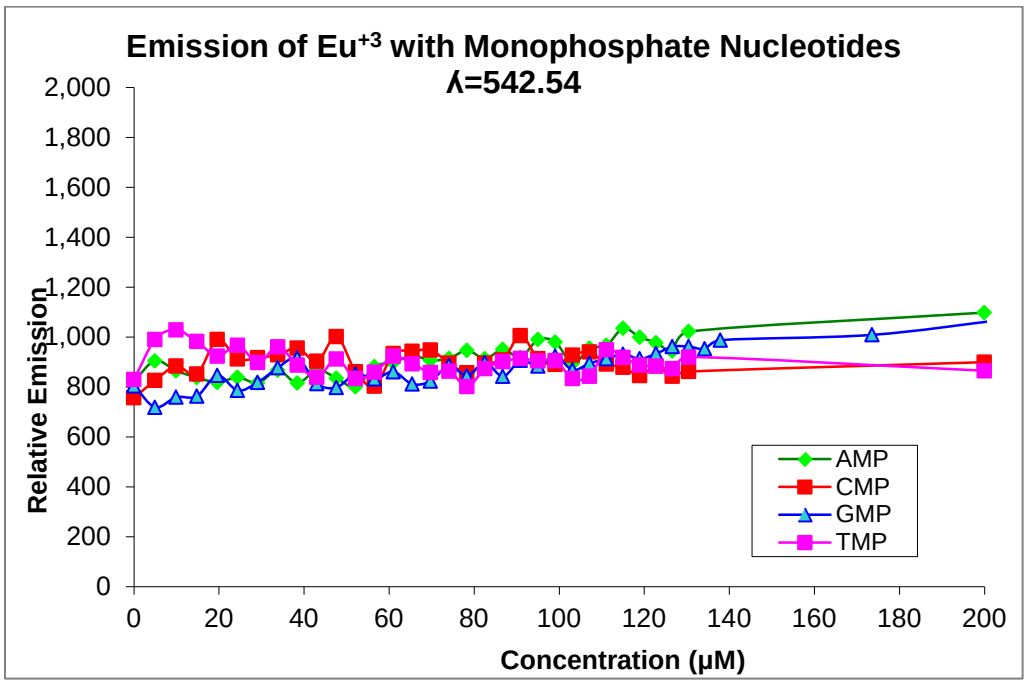


Figure 19. Emission of Eu (III) with monophosphate nucleotides λ =486.82 nm

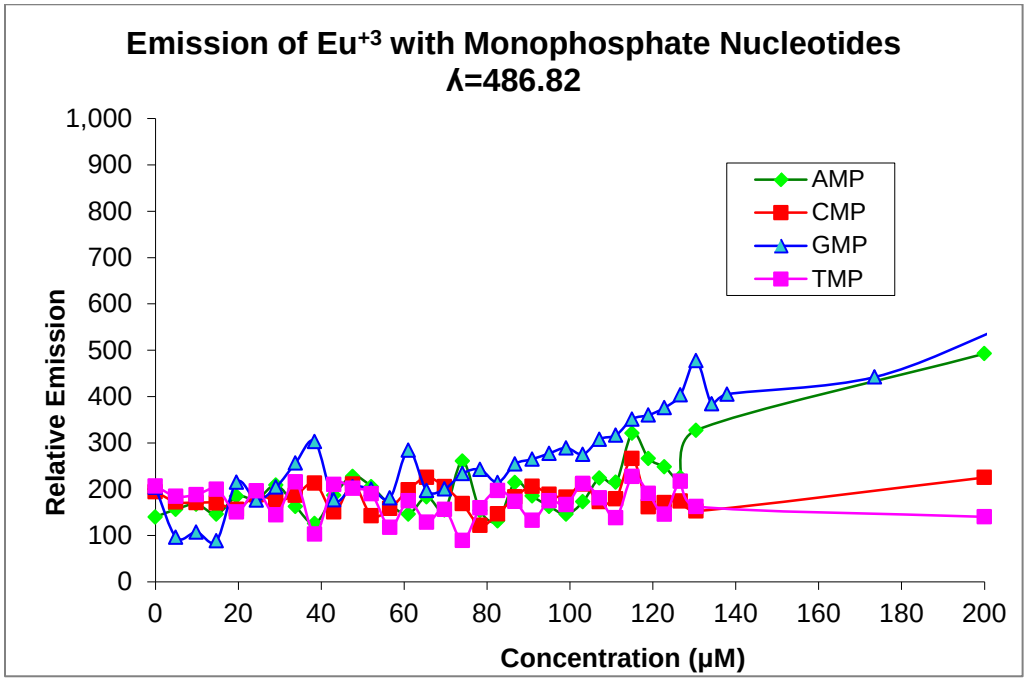


Figure 20. Emission of Tb (III) with monophosphate nucleotides λ =542.54 nm

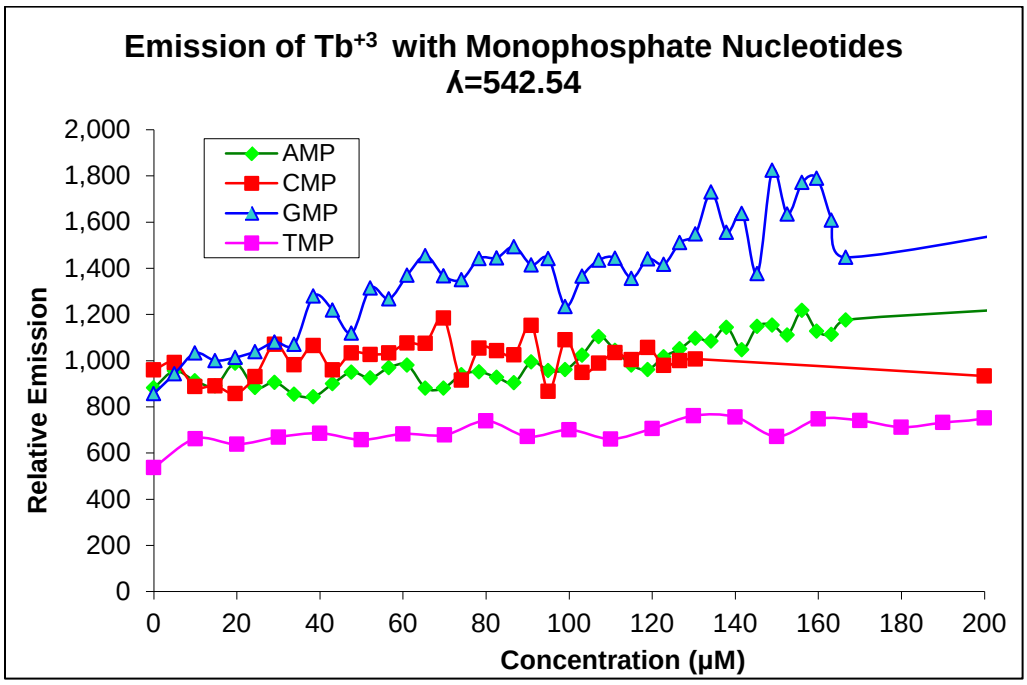


Figure 21. Emission of Tb (III) with monophosphate nucleotides λ =486.82 nm

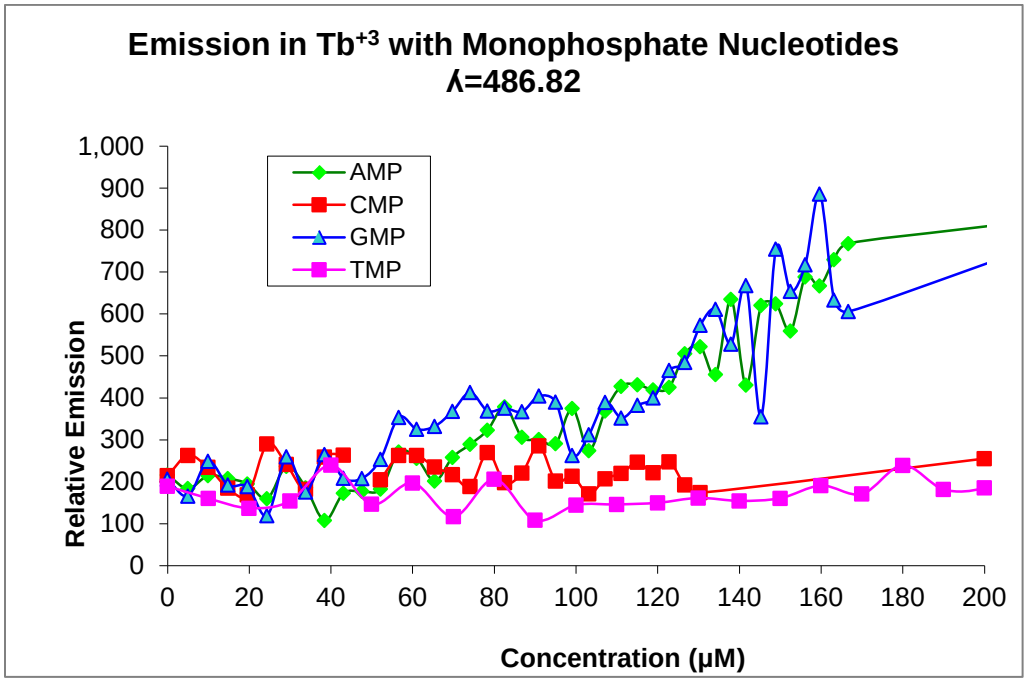
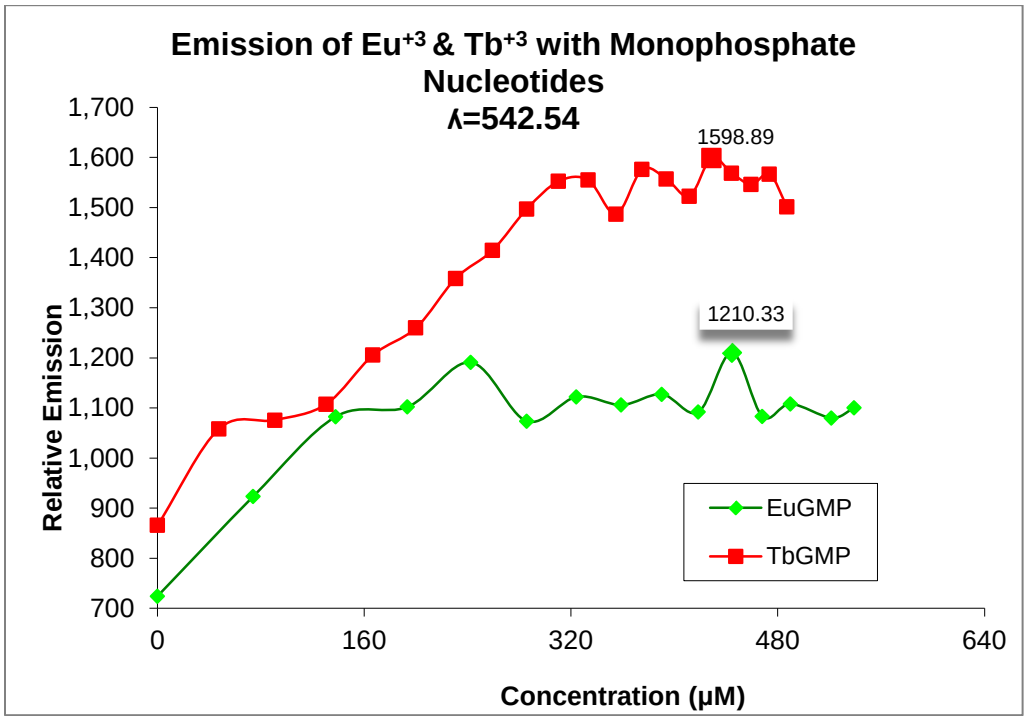


Figure 22. Energy transfer of complex Eu (III) + GMP and Tb (III) +GMP



3.d. Design of Nanoparticles

After finding the best ratio for efficient EnT, the fluorescence biosensor has to be transported into the cells. As a means of delivery system, nanoparticles can encapsulated and protect the biosensor. One way to create the nanoparticles is through the formation of nanoemulsions. Nanoemulsions are a class of emulsions with a droplet size between 20 and 500 nm [38]. Their droplets are stabilized by surfactants. They are not formed spontaneously; their properties depend not only on thermodynamic conditions but on preparation methods and the order of addition of components. Nanoemulsions can be used as micro reactors of controlled size for the preparation of mono-disperse particles [38].

We followed two different design schemes. The first one was based on Governors State University thesis research about Rifampicin, an antibiotic that has been widely used as an anti-tubercular drug. It has been shown that the nanoparticles with PLGA-PEG polymer combination increase the hydrophilicity of Rifampicin (RIF), a hydrophobic drug. Using the highly fluorescence property of Rifampicin as a tool, it was proven that the drug was encapsulated successfully [(26)]. The thesis work, "Photo Physical Properties of Non- Encapsulated and Encapsulated Rifampicin: A comparative study", was the base for becoming familiar with the methods and measurement of fluorescence nanoparticles. A TEM picture of the encapsulated RIF inside the NP is shown in figure 28.

The second scheme was the design of double or inverse emulsions (w/o/w) to create nanoparticles (Figure 29). Several surfactants were tested to encapsulate the hydrophilic biosensor. After extensive literature research and experimentation, two surfactants were chosen for the nanoparticles production. One surfactant was palmitic acid and the other L-α phosphaditylcholine (known as lecithin).

We selected these two surfactants based mainly on their compatibility with biological systems, their stability during storage, and efficacy after administration into the body. First, L-α phosphaditylcholine, is a major structural component of cellular membranes in eukaryotic cells. Secondly, phosphatidylcholine serves as a reservoir for several lipid messengers. Finally phosphatidylcholine is used for

preparation of vesicle suspensions commonly called liposomes or as monolayers [39].

Figure 23. Hydrophobic nanoparticles

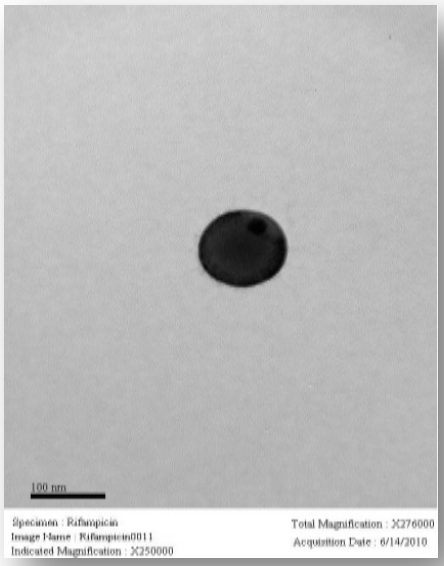
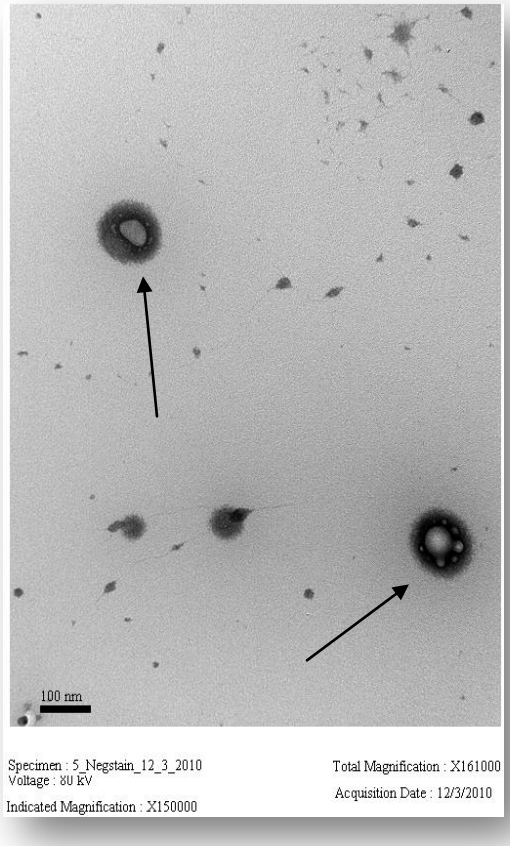


Figure 24. Hydrophilic nanoparticles



Similarly, palmitic acid or hexadecanoic acid is one of the most common saturated fatty acids found in animals and plants and has low toxicity. We sent our nanoparticles with the encapsulated biosensor to University of Illinois at Chicago for proper assessment of particle size due to the lack of TEM equipment (transmission electronic microscope). Our findings can be visualized in Figure 29, which shows the encapsulation of the biosensor into 75 nm particles made of palmitic acid.

We tested the efficiency of the nanoparticles delivery into the cells. The hydrophobic nanoparticles, made of a combination of PLGA-PEG polymer, were added into cells we had previously grown.

The initial checks for emission were hampered due to the difficulty in the cells lysing. After sonication and shaking for several days, two separate samples (Figure 30 and 31) that displayed emission were obtained. Figure 30 exhibited the fact the NP had entered into the cells. Figure 31 showed such a high emission that the NP's themselves had fragmented besides the cells bursting. The highly fluorescence property of Rifampicin as a tool proved that the drug is encapsulated successfully.

Figure 25. Emission of hydrophobic nanoparticles.

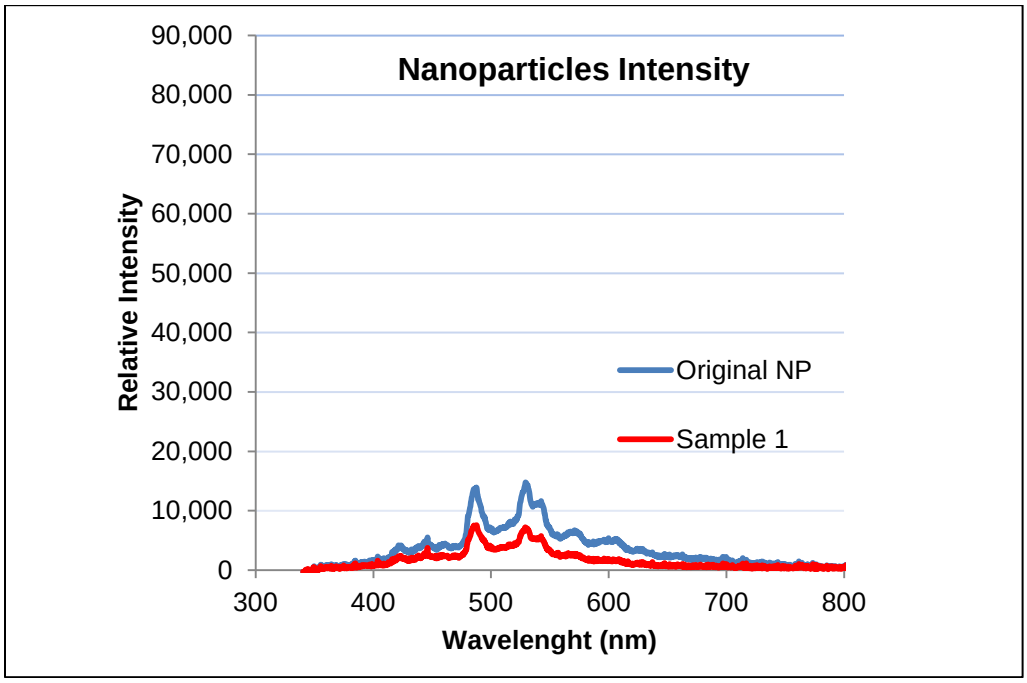
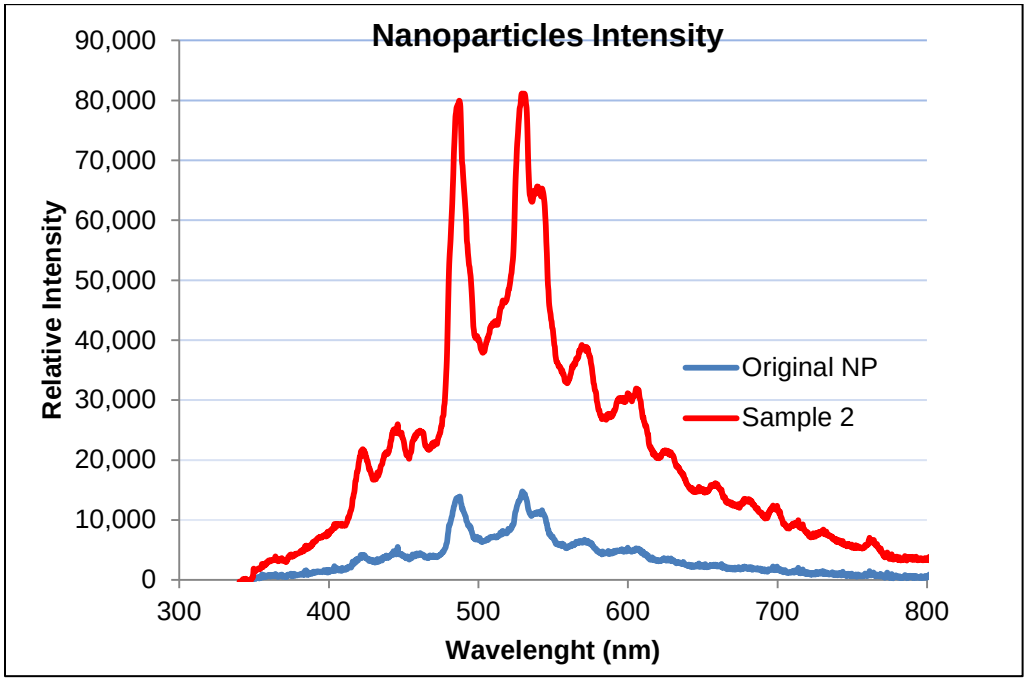


Figure 26. Bursting of hydrophobic nanoparticles.

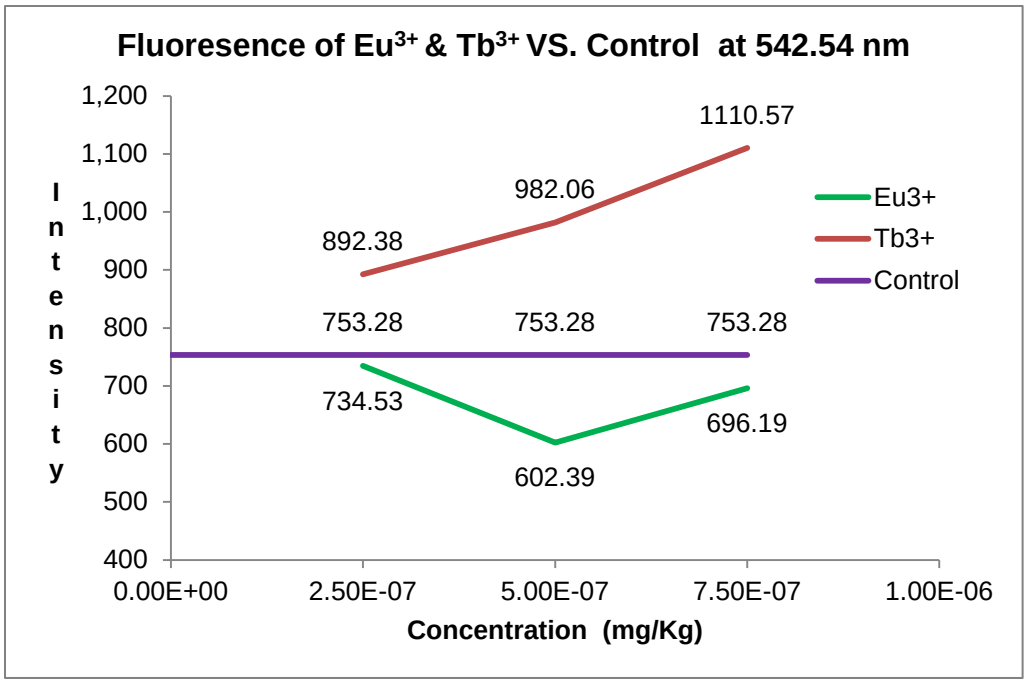


Similarly, the emission of the palmitic acid nanoparticles that encapsulated the hydrophilic biosensor was tested. Unfortunately, the particle size for the L-α phosphaditylcholine nanoparticle was not appropriate for analysis. The emission result for the palmitic acid nanoparticles is shown in Figure 32. It was clear that GMP enhanced the emission of the lanthanide Tb (III) in a higher degree than Eu (III). The addition of a surfactant like palmitic acid to the solution aided the luminescence enhancement of the ligand-lanthanide complex. Adding this surfactant created inverse micelles that helped in the formation of nonpolar regions in an aqueous solution. When the complex was added in vitro, there was a sustained release. The initial burst released the luminescence biosensor effectively, confirming that the nanoparticles burst and the protein was released. In contrast, since GMP led to a lesser degree of enhancement of Eu (III), the complex added in vitro did not show release of the biosensor. This behavior suggested that most of the proteins remained in the nanoparticles.

The performance of Eu (III) complex can be explained by the EnT mechanism and impurities that may affect the biosensor emission. It is believed that GMP is usually remote relative to bounding the lanthanide Eu (III), so the effect in EnT is weaker than in Tb (III) and the emission fluorescence effect is reduced. Also, the formation of impurities such as Eu (II) ion may diminish the emission. The in vitro results were in agreement with the findings of the emission studies. Tb (III) was a better quencher than Eu (III) when bound to dGMP, and its emission in

vitro studies reinforced this fact. Encapsulation of the biosensor offered successful delivery of it inside the cells.

Figure 27. Fluorescence of encapsulated Eu (III) and Tb (III)



Finally to confirm the fluorescence release of the biosensor into the cells, pictures of the emission of the nanoparticles were taken. This was done using the fluorescence capturing capability of the Olympus BX-51 microscope in conjunction with the DP 70 camera , the picture was taken under oil (oil is place on the slide), and the size magnification means 100X multiplied by 10X equaled 1000X (times) magnification. Figure 33 shows the fluorescent L-α phosphaditylcholine nanoparticles, in which the nanoparticles' size, were not reach. Figure 34 shows the emission of 75 nm palmitic acid nanoparticles.

Figure 28. Fluorescence of L-α phosphaditylcholine NPs into the cells

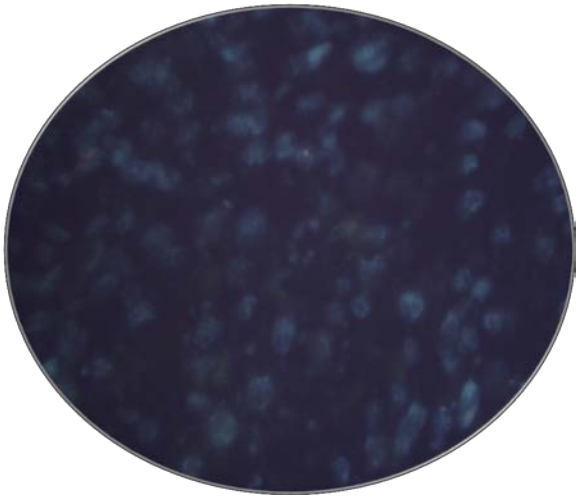
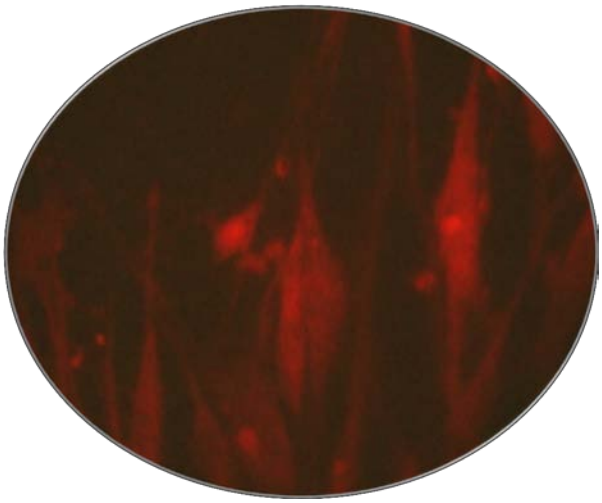


Figure 29. Fluorescence of palmitic acid NPs into the cells



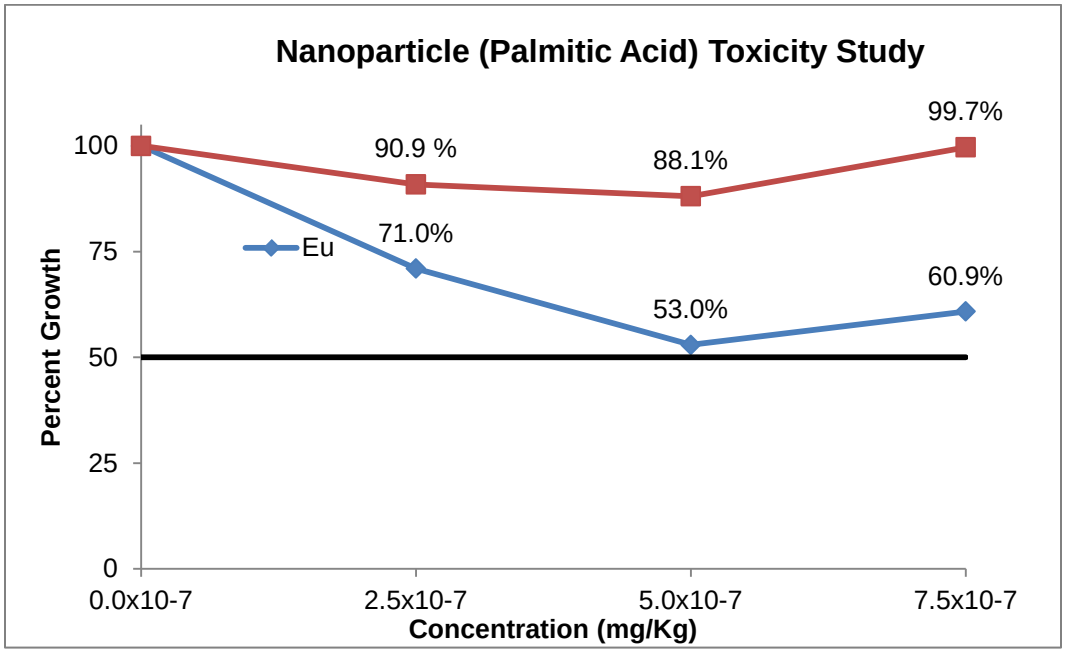
3.e. Toxicity Study

The in vitro toxicity of the nanoparticles, with both luminescence biosensors, was assessed by BCA assay. As shown in Figure 35, the results were satisfactory. The nanoparticles were non toxic and all their values were above the

LD50 (Lethal Dose 50%). An LD50 is a standard measurement of acute toxicity that is shown as percentage of cells growth as concentration dependant in milligrams (mg) of biosensor per kilogram (kg) of nanoemulsion.

The acute toxicity values of LD50 represented the individual dose required to kill 50 percent of a population of cells. Because LD50 values are standard measurements, it might be possible to compare relative toxicities among biosensors. The lower the LD50 dose, the more toxic the biosensor will be. .Using the absorbance results obtained by the micro plate reader, a ratio of each sample concentration versus the three control wells' absorbance would be determined. This ratio was translated to a percentage. The biosensor of Eu (III) 53% value represents that almost half of the cell population still alive, at the same concentration, the biosensor of Tb (III) showed 88% of the cell population survived. Results supported that both luminescence nanoparticles biosensors were non toxic. Nanoparticles of Eu (III) seemed to be more toxic than the ones of Tb (III).

Figure 30. Biosensor Acute Toxicity



4. CONCLUSIONS

The lanthanides trivalent ions, Eu (III) and Tb (III), prove their importance as luminescent biosensors. The luminescence enhancement of Eu (III) and Tb (III) emissions was utilized to probe the interaction between nucleic bases and the trivalent ions. It was found that guanine enhances both of the trivalent ions emissions with Tb (III) being a better acceptor.

The unanticipated results of lacking enhancement for the base cytosine bounded to Tb (III) and Eu (III) is difficult to explain. First, minor fluorescent impurities can make significant contributions and may contribute to wrong results. Second, unpolarized excitation light, such as “natural light” will lead to a photo selection of fluorophores. Finally, as discussed in the poster presentation in Hawaii, problem with luminescence in an aqueous solution is that another pathway is available for deactivation of the excited state of the lanthanide, in the form of vibrational energy transfer to water molecules in particular. This quenching of luminescence was minimized by using ligands which tended to encapsulate the lanthanide ion. Furthermore, supported by literature findings, energy transfer from intrinsic fluorophores, such as nucleic acids, generally give rise to enhanced Tb (III) emission. This is often not the case with Eu (III) if charge transfer occurs [15].

The Luminescence enhancement of Eu (III) and Tb (III) was used to probe the interaction between the nuclei bases and the deoxytriphosphate bases. It was

found the base Guanine enhanced the luminescence of both lanthanides but dGTP did not, indicating that the lanthanide bound to the phosphate group is too far away from the base for effective energy transfer. Eu (III) is generally found to be a more effective quencher than Tb (III).

The advantage offered by fluorescence measurements (over the absorption) are the greater sensitivities and lower concentration limits achievable with the excitation spectra.

The w/o/w double emulsion technique successfully created nanoparticles which encapsulated the water soluble biosensor. The particle size achieved was 75nm, which falls into the nanoemulsions range. Accomplished was the release of the biosensor in vitro and the stability of the emulsion was proved. The nanoparticles could be easily concentrated in the lymphatic system by intramuscular injection with no toxic effects.

5. FUTURE GOALS

A researcher could gain further information about the topography of the biosensor, by variation of conditions, such as pH, ionic strength, viscosity and temperature. Also, a good measurement of efficient EnT would be the determination of fluorescent lifetime. In addition, the designing of an emission system to maximize the energy transfer by single strand DNA, (SSDNA), encapsulation instead of inverse micelles nanoparticles.

In the area of nanoparticles characterization, it would be valuable to analyze particle size of the nanoparticles by dynamic light scattering (DSL) technique, to get a more accurate idea of particle size distribution.

A researcher could assess the interaction of the nanoparticle with lipoproteins in blood that may lead to premature release of the marker. By increasing the permanence of the phospholipids nanoparticles could increase blood circulation times and with the enhanced stability could control in vivo release of the biosensor.

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APPENDIX

Nanoparticles Surfactants MSDS

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

L-α-Phosphatidylcholine

Product Number **P7443**
Storage Temperature -20 °C

Product Description
CAS Number: 8002-43-5
Synonyms: L-α-Lecithin, 3-*sn*-Phosphatidylcholine,

Typical lots of soybean phosphatidylcholine have fatty acid contents of approximately 13% C16:0 (palmitic), 4% C18:0 (stearic), 10% C18:1(oleic), 64% C18:2 (linoleic), and 6% 18:3 (linolenic) with other fatty acids being minor contributors This would calculate to an average molecular weight of approximately 776.

Phosphatidylcholine is the major membrane phospholipid in eukaryotic cells. In addition to being the major structural component of cellular membranes, phosphatidylcholine serves as a reservoir for several lipid messengers. It is the source of the bioactive lipids lysophosphatidylcholine, phosphatidic acid, diacylglycerol, lysophosphatidylcholine, platelet-activating factor, and arachidonic acid.[1] An understanding of the control and regulation of the several metabolic pathways involved in the formation of these bioactive lipids is an ongoing science.

Phosphatidylcholine is used for preparation of vesicle suspensions commonly called liposomes or as monolayers. There have been several books published on liposomes and their applications.[2,3] Monolayers have been formed using a solution of 1% soybean phosphatidylcholine in hexane.[4]

Valinomycin induced changes in membrane potentials of red blood cell and phospholipid (phosphatidylcholine from egg yolk plus cholesterol) vesicle suspensions have been measured using positively-charged, cyanine dyes that fluorimetrically responded to the change in potential.[5]

Purified rhodopsin has been incorporated into soybean phosphatidylcholine vesicles.[6]

A procedure for determination of the amount of oxidation of egg phosphatidylcholine in a liposome preparation by measurement of the oxidation index has been published.[7] The oxidation index is the ratio of the absorbance at 233 nm to the absorbance at 215 nm. The latter wavelength was chosen since there is little contribution of the fatty acid carbonyl to the absorbance at this wavelength, allowing Beer's Law to be followed. A method of determining choline content has been published.[8]

Phosphatidylcholine is purified by a modification of the chromatography method of Singleton by passing an extract of egg yolk through an alumina column.[9]

Precautions and Disclaimer
For Laboratory Use Only. Not for drug, household or other uses.

Preparation Instructions
Purified soybean L-α-phosphatidylcholine is soluble (100 mg/ml) at room temperatue in chloroform, ethanol, and hexane containing 3% ethanol. These solubility characteristics should generally apply to other purified natural product phosphatidylcholine products such as from egg, soybean, and brain that contain unsaturated fatty acids.

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Palmitic Acid

sc-203175

Material Safety Data Sheet

Hazard Alert Code Key: EXTREME | HIGH | MODERATE | LOW

Section 1 - CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

PRODUCT NAME
Palmitic Acid

STATEMENT OF HAZARDOUS NATURE
CONSIDERED A HAZARDOUS SUBSTANCE ACCORDING TO OSHA 29 CFR 1910.1200.

NFPA

SUPPLIER
Santa Cruz Biotechnology, Inc.
2145 Delaware Avenue
Santa Cruz, California 95060
800.457.3801 or 831.457.3800
EMERGENCY:
ChemWatch
Within the US & Canada: 877-715-9305
Outside the US & Canada: +800 2436 2255
(1-800-CHEMCALL) or call +613 9573 3112

SYNONYMS
C16-H32-O2, CH3(CH2)14COOH, "cetylic acid", "hexadecanoic acid", "n-hexadecanoic acid", "n-hexadecoic acid", "hexadecylic acid", "1-pentadecanecarboxylic acid", Emersol, Hydrofol, Hystrene, Industrene

Section 2 - HAZARDS IDENTIFICATION

CHEMWATCH HAZARD RATINGS

		Min	Max
Flammability:	1		
Toxicity:	0		
Body Contact:	2		
Reactivity:	1		
Chronic:	2		

Min/Nil=0
Low=1
Moderate=2
High=3
Extreme=4

CANADIAN WHMIS SYMBOLS

EMERGENCY OVERVIEW

RISK

Irritating to eyes, respiratory system and skin.

POTENTIAL HEALTH EFFECTS

ACUTE HEALTH EFFECTS

SWALLOWED

■ The material has NOT been classified as "harmful by ingestion". This is because of the lack of corroborating animal or human evidence.
<\p>.

EYE

■ This material can cause eye irritation and damage in some persons.

SKIN

■ The material may cause mild but significant inflammation of the skin either following direct contact or after a delay of some time. Repeated exposure can cause contact dermatitis which is characterized by redness, swelling and blistering.
■ Skin contact is not thought to have harmful health effects, however the material may still produce health damage following entry through wounds, lesions or abrasions.
■ Molten material is capable of causing burns.
■ Open cuts, abraded or irritated skin should not be exposed to this material.
■ Entry into the blood-stream, through, for example, cuts, abrasions or lesions, may produce systemic injury with harmful effects. Examine the skin prior to the use of the material and ensure that any external damage is suitably protected.

INHALED

■ The material can cause respiratory irritation in some persons. The body's response to such irritation can cause further lung damage.
■ Persons with impaired respiratory function, airway diseases and conditions such as emphysema or chronic bronchitis, may incur further disability if excessive concentrations of particulate are inhaled.
■ Processing for an overly long time or processing at overly high temperatures may cause generation and release of highly irritating vapors, which irritate eyes, nose, throat, causing red itching eyes, coughing, sore throat.
■ Usually handled as molten liquid which requires worker thermal protection and increases hazard of vapor exposure.CAUTION: Vapors may be irritating.

CHRONIC HEALTH EFFECTS

■ Long-term exposure to respiratory irritants may result in disease of the airways involving difficult breathing and related systemic problems.
Limited evidence suggests that repeated or long-term occupational exposure may produce cumulative health effects involving organs or biochemical systems.
Long term exposure to high dust concentrations may cause changes in lung function i.e. pneumoconiosis; caused by particles less than 0.5 micron penetrating and remaining in the lung.
<\p>.
When fed to weanling mice, palmitic acid (5-40% diet) depressed growth at all but the lowest level. High mortality rates were recorded at 20-40% diet and 100% mortality was recorded at 40% diet.

Section 3 - COMPOSITION / INFORMATION ON INGREDIENTS

NAME	CAS RN	%
palmitic acid	57-10-3	> 99

Section 4 - FIRST AID MEASURES

SWALLOWED

· Immediately give a glass of water. · First aid is not generally required. If in doubt, contact a Poisons Information Center or a doctor.

EYE

■ If this product comes in contact with the eyes: · Wash out immediately with fresh running water. · Ensure complete irrigation of the eye by keeping eyelids apart and away from eye and moving the eyelids by occasionally lifting the upper and lower lids. For THERMAL burns: · Do NOT remove contact lens · Lay victim down, on stretcher if available and pad BOTH eyes, make sure dressing does not press on the injured eye by placing thick pads under dressing, above and below the eye. · Seek urgent medical assistance, or transport to hospital.

SKIN

■ If skin contact occurs: · Immediately remove all contaminated clothing, including footwear · Flush skin and hair with running water (and soap if available). In case of burns: · Immediately apply cold water to burn either by immersion or wrapping with saturated clean cloth. · DO NOT remove or cut away clothing over burnt areas. DO NOT pull away clothing which has adhered to the skin as this can cause further injury. · DO NOT break blister or remove solidified material. · Quickly cover wound with dressing or clean cloth to help prevent infection and to ease pain. · For large burns, sheets, towels or pillow slips are ideal; leave holes for eyes, nose and mouth. · DO NOT apply ointments, oils, butter, etc. to a burn under any circumstances. · Water may be given in small quantities if the person is conscious. · Alcohol is not to be given

under any circumstances. · Reassure. · Treat for shock by keeping the person warm and in a lying position. · Seek medical aid and advise medical personnel in advance of the cause and extent of the injury and the estimated time of arrival of the patient.

INHALED

· If fumes or combustion products are inhaled remove from contaminated area. · Lay patient down. Keep warm and rested.

NOTES TO PHYSICIAN

■ Treat symptomatically.

Section 5 - FIRE FIGHTING MEASURES

Vapour Pressure (mmHG):	Negligible.
Upper Explosive Limit (%):	Not available.
Specific Gravity (water=1):	0.84
Lower Explosive Limit (%):	Not available.

EXTINGUISHING MEDIA

· Do NOT direct a solid stream of water or foam into burning molten material; this may cause spattering and spread the fire.
· Foam.
· Dry chemical powder.

FIRE FIGHTING

· Alert Emergency Responders and tell them location and nature of hazard.
· Wear breathing apparatus plus protective gloves.

GENERAL FIRE HAZARDS/HAZARDOUS COMBUSTIBLE PRODUCTS

· Combustible solid which burns but propagates flame with difficulty.
· Avoid generating dust, particularly clouds of dust in a confined or unventilated space as dusts may form an explosive mixture with air, and any source of ignition, i.e. flame or spark, will cause fire or explosion. Dust clouds generated by the fine grinding of the solid are a particular hazard; accumulations of fine dust may burn rapidly and fiercely if ignited.
Combustion products include: carbon monoxide (CO), carbon dioxide (CO_2), other pyrolysis products typical of burning organic material.
May emit poisonous fumes.
May emit corrosive fumes.
CARE: Contamination of heated / molten liquid with water may cause violent steam explosion, with scattering of hot contents.

FIRE INCOMPATIBILITY

■ Avoid contamination with oxidizing agents i.e. nitrates, oxidizing acids,chlorine bleaches, pool chlorine etc. as ignition may result.

PERSONAL PROTECTION

Glasses:
Chemical goggles.
Gloves:
1.NATURAL RUBBER 2.NEOPRENE
Respirator:
Type A-P Filter of sufficient capacity

Section 6 - ACCIDENTAL RELEASE MEASURES

MINOR SPILLS
· Clean up all spills immediately.
· Avoid breathing dust and contact with skin and eyes.
MAJOR SPILLS
■ Moderate hazard.
· CAUTION: Advise personnel in area.
· Alert Emergency Responders and tell them location and nature of hazard.

Section 7 - HANDLING AND STORAGE

PROCEDURE FOR HANDLING

· The greatest potential for injury caused by molten materials occurs during purging of machinery (moulders, extruders etc.)
· It is essential that workers in the immediate area of the machinery wear eye and skin protection (such as full face, safety glasses, heat resistant gloves, overalls and safety boots) as protection from thermal burns.
· Avoid all personal contact, including inhalation.
· Wear protective clothing when risk of exposure occurs.
Empty containers may contain residual dust which has the potential to accumulate following settling. Such dusts may explode in the presence of an appropriate ignition source.
· Do NOT cut, drill, grind or weld such containers.
· In addition ensure such activity is not performed near full, partially empty or empty containers without appropriate workplace safety authorisation or permit.

RECOMMENDED STORAGE METHODS

■ Glass container.

· Polyethylene or polypropylene container.
· Check all containers are clearly labelled and free from leaks.

STORAGE REQUIREMENTS

· Store in original containers.
· Keep containers securely sealed.

Section 8 - EXPOSURE CONTROLS / PERSONAL PROTECTION

EXPOSURE CONTROLS

Source	Material	TWA ppm	TWA mg/m³	STEL ppm	STEL mg/m³	Peak ppm	Peak mg/m³	TWA F/CC	Notes
Canada - Alberta Occupational Exposure Limits	palmitic acid (Kerosene/Jet fuels, as total hydrocarbon vapour)		200						
Canada - Alberta Occupational Exposure Limits	palmitic acid (Diesel fuel, as total hydrocarbons)		100						
Canada - Saskatchewan Occupational Health and Safety Regulations - Contamination Limits	palmitic acid (Diesel fuel as total hydrocarbons, (vapour))		100		150				Skin
Canada - British Columbia Occupational Exposure Limits	palmitic acid (Diesel fuel, as total hydrocarbons, Inhalable)		100 (V)						Skin

ENDOELTABLE

PERSONAL PROTECTION

RESPIRATOR

Type A-P Filter of sufficient capacity
Consult your EHS staff for recommendations

EYE

· Safety glasses with side shields.
· Chemical goggles.

HANDS/FEET

■ Suitability and durability of glove type is dependent on usage. Important factors in the selection of gloves include: such as:
· frequency and duration of contact,
· chemical resistance of glove material,
· glove thickness and
· dexterity

Select gloves tested to a relevant standard (e.g. Europe EN 374, US F739).
· When prolonged or frequently repeated contact may occur, a glove with a protection class of 5 or higher (breakthrough time greater than 240 minutes according to EN 374) is recommended.
· When only brief contact is expected, a glove with a protection class of 3 or higher (breakthrough time greater than 60 minutes according to EN 374) is recommended.
· Contaminated gloves should be replaced.

Gloves must only be worn on clean hands. After using gloves, hands should be washed and dried thoroughly. Application of a non-perfumed moisturiser is recommended.
· When handling hot materials wear heat resistant, elbow length gloves.
· Rubber gloves are not recommended when handling hot objects, materials.
· Protective gloves eg. Leather gloves or gloves with Leather facing.

Experience indicates that the following polymers are suitable as glove materials for protection against undissolved, dry solids, where abrasive particles are not present.
· polychloroprene
· nitrile rubber
· butyl rubber
· fluorocaoutchouc
· polyvinyl chloride
Gloves should be examined for wear and/ or degradation constantly.

OTHER

· When handling hot or molten liquids, wear trousers or overalls outside of boots, to avoid spills entering boots.
Usually handled as molten liquid which requires worker thermal protection and increases hazard of vapor exposure.CAUTION: Vapors may be irritating.
· Overalls.
· P.V.C. apron.
· Barrier cream.
· Skin cleansing cream.
· Eye wash unit.

ENGINEERING CONTROLS

■ For molten materials:
Provide mechanical ventilation; in general such ventilation should be provided at compounding/ converting areas and at fabricating/ filling work stations where the material is heated. Local exhaust ventilation should be used over and in the vicinity of machinery involved in handling the molten material.
· Local exhaust ventilation is required where solids are handled as powders or crystals; even when particulates are relatively large, a certain proportion will be powdered by mutual friction.
· Exhaust ventilation should be designed to prevent accumulation and recirculation of particulates in the workplace.

Section 9 - PHYSICAL AND CHEMICAL PROPERTIES

PHYSICAL PROPERTIES

Solid.
Does not mix with water.
Floats on water.

State	Divided solid	Molecular Weight	256.48
Melting Range (°F)	145.22	Viscosity	Not Available
Boiling Range (°F)	664.7	Solubility in water (g/L)	Immiscible
Flash Point (°F)	Not Available	pH (1% solution)	Not applicable.
Decomposition Temp (°F)	Not available.	pH (as supplied)	Not applicable
Autoignition Temp (°F)	Not available.	Vapour Pressure (mmHG)	Negligible.
Upper Explosive Limit (%)	Not available.	Specific Gravity (water=1)	0.84
Lower Explosive Limit (%)	Not available.	Relative Vapor Density (air=1)	Not available.
Volatile Component (%vol)	Negligible	Evaporation Rate	Not applicable

APPEARANCE

White crystalline flakes or powder. Insoluble in water. Sparingly soluble in cold alcohol or petroleum ether. Freely soluble in hot alcohol, ether, propyl alcohol and chloroform.

Section 10 - CHEMICAL STABILITY

CONDITIONS CONTRIBUTING TO INSTABILITY

· Presence of incompatible materials.
· Product is considered stable.

STORAGE INCOMPATIBILITY

■ Avoid reaction with oxidizing agents.

For incompatible materials - refer to Section 7 - Handling and Storage.

Section 11 - TOXICOLOGICAL INFORMATION

PALMITIC ACID

TOXICITY AND IRRITATION

PALMITIC ACID:
■ unless otherwise specified data extracted from RTECS - Register of Toxic Effects of Chemical Substances.

TOXICITY IRRITATION

Skin (human): 75 mg/3d-I Mild

■ Asthma-like symptoms may continue for months or even years after exposure to the material ceases. This may be due to a non-allergenic condition known as reactive airways dysfunction syndrome (RADS) which can occur following exposure to high levels of highly irritating compound. Key criteria for the diagnosis of RADS include the absence of preceding respiratory disease, in a non-atopic individual, with abrupt onset of persistent asthma-like symptoms within minutes to hours of a documented exposure to the irritant. A reversible airflow pattern, on spirometry, with the presence of moderate to severe bronchial hyperreactivity on methacholine challenge testing and the lack of minimal lymphocytic inflammation, without eosinophilia, have also been included in the criteria for diagnosis of RADS. RADS (or asthma) following an irritating inhalation is an infrequent disorder with rates related to the concentration of and duration of exposure to the irritating substance. Industrial bronchitis, on the other hand, is a disorder that occurs as result of exposure due to high concentrations of irritating substance (often particulate in nature) and is completely reversible after exposure ceases. The disorder is characterised by dyspnea, cough and mucus production.

The material may cause skin irritation after prolonged or repeated exposure and may produce on contact skin redness, swelling, the production of vesicles, scaling and thickening of the skin.

SKIN

palmitic acid	Canada - Alberta Occupational Exposure Limits - Skin	Substance Interaction	1

Section 12 - ECOLOGICAL INFORMATION

No data

Ecotoxicity

Ingredient	Persistence: Water/Soil	Persistence: Air	Bioaccumulation	Mobility
palmitic acid	LOW		LOW	MED

GESAMP/EHS COMPOSITE LIST - GESAMP Hazard Profiles

Name / EHS TRN A1a A1b A1 A2 B1 B2 C1 C2 C3 D1 D2 D3 E1 E2 E3 Cas No / RTECS No _____ Fatty 226 277 (4) NI (4) R (4) (1) (0) (0) (1) (1) (1) Fp 3 acids, 0 9 linear, C8- C18 saturated with C18 unsaturat ed / CAS:57- 10- 3 /

Legend: EHS=EHS Number (EHS=GESAMP Working Group on the Evaluation of the Hazards of Harmful Substances Carried by Ships) NRT=Net Register Tonnage, A1a=Bioaccumulation log Pow, A1b=Bioaccumulation BCF, A1=Bioaccumulation, A2=Biodegradation, B1=Acuteaquatic toxicity LC/ECIC50 (mg/l), B2=Chronic aquatic toxicity NOEC (mg/l), C1=Acute mammalian oral toxicity LD50 (mg/kg), C2=Acutemammalian dermal toxicity LD50 (mg/kg), C3=Acute mammalian inhalation toxicity LC50 (mg/kg), D1=Skin irritation & corrosion, D2=Eye irritation& corrosion, D3=Long-term health effects, E1=Tainting, E2=Physical effects on wildlife & benthic habitats, E3=Interference with coastal amenities, For column A2: R=Readily biodegradable, NR=Not readily biodegradable. For column D3: C=Carcinogen, M=Mutagenic, R=Reprotoxic, S=Sensitising, A=Aspiration hazard, T=Target organ systemic toxicity, L=Lunginjury, N=Neurotoxic, I=Immunotoxic. For column E1: NT=Not tainting (tested), T=Tainting test positive. For column E2: Fp=Persistent floater, F=Floater, S=Sinking substances. The numerical scales start from 0 (no hazard), while higher numbers reflect increasing hazard. (GESAMP/EHS Composite List of Hazard Profiles - Hazard evaluation of substances transported by ships)

Section 13 - DISPOSAL CONSIDERATIONS

Disposal Instructions

All waste must be handled in accordance with local, state and federal regulations.

¦ Legislation addressing waste disposal requirements may differ by country, state and/ or territory. Each user must refer to laws operating in their area. In some areas, certain wastes must be tracked.

A Hierarchy of Controls seems to be common - the user should investigate:

· Reduction
· Reuse
· Recycling
· Disposal (if all else fails)

This material may be recycled if unused, or if it has not been contaminated so as to make it unsuitable for its intended use. Shelf life considerations should also be applied in making decisions of this type. Note that properties of a material may change in use, and recycling or reuse may not always be appropriate.

DO NOT allow wash water from cleaning equipment to enter drains. Collect all wash water for treatment before disposal.

· Recycle wherever possible.
· Consult manufacturer for recycling options or consult Waste Management Authority for disposal if no suitable treatment or disposal facility can be identified.

Section 14 - TRANSPORTATION INFORMATION

NOT REGULATED FOR TRANSPORT OF DANGEROUS GOODS: DOT, IATA, IMDG

Section 15 - REGULATORY INFORMATION

palmitic acid (CAS: 57-10-3) is found on the following regulatory lists;
"Canada Domestic Substances List (DSL)","Canada Toxicological Index Service - Workplace Hazardous Materials Information System - WHMIS (English)","International Council of Chemical Associations (ICCA) - High Production Volume List","International Fragrance Association (IFRA) Survey: Transparency List","OECD Representative List of High Production Volume (HPV) Chemicals","US Cosmetic Ingredient Review (CIR) Cosmetic ingredients found safe as used","US DOE Temporary Emergency Exposure Limits (TEELs)","US EPA High Production Volume Program Chemical List","US EPA Master Testing List - Index I Chemicals Listed","US Food Additive Database","US Toxic Substances Control Act (TSCA) - Inventory"

Section 16 - OTHER INFORMATION

LIMITED EVIDENCE
■ Cumulative effects may result following exposure*.
* (limited evidence).

ND
Substance CAS Suggested codes palmitic acid 57- 10- 3

Reasonable care has been taken in the preparation of this information, but the author makes no warranty of merchantability or any other warranty, expressed or implied, with respect to this information. The author makes no representations and assumes no liability for any direct, incidental or consequential damages resulting from its use. For additional technical information please call our toxicology department on +800 CHEMCALL.

■ Classification of the preparation and its individual components has drawn on official and authoritative sources as well as independent review by the Chemwatch Classification committee using available literature references.
A list of reference resources used to assist the committee may be found at:
www.chemwatch.net/references.

■ The (M)SDS is a Hazard Communication tool and should be used to assist in the Risk Assessment. Many factors determine whether the reported Hazards are Risks in the workplace or other settings. Risks may be determined by reference to Exposures Scenarios. Scale of use, frequency of use and current or available engineering controls must be considered.

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