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Synthesis and Analytical Evaluation of Folate Conjugates for Use in Cancer Cell Detection

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Governors State University

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**Synthesis and Analytical Evaluation of Folate Conjugates
for use in Cancer Cell Detection**

A Project

Submitted

to

Governors State University

By

Rakeshkumar V Desai

In Partial Fulfillment of the

Requirements for the Degree

of

Masters in Science

May, 2011

Governors State University

University Park, Illinois

**Dedicated to
My Family**

ACKNOWLEDGEMENTS

My Sincere and deepest gratefulness to Dr. Walter Henne who has been a great person and a great mentor. Without his lab, instruments, and kind support this project would not have been possible. My deepest thanks to him for this project which will be helpful in my future.

I appreciate my committee members Dr. Patty Fu and Professor Kent, for their support, guidance, and provision of some instruments during project. I wish to thank my graduate teachers, Dr. Kumar, Dr. Addison, Dr. Fu, Dr. D'Arcy, Prof. Kent and Prof. Nancy for their invaluable support and advice. I am grateful to the program secretary, Nancy for assisting me in many different ways.

Thank you so much Governors State University for giving me opportunity to fulfill my dream and laboratory facilities for conducting our research project.

Special thanks to my project partner Sneha Reddy Kuthuru for active participation throughout the entire project. Thanks to my special friends who were with me during my project. I learned lot from the discussion with my friends about solving practical difficulties.

I would like to thank my family for their love, motivation support, and encouragement to go overseas to complete my education.

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Abstract

Folate receptors have two glycosyl phosphatidylinositol anchored isomers, alpha and beta. Folate receptor alpha binds with high affinity for folic acid and act as a receptor for mediated transport of folate into the cells. Folate is necessary for DNA metabolism and thus it is speculated that rapidly dividing cancer cells have an increased necessity for folic acid. Folate receptor alpha levels eminent in specific malignant diseases (like solid tumors and leukemia) and thus folate receptors serve in the detection of FR+ (Folate receptor positive) and diagnosis of cancers¹⁻³.

In general, liposomes have the capacity intake the most imaging and toxic agents due to their large diameter size (100nm). In the liposomal system, the most important thing for therapeutic activity is the conjugation between the liposome and folic acid due to the:

1. Need to present folate to the cancer cell surface unfettered from the bulky liposome (in order to bind to the folate surface).
2. Need to have sufficient folate ligands for proficient binding to cell but not much more folate molecules that could result in non-specific binding.

In the liposomal system, folate is attached to poly ethylene glycol (PEG), which is incorporated into the lipid membrane by way of a hydrophobic tail. Although widely established, liposomes require a fair degree of technical ability to synthesize and analyze. Recently, it has been revealed that apoferritin (iron transport protein), 450 kD polymeric protein (at 70 nm in diameter), is capable of being dissociated into its respective subunits at low pH 2 and re-associated at pH around 8.5 to restructure the apoferritin cage for therapeutic purposes. Based on all these results, our project aim is to synthesize a folate based apoferritin probe⁴⁻⁵. The type of folate conjugation to the apoferritin, the degree of folate labeling to the protein, the quantity of

dye incorporation into the protein cage and type of dye, drugs and other agents will be assessed and ultimately be tested for cell uptake. These types of cages are useful for the production of radio-imaging agents, MRI contrast agents and some drug delivery systems. The main goal of this project is to make an economical and easily produced folate probe that will be substituted for more costly and cumbersome liposome delivery vehicles⁶.

Introduction

New Clinical research presents the importance of folate receptor in the targeted drug delivery system. Targeted deliveries via selective cellular marker can increase the usefulness and diminish the toxicity of therapeutic drugs. Folate receptors are richly present in cancer cells, helping to deliver the chemotherapeutic agents at specific sites of action and also preventing to rich in to the healthy cell. The most important thing for therapeutic activity of the drug is the folate receptor drug delivery system link between the drug and folic acid. Strong bonds like amide show no or little activity whereas weak bonds like disulfide bonds show strong activity⁷⁻⁸.

Folic Acid:

Folic acid (vitamin B9) is a water soluble vitamin (see figure 1). The human body requires folic acid to generate healthy red blood cells and prevent anemia. Folic acid is important for cell division and growth. Folic acid itself is inactive in the body but the reduced form of folic acid is tetrahydrofolate that essentially important in the metabolic reactions of the body. It helps in fetal development in pregnant women as it develops cells of the fetus. It is also important as a coenzyme in the synthesis of DNA and RNA⁹⁻¹⁰.

Folate is required in the metabolism of amino acids like histidine, serine, glycine and methionine. If folate is not present in methionine metabolism, homocysteine builds up. Excess

amount of homocysteine increases the risk of various diseases, particularly cardiovascular disease. Homocysteine metabolism also needs vitamin B12 and vitamin B6. Folic acid with vitamin B12 causes the conversion of homocysteine to methionine, reducing the blood levels of homocysteine and decreasing the risk of heart diseases¹¹. Deficiency of folic acid may create many health problems such as impaired DNA synthesis, cancer development, and cardiovascular disease¹². Normal diets including leafy foods, liver products, and cereals contain high amounts of natural folate and may decrease the risk of cancer¹³.

Apoferritin cages:

Apoferritin is nothing but ferritin not combined with iron. Apoferritin is composed of the same protein shell as ferritin, but its core is empty. Apoferritin has protein spheres found mainly in the liver and spleen, research interests has increased due to interesting structure features and biological properties. Apoferritin is a native protein containing 24 subunits of polypeptide that interact to gather to form a hollow cage like structure with a 12.5 nm diameter. The interior cavity of apoferritin has approximately an 8 nm diameter and an interior volume capacity up to 4500 iron atoms as an iron oxide hydroxide mineral. Apoferritin channels formed by subunit interactions with diameter of 3-4 Å, connect with each other at the outside of the apoferritin molecule^{6, 14}.

The apoferritin cage can be dissociated into 24 subunits at pH 2 and subunits reconstitute at high pH 8.5. Apoferritin, having unique cavity structure as well as its dissociation and reconstitution characteristics, has been widely used as a protein cage to synthesize size restricted bioinorganic nanocomposite, e.g., cobalt, manganese, iron sulfide, iron phosphate, cadmium sulfide, uranium. The application of the synthesized bioinorganic nanocomposite include

magnetic resonance imaging, radio pharmaceutical, quantum dots, nanobatteries, uranium neutron captured therapy, photo catalysts and magnetic memory devices¹⁵.

Appearance of folate receptor:

The α , β and γ are the three main isoforms of folate receptor. The most widely expressed isoforms of folate receptor are α and β . The folate receptor α is expressed at high levels in several tissues that include; proximal tubules in the kidney, pneumocytes, intestinal membrane and placenta¹⁶⁻¹⁷. The folate receptor β is expressed in low to reasonable amounts in normal tissues.

Folate receptors in the cells

Folate receptor α is a glycoposphatidylinositol (GPI) membrane bound protein having high affinity for folic acid and transport receptor mediated folate in the cells (see figure-2). Folate is a basic component for DNA synthesis and its repair. It is hypothesized that rapidly dividing cancer cells have an increased requirement for folate to maintain DNA synthesis¹⁸. Folate receptors α level are high in specific malignant tumors of epithelial origin compared to normal cells. Folate receptors are over expressed on some cancer cell types but present in low abundance in non cancerous tissue. Based on this over expression of the folate receptor, folate based drug delivery and diagnostic systems are in several stages of development¹⁹⁻²⁰.

Folate targeted Apoferritin cages:

Folate targeted apoferritin cages can be made in two different ways;

1. First, apoferritin cages are loaded with folate cysteine and then the pH is lowered to 2 by adding 0.1M HCl. Fluorescein sample is loaded into it and pH rises to 8.5 by adding 0.1M NaOH, The apoferritin cages would trap the fluorescein during reconstitution (see figure-13).

2. Another way is the apoferritin cage can be dissociated by lowering pH to 2 by adding 0.1M HCl and loading it with fluorescein sample and then increasing the pH by adding 0.1M NaOH and loading it with folate cysteine, as the last step.

The apoferritin surface has exposed lysine residue. The lysine residue can be coupled with folate by combination of a hetero bifunctional crosslinker like SPDP with subsequent addition of folate cysteine. We established specific strategy to join folic acid to the apoferritin molecule.

Materials, Methods and Instruments

Materials and reagents:

Apoferritin: Apoferritin from Equine spleen, 2.2 ml – 48mg protein/ml, Lot # A3641

Apoferritin from Horse spleen, 25 mg/ml in 50% glycerol & 0.075M NaCl.

Phosphate Buffer Saline (PBS): Mediatech Inc, Manassas, VA 20109, Lot # 21040174

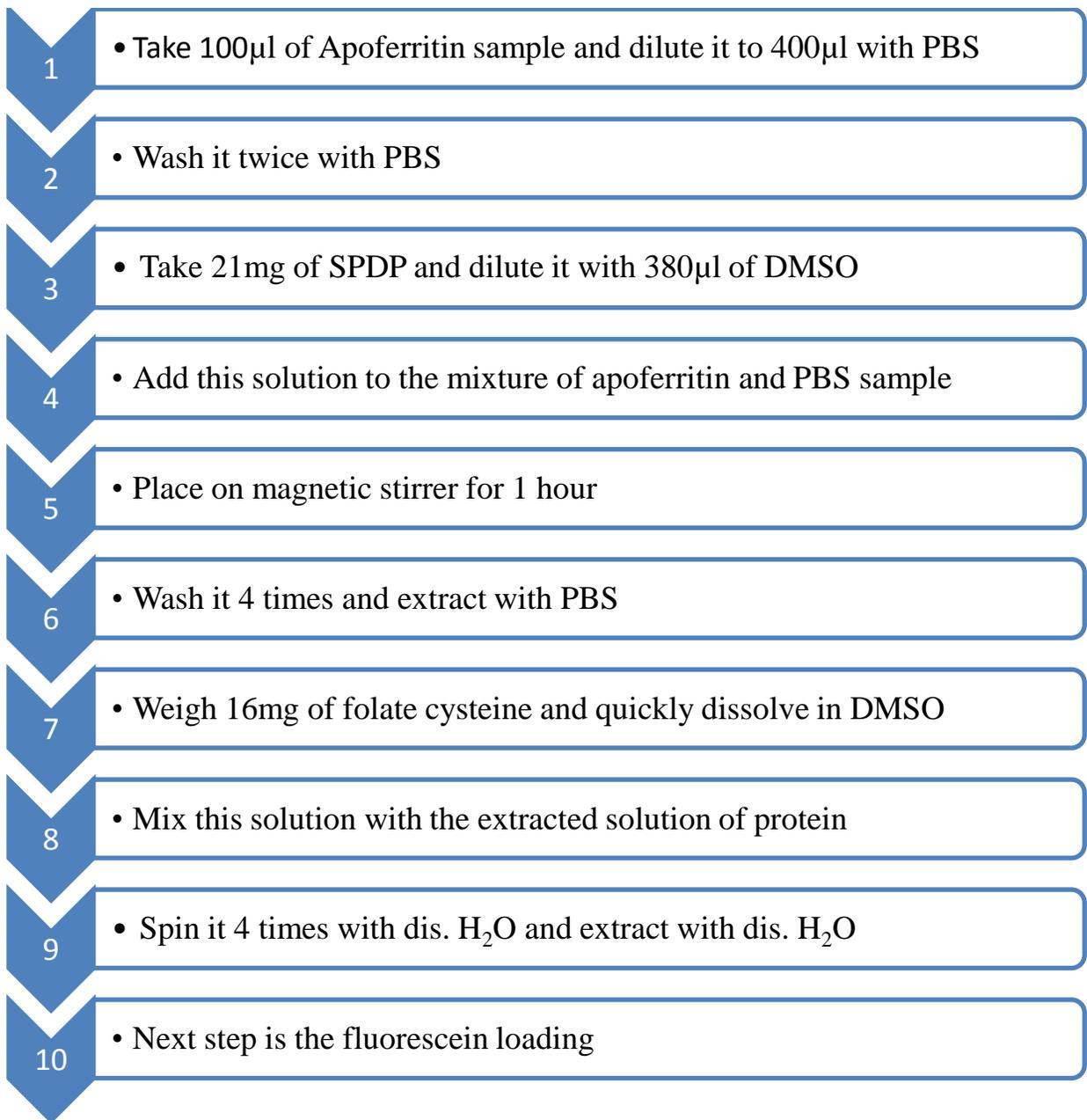
Cat no. 21-040-CV; BCA 1-1 kit: Bicinchoninic Acid kit for protein determination, Sigma Aldrich, Lot # 059K9801, Bicinchonic Acid Solution: Batch # 118K5300, Copper (II) Sulfate Solution: Batch # C2282 (25ml); SPDP (N- Succinimidyl – 3-(2-Pyridyldithio) Propionate, Pierce, Lot # HB101395, Prod # 21857; DMSO: Sigma Aldrich, 99% purity, Batch # 11696 DK; Fluorescence Sodium Salt ($C_{20}H_{10}Na_2O_5$): Sigma-Aldrich, Lot # 079K0141V; Folate Cysteine: Provided by Dr. Henne; Water: B & J brand, Lot # CZ 360; Multichannel Pipette : RAININ, 20-300 μ l; UV Plate: BD Falcon micro test 96 well 370 μ l clear plate, UV – VIS transparent film bottom, Non-sterile, Lot # E10002007; Regular plate: Generic Bio-One – micro plate, 96 each well with flat bottom, Lot # E091006L (see figure-11); Spin Filters: Sigma Aldrich, Amicon ultra 0.5 centrifugal filter, Batch # 3110; PD-10 Column: Sephadex G-25M column, contains

0.15% Kathon CG in distilled water, Lot # 393861; Centrifuge: Beckman CS-15R and new one Thermo scientific Sorvall Legend Micro 17 Ventilated micro centrifuge with 24 place rotor, 120VAC (see figure-7); UV plate reader and BCA assay Software: Gen 5.1.10 Biotech, Epoch; Ocean optics USB 2000+ (for fluorescence study); Ocean optics PX-2, pulsed xenon light source; 1 cm cuvette holder .

Methodology

SPDP labeling with folate cysteine:

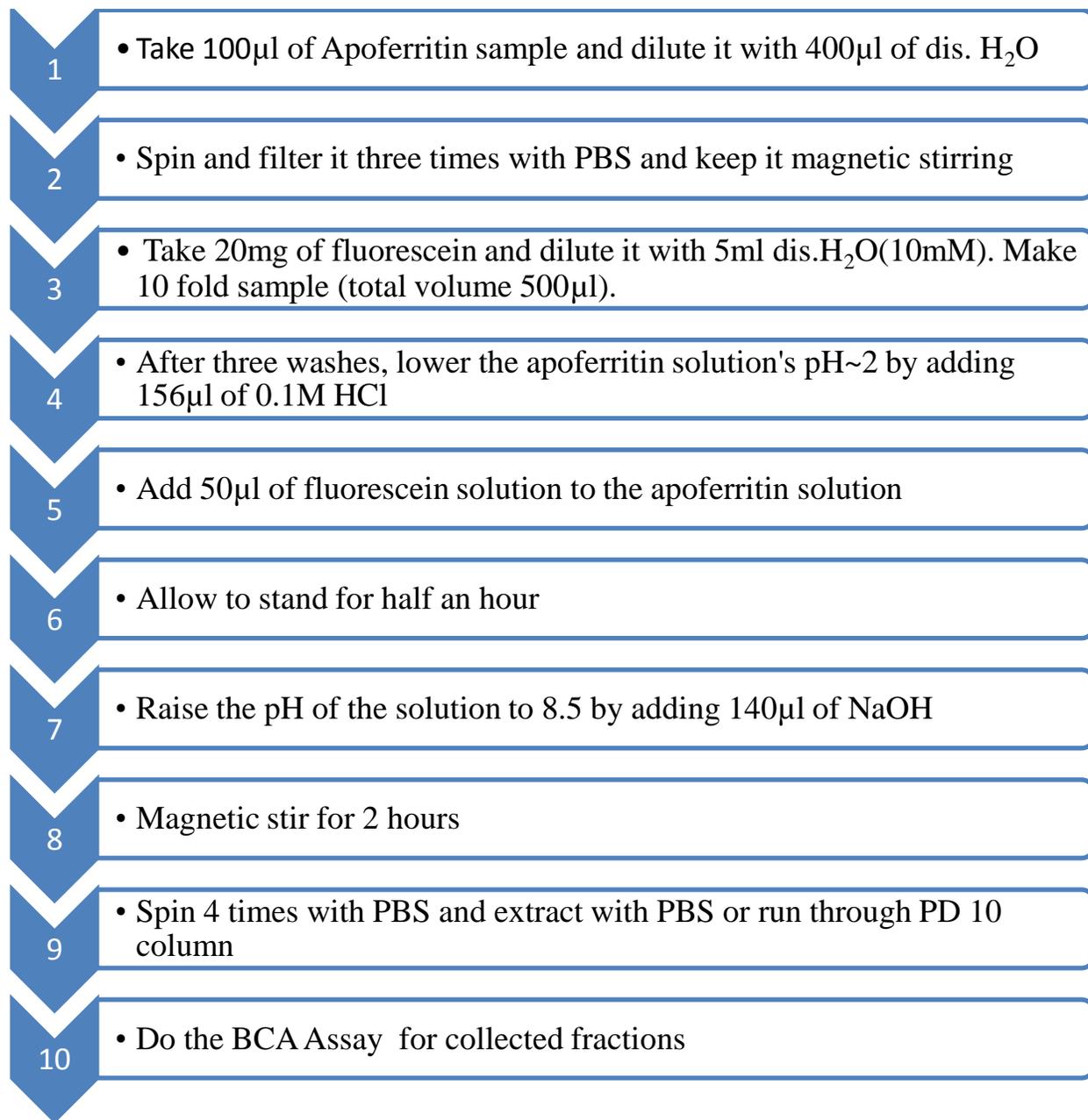
SPDP (N-Succinimidyl-3-(2-Pyridyldithio)-propionate) reagent is an amine and sulfhydryl reactive heterobifunctional crosslinker. It forms amine-amine or amine-sulfhydryl crosslinker with molecules. Folic acid links with apoferritin cage by heterobifunctional crosslinker SPDP (see figure-12). Weight 0.0021gm of SPDP dissolved in 380 μ l of DMSO. Take only 50 μ l of this solution (SPDP+DMSO) and add to apoferritin sample (100 μ l of Apoferritin + 400 μ l of PBS). It is allowed to react for 1 hour. After completion of this reaction, the protein cage purifies from excess SPDP using spin filters. Spin it for 4 to 5 times with PBS and extracted with PBS (Sample-1). Weigh 1.6mg of folate and quickly dissolve in DMSO. Mix this sample with sample-1. Again spin it for 4-5 times and extract with H₂O. Determination of folic acid on the protein was measured in UV-Vis plate at 363nm.



Fluorescein Loading:

Fluorescein loading can be done either with folate or without folate for different purposes. First dilute 100µl of apoferritin sample into 400µl of PBS. Wash 3 times with PBS to get rid of glycerol. Lower the pH of the solution by 2 by adding 0.1M HCl. Load the fluorescein and stand

the solution for approximately 15 minutes. Again raise the pH to 8.5 by adding 0.1M NaOH. The next step is BCA assay.

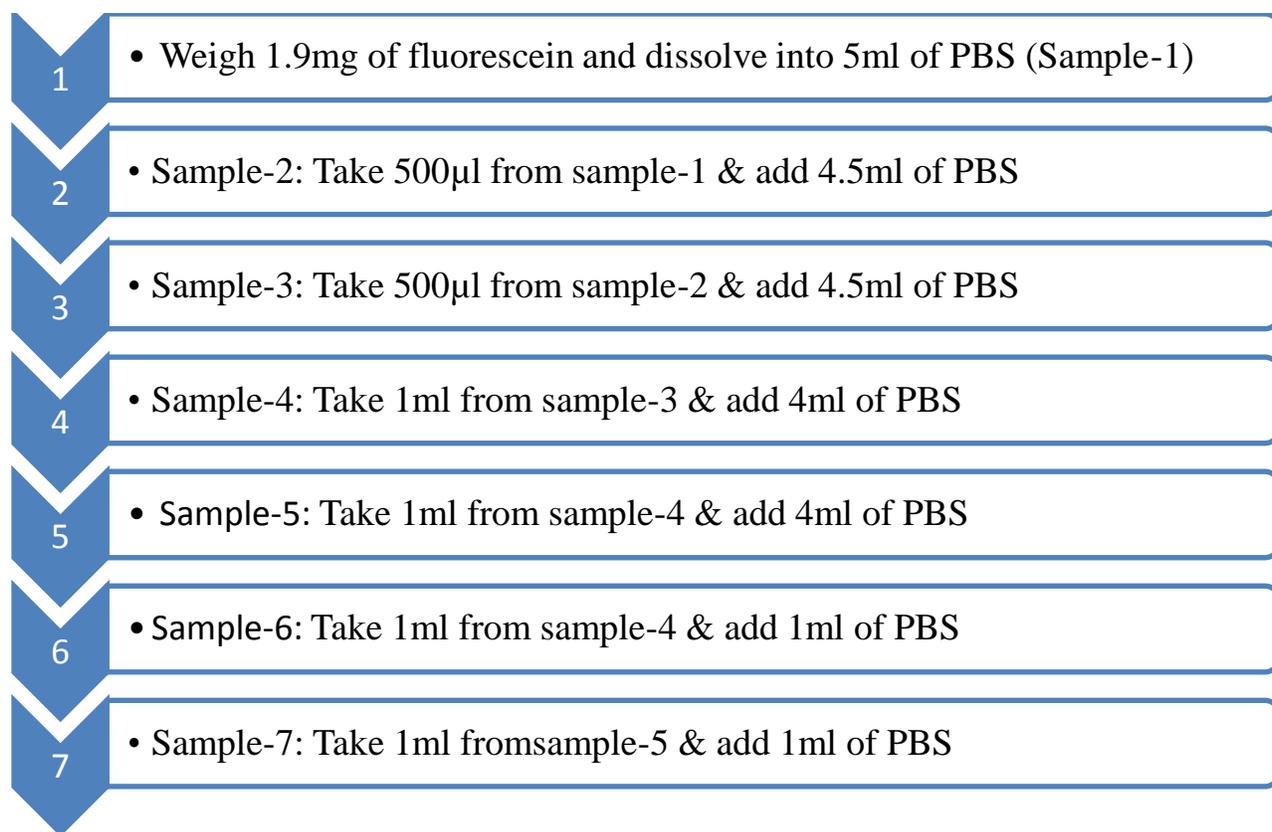


Fluorescence study:

Determination of loading amount of marker (fluorescence sodium salts) in apoferritin can be done by simple fluorescence study. The number of entrapped fluorescence was estimated by

calibrating the ratio of concentration of fluorescence and apoferritin. Bicinchoninic acid method can be used for the apoferritin concentration measurement. The fluorescein content in apoferritin can be determined by comparing fluorescence intensities of the fluorescein Marker-Loaded apoferritin nanoparticles with standard fluorescein solution. By measuring fluorescence intensities of fluorescein solution at different concentrations, a calibration curve for the standard fluorescein was developed⁶.

Preparation of different dilutions of fluorescein sodium salt:



BCA Assay:

The apoferritin concentration can be measured by using the bicinchoninic acid method. BCA protein assay is a detergent compatible formulation based on BCA for colorimetric

detection and quantitation of protein. Bovine serum albumin (BSA) stock solution was taken from ampoule and different dilutions prepared as per Thermo Scientific Pierce BCA protein assay instructions. Prepare different dilutions 2000µg/ml, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 25µg/ml and 0µg/ml for BCA assay. By using 96-microwell plate, take 10µl of each standard solution as well as unknown by duplicates in each well. Then added 200µl of the working prepared solution (10ml of BCA solution and 200µl of Cu (II) sulphate solution and mixed it in a tube). Incubate the 96-microwell plate for half an hour and find out the concentration by using the BCA Assay Gen 5.0 software (see figure-11).

Results and Discussion

Folate Labeling:

The method used here for folate labeling with SPDP crosslinker was straightforward and quick. Labeling of protein with folate was carried out with specific concentration of folate by using SPDP crosslinker. With the molar extinction coefficient of folic acid at pH 7.5, we determined the degree of folate labeling was approximately 100. There were 4 exposed lysine units found on the apoferritin cage surface which means approximately 96 (4*24) residues were conjugatable. Our results explain that we could label approximately 96 sites and almost got full folate labeling (see figure-13).

BCA Assay:

Bicinchoninic acid method was used for the measurement of apoferritin concentration. This method employs the reduction of Cu^{+2} to Cu^{+1} by protein in alkaline medium with the highly selective and sensitive colorimetric detection of the Cu^{+1} by using bicinchoninic acid reagent. The chelation of two molecules of BCA with Cu^{+1} creates a purple colored reaction that

absorbs at 562nm. The amount of product formed is nearly linear with increasing protein concentrations over a broad working range (0 - 1500 μ g/ml).

Upon final purification using the spin filter method, the compound was analyzed for protein concentration using BCA assay. Based on the BCA assay reading, we determined the apoferritin concentration to be 2191mg/ml (see figure-11 and figure-14).

Fluorescein Loading (Fluorescence Study):

The ratio of the concentrations of markers (fluorescein sodium salt and Methylene blue) and apoferritin cage can help to estimate the number of entrapped markers. Attempts to increase the loading of fluorescein sodium salt have been undertaken.

By using the concentration of protein from BCA assay and concentration of fluorescein from fluorescence study, we determined fluorescein loading of 1 or 2

A calibration curve for the standard fluorescein was developed by measuring fluorescence intensities at the different concentration of fluorescein. We got liner graph ($R^2 = 0.9992$) with fluorescence study (see figure-18 and figure-19).

Conclusions:

Folate labeling and fluorescein loading with apoferritin is prepared using a very straightforward and secure methodology using very simple instrumentations. First we conjugated folate to the apoferritin cage and then determined the degree of labeling to the protein. Purified protein cages were loaded with folate using spin filters. We attempted to load the protein with fluorescein dye and determined the degree of dye incorporation. We tried to trap the fluorescein with apoferritin cage by using different concentrations. We succeeded in fluorescence spectroscopy by using different concentration of fluorescein.

Future Studies

Increase fluorescein loading and test the apoferritin protein cages with folate receptor positive cancer cells.

Load the apoferritin cage with other agents such as methylene blue for photodynamic therapy and/or chemotherapeutic agents such as cis-platin.

Funding

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List of Figures:

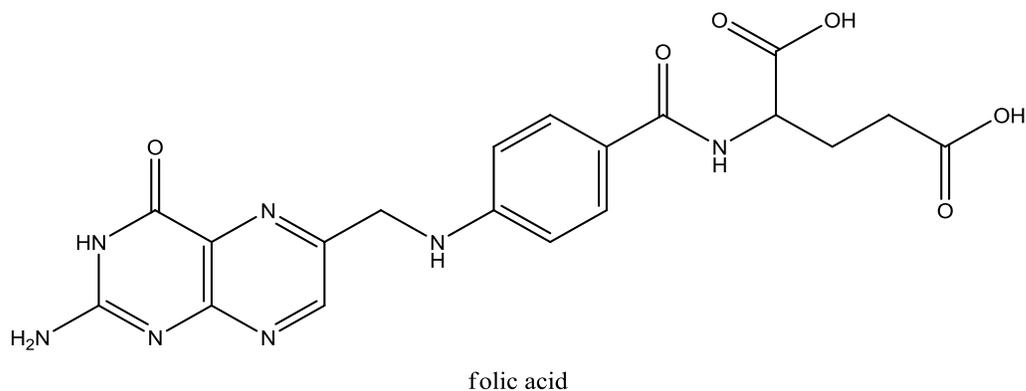


Figure 1: Structure of folic acid

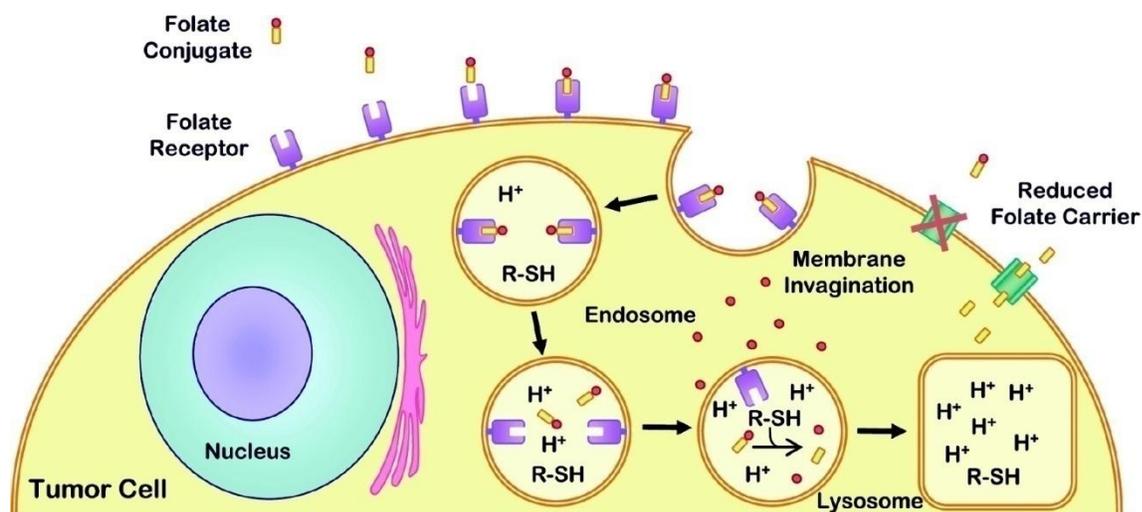


Figure 2: Folate receptor pathway and FR-mediated endocytosis of a folic acid drug conjugates.

Folate conjugates bind folate receptor with high affinity and then internalized into endosomes that can reduce disulfide bonds. (Figure taken from Accounts of chemical research 120-129 January 2008 Vol. 41, No.1)

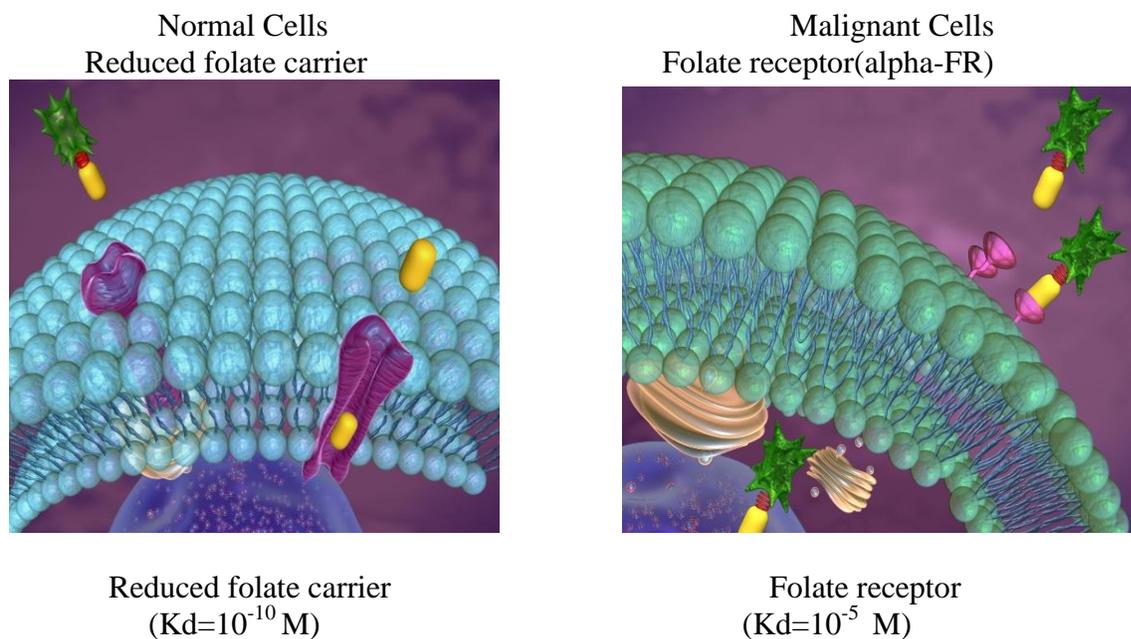


Figure 3: Comparison of alpha-FR on normal cells and malignant cells. (Endocyte Inc.)

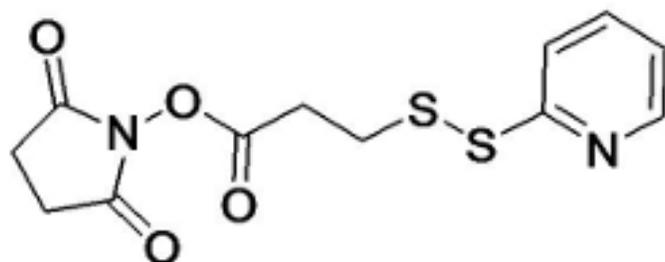


Figure 4: SPDP Structure

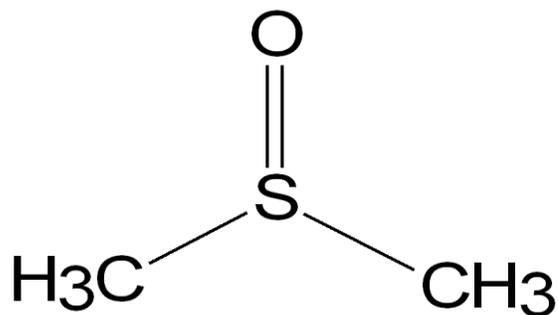


Figure 5: DMSO (Dimethyl Sulfoxide)

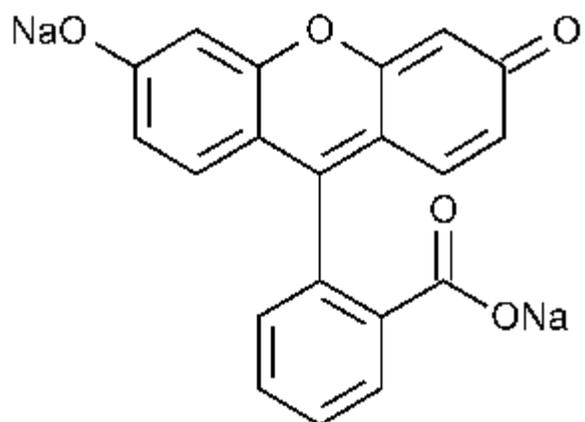


Figure 6: Fluorescein Sodium Salt ($C_{20}H_{10}Na_2O_5$)



Figure 7: Thermo scientific Sorvall Legend Micro 17 Ventilated micro centrifuge with 24 place rotor, 120VAC (8898 RPM for 12 minutes)



Figure 8: pH meters



Figure 9: Magnetic Stirrer



Figure 10: Folate labeling and fluorescein loading to apoferritin cage after final spin filter.

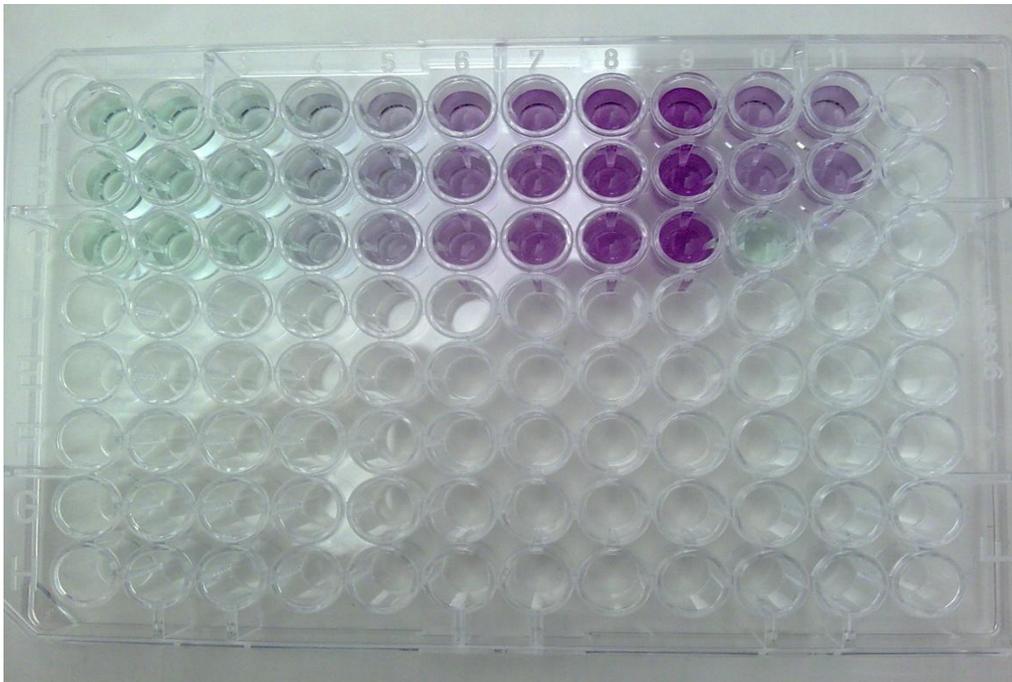


Figure 11: Generic Bio-One, 96-microwell plate, after 1 hour incubation while doing BCA Assay

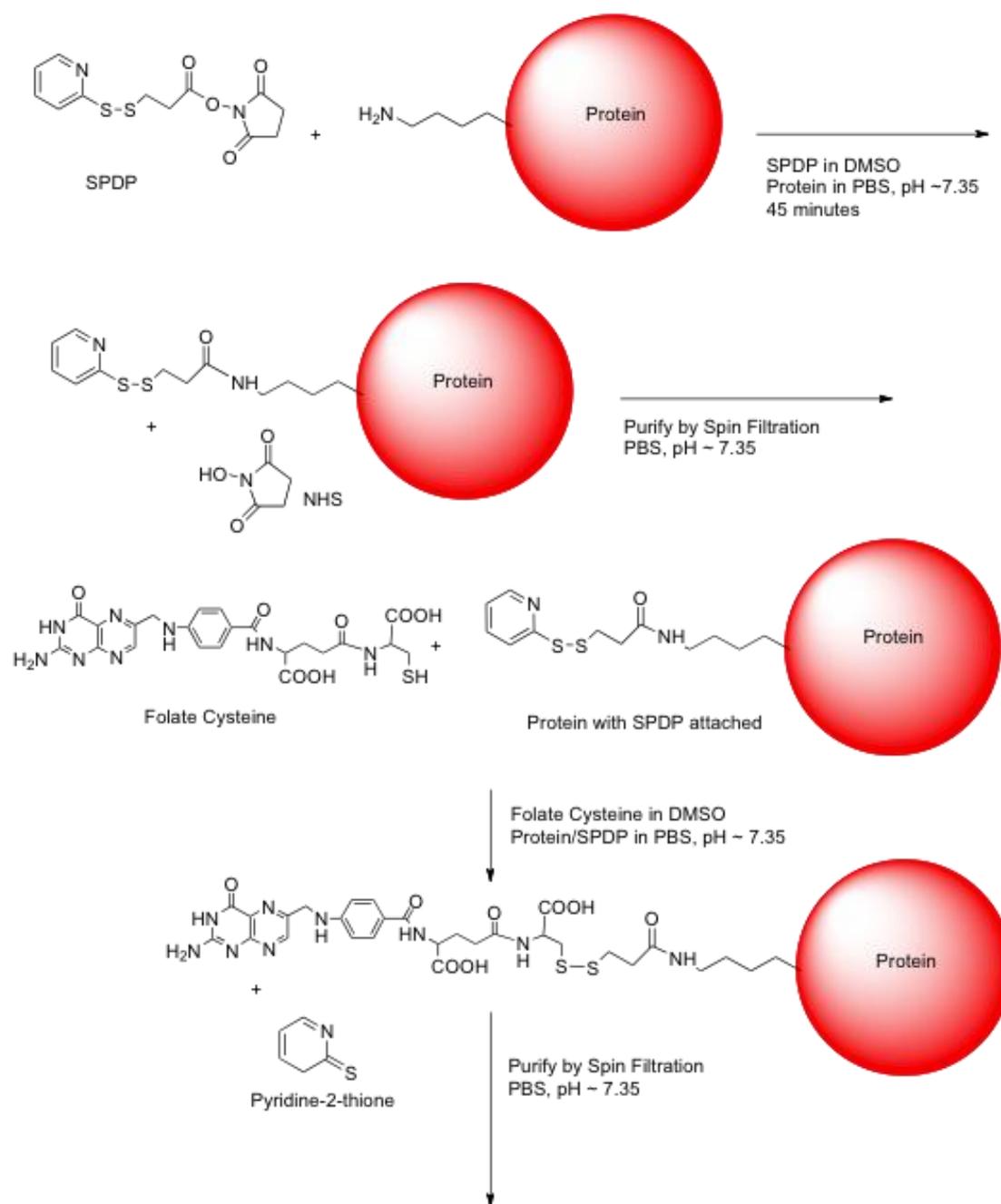


Figure 12: SPDP Labeling and folate labeling

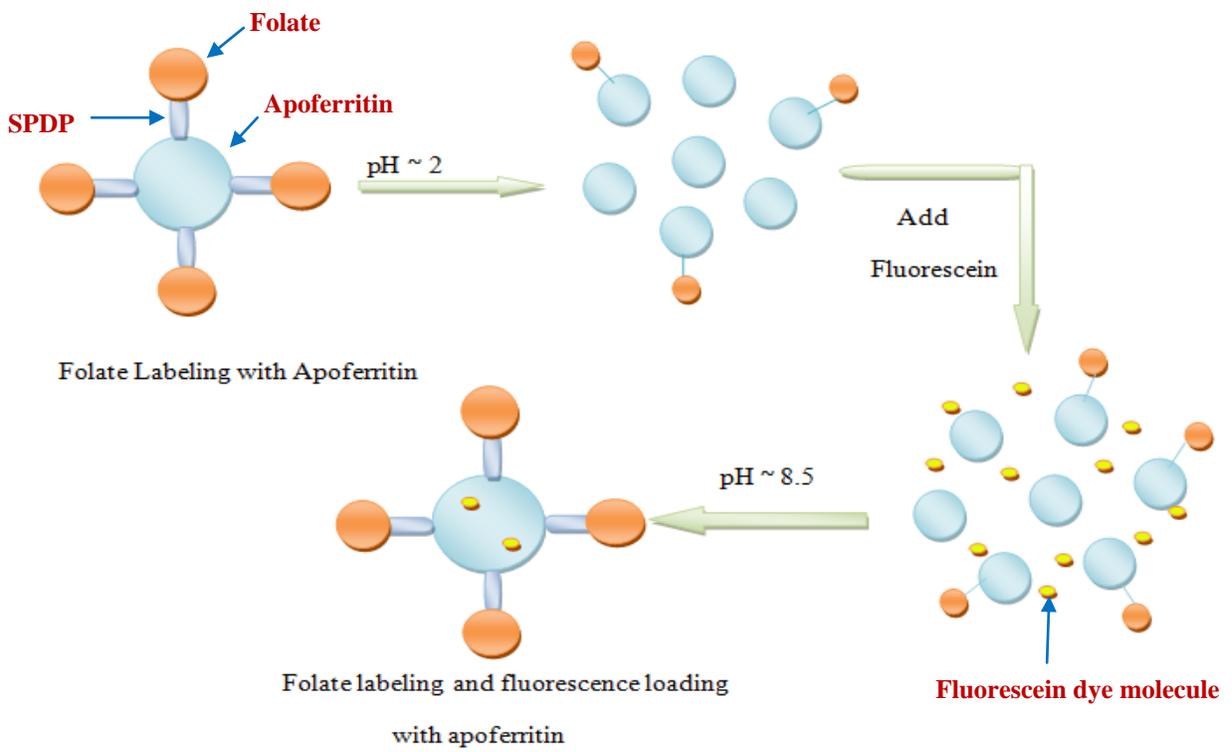


Figure 13: Folate labeling and fluorescein loading with apoferritin

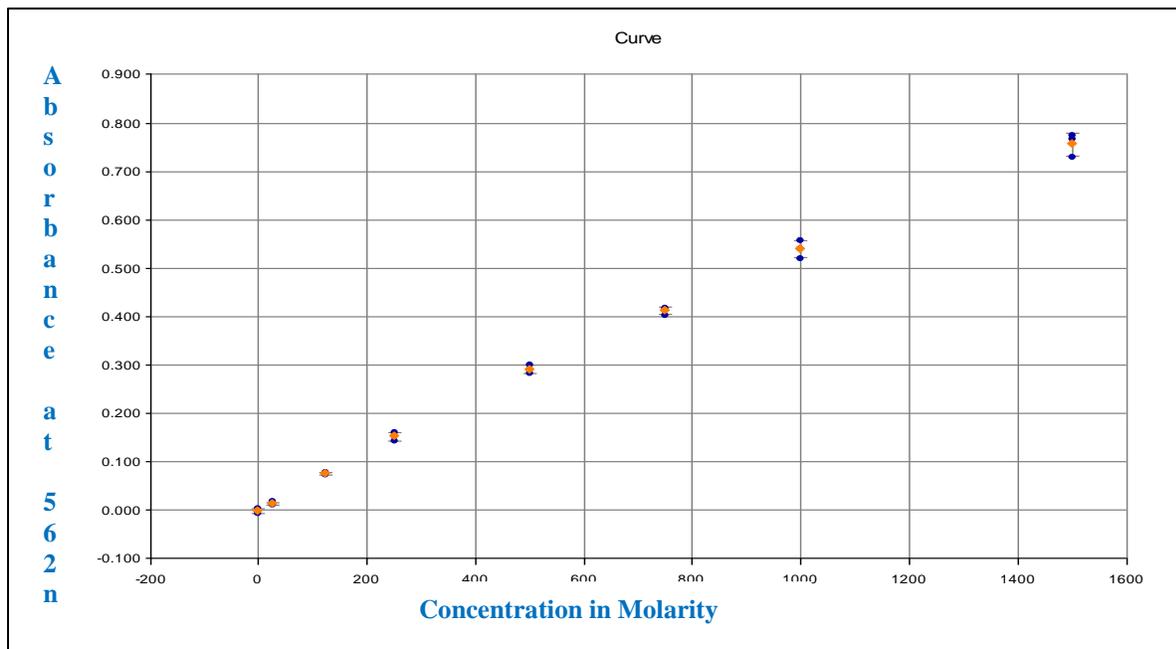


Figure 14: BCA Assay

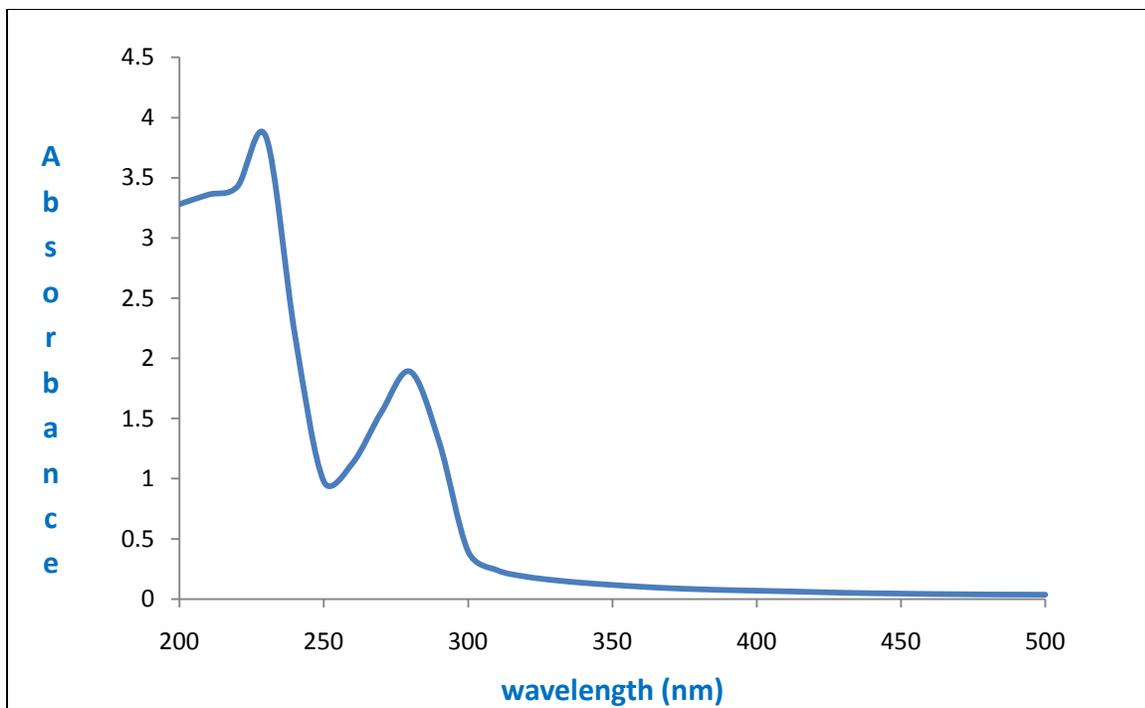


Figure 15: UV-Vis Spectrum of apoferritin

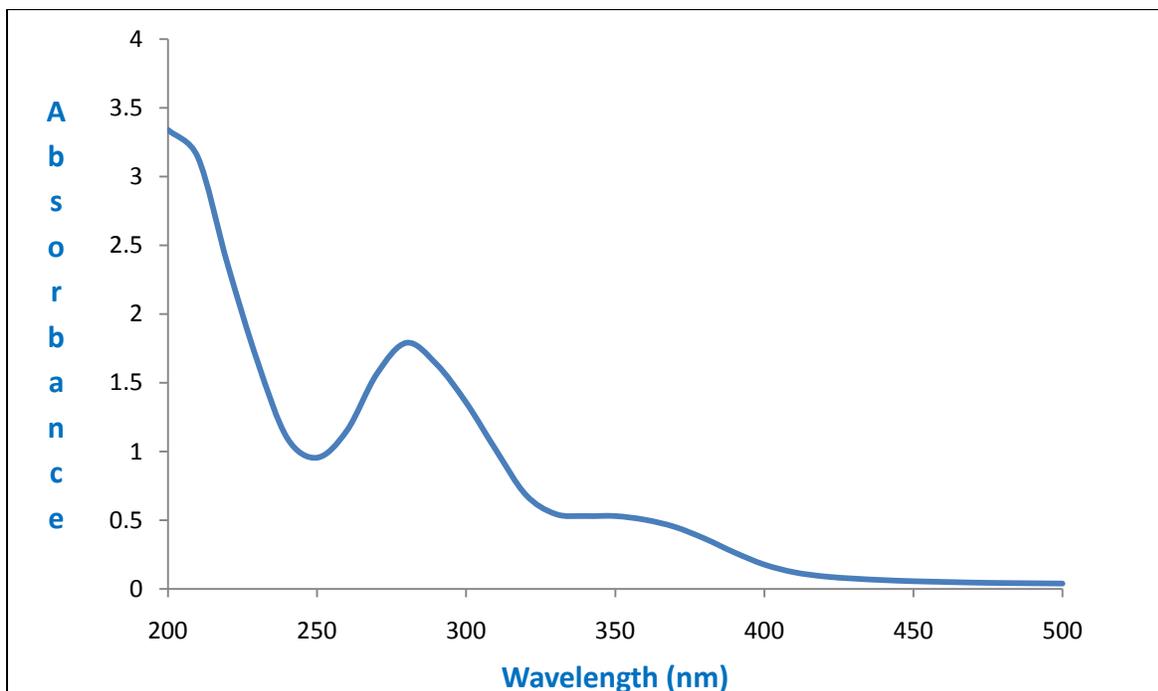


Figure 16: UV-Vis Spectrum of Folate

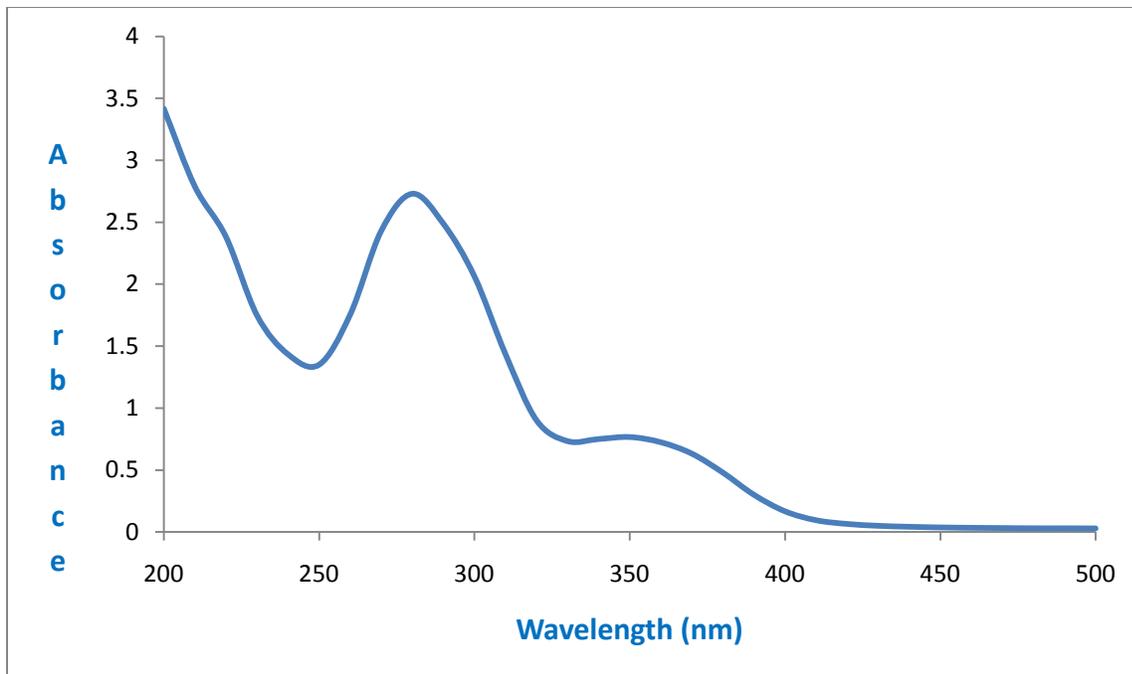


Figure 17: UV-Vis Spectrum of apoferritin + folate

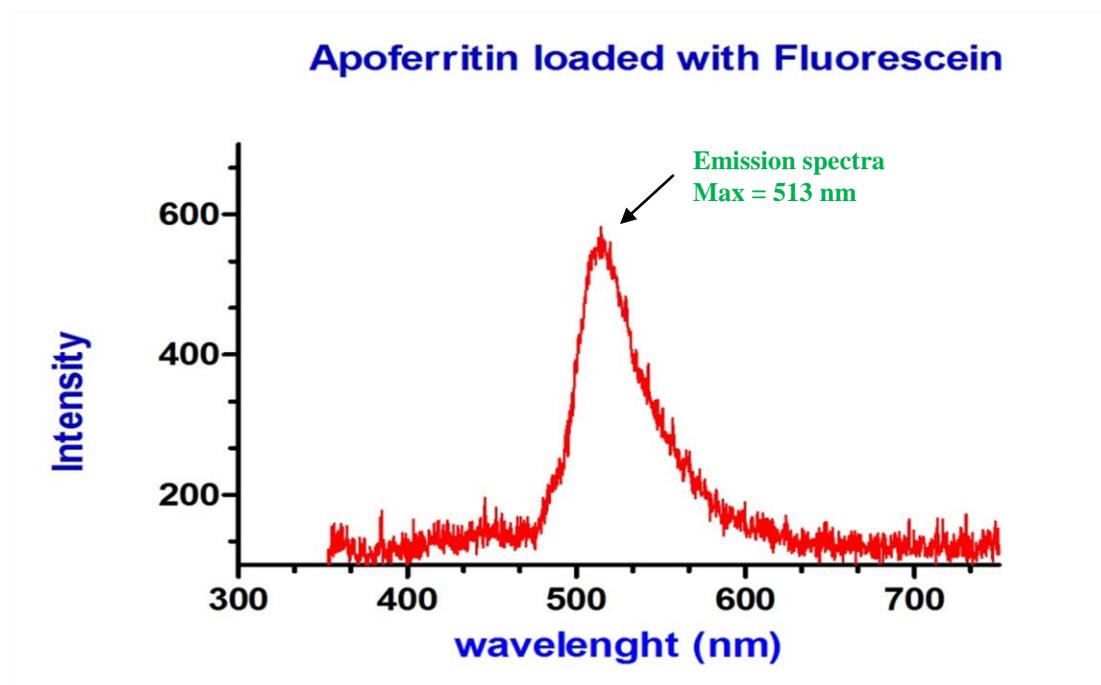


Figure 18: Apoferritin loaded with fluorescein

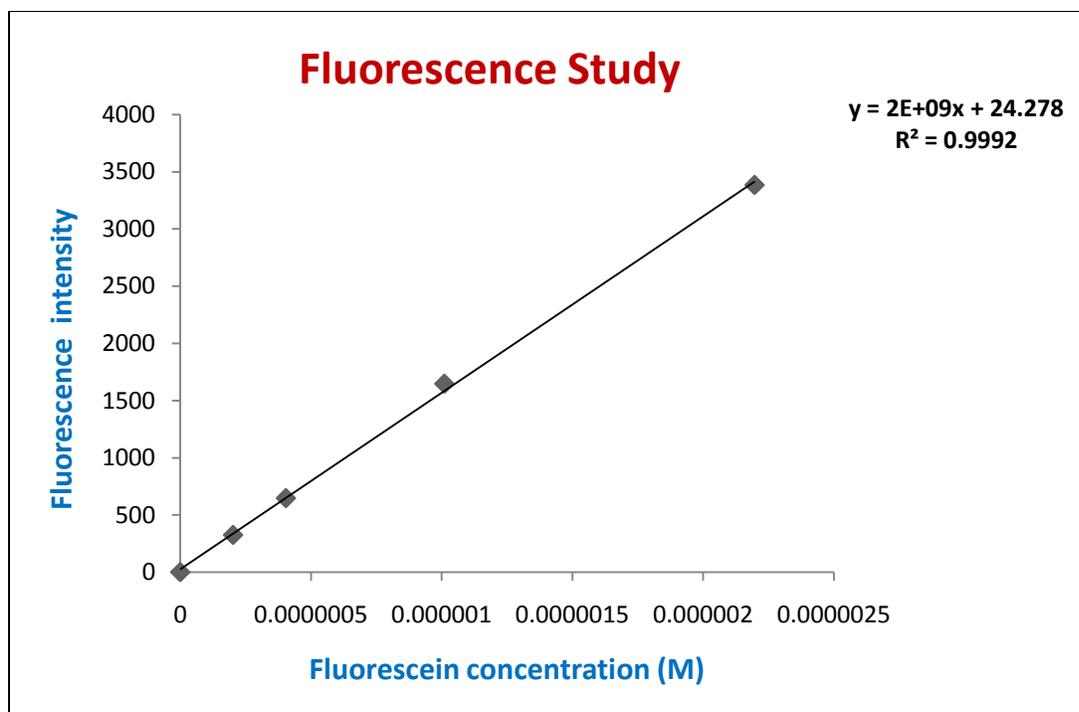


Figure 19: Fluorescence study