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

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

A Project

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By

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Dedicated to

My Family

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My deepest gratitude to my committee members Dr. Patty Fu and Prof. Kent for their continuous assistance throughout the project work.

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Abstract:

Recent clinical studies have shown the importance of folate receptor in drug delivery system as they increase the potency and reduce toxicity of many cancer therapies. The folate receptor alpha (FR-a) binds with high affinity for folic acid and serves for receptor mediated transport of folate into cells. Folate is necessary for DNA metabolism and thus it is speculated that rapidly dividing cancer cells have an increased requirement for folic acid. It is known that FR-a levels are elevated in specific malignant diseases (solid tumors, leukemia) and thus the FR receptor serves as useful targeting moiety for the diagnosis and detection of FR+ cancers.[1] Drugs that have been attached to the folate include protein toxins, chemotherapeutic DNA, radioimaging agents, magnetic resonance imaging agents and liposomes with entrapped drugs.[2-3]

In liposomal systems, the overall conjugation between the liposome and folic acid is very important for therapeutic activity due to

1) The need to present folate to the cancer cell surface unencumbered from bulky liposome(in order to bind to the folate surface)
2) The need to have adequate folate ligands for efficient binding to cell but not too many spacers/folate molecules that could result in non-specific binding.

In traditional liposomal systems, folate is attached to a PEG (poly ethylene glycol), which is incorporated into the lipid membrane via a hydrophobic tail. Although widely established, liposomes require a fair degree of technical ability to synthesize and analyse.

Recently, it has been discovered that apoferritin (iron transport protein), a 440kD polymeric protein, is capable of being dissociated into its respective subunits at low pH (pH ~ 2) and re-associated at pH ~ 7 to reform the apoferritin cage for the therapeutic purposes. Based on these results, our project aim is to synthesize a folate based apoferritin probe.[4] The type of folate

conjugation to the protein (i.e. synthesis of the folate spacer arm), the degree of folate labeling to the protein, the amount of dye incorporation into the protein cage and the types of dyes, drugs or other agents will also be assessed which would be eventually be tested for cell uptake. These type of these cages may be useful for the production of radio-imaging agents, MRI contrast agents, and other drug delivery platforms similar to liposomes.[4]

Introduction

Folic Acid:

Folic acid or vitamin B9 is a water soluble vitamin. Folic acid itself is inactive in the body. The reduced form i.e. Tetrahydrofolate (THF) plays an important role in the metabolic reactions of the body.

It is necessary for the normal growth and maintainance of cells because it acts as a coenzyme for DNA and RNA synthesis.[5] Folate is very important for pregnant women as they help in the reproduction of cells in the foetus. Deficiency of folic acid affects the cell division and protein synthesis and therefore causes impairment of growth. Folic acid (Vit B9) along with Cyanacobalmine (Vit B12) causes conversion of homocysteine to methionine and thereby reducing the bloodlevels of homocysteine and thus decreasing the risk of heart diseases.[6-7] It maintains nervous system's integrity by involving in the production of neurotransmitters like serotonin which regulate mood, sleep and appetite. It also decreases urinary tract infections.

Neural tube effects can be decreased if the pregnant women include folic acid into their dietary supplement. Low levels of folic acid cause cancers of cervix, colon and lung. High homocysteine levels also weakens the bones causing the bones to fracture. Red blood cells are particularly

susceptible to folic acid deficiency, hence causing anaemia.[8-9] Folate is also required in the metabolism of amino acids like histidine, serine, glycine and methionine.

Apoferritin Cages:

Apoferritin is a native protein composed of 24 polypeptide subunits that interact to form a hollow cagelike structure 12.5nm in diameter. The interior cavity of apoferritin is ~8 nm in diameter and has an interior volume that can store upto 4500 iron atoms as an iron oxide-hydroxide mineral. Apoferritin has 14 channels, which are formed at subunit intersections with diameters of 3-4A^0 and which connect the outside of the apoferritin molecule with its interior.[10]

The protein cage of apoferritin can be disassociated into 24 subunits at low pH (2.0), and the subunits reconstitute in a high pH (8.5) environment.[4] Because of its unique cavity structure as well as its disassociation and reconstitution charecterstics, apoferritin is widely used as a protein cage to synthesize a size restricted bioinorganic nanocomposite. E.g., cobalt,[11] manganese,[12] iron sulfide,[13] iron phosphate, cadmium sulfide, uranium,[14] Prussian blue,[15] cobalt and platinum, nickel, chromium, and magnetite. Small molecules, such as the pH indicator neutral red and the gadolinium complex, have been captured in the cavities of apoferritin. The applications of the synthesized bioinorganic nanocomposite include magnetic resonance imaging, uranium neutron-captured therapy, radio-pharmaceuticals, quantum dots and nanobatteries, photocatalysts, and magnetic memory devices.

Folate targeted Apoferritin Cages:

There are two ways in which folate targeted apoferritin cages could be made.

1) First the apoferritin cages were dissociated by lowering it to pH~2 by adding 0.1M HCl and loading it with the fluorescein. Then increasing the pH~8.5 by adding 0.1M NaOH and loading it with the folate cysteine.
2) First load the apoferritin cages with folate cysteine and then lowering it to pH~2 and then adding fluorescein to it so that when the pH is again made to ~8.5 the apoferritin cages would trap the fluorescein while reconstitution.

In a Apoferritin molecule, the surface has several exposed lysine residues. Folate can be coupled to these lysine residues by a combination of a hetero bifunctional crosslinker. SPDP with subsequent addition of folate cysteine. In this project, we established this labeling strategy to tether folic acid to the apoferritin molecule.

Materials and Methods

Materials and reagent:

Apoferritin: Apoferritin from Equine spleen, 48mg protein/ml, Lot No: A3641; Phosphate-Buffered Saline(PBS),Mediatech, Inc, Manassas, VA 20109, Lot No. 21040174;

BCA Assay: Bicinchonic Acid Kit for Protein Determination, SIGMA ALDRICH, Bicinchonic Acid Solution: Batch No: 118K5300, Copper(II) Sulfate Solution: Batch No: C2284 (25ml);

SPDP(N-Succinimidyl – 3-(2- Pyridyldithio) Propionate , Pierce , Lot No: 21857;

DMSO: Sigma Aldrich, 99% purity; Folate Cysteine: Provided by Dr. Henne ; Fluorescein sodium salt($C_{20}H_{10}Na_2O_5$) : Lot# 079K0141V; Multichannel pipetter : RAININ , 20-300µl;

UV Plates: BD Falcon microtest 96 well 370µl clear plate, UV Vis transparent film bottom,

Non-sterile, no lid, lot No:E1002007; Regular Plates: Generic Bio-One, microplate, 96well flat bottom, Lot No: E091006L; Spin Filters : Sigma Aldrich, Amicon ultra 0.5 centrifugal filter, 3 kDa, Batch No:3110; PD-10 Columns: sephadex G-25M columns, contains 0.15% Kathon CG in DI H20, lot no. 393861; Centrifuge: Beckman CS-15R Centrifuge at 8898 RPM for 12 mins. UV Plate Reader and BCA assay Software: Gen 5.1.10 Biotek, Epoch.

Methodology:

SPDP Labelling:

Folic acid was linked to Apoferritin cages using heterobifunctional crosslinker SPDP(N-Succinimidyl – 3-(2- Pyridyldithio) Propionate).[16] Approximately 100fold excess of SPDP dissolved in 25µl of DMSO was added to 5mg of Apoferritin cage(0.5ml PBS pH~ 7.35). it was allowed to react for 45mins. Upon completion of this reaction, the protein cage was purified from excess SPDP using spin filters (x 5 washes with PBS). The protein(0.5ml) was next reacted with a 200fold excess of folate cysteine in 50µl of DMSO and was allowed to react overnight. The protein was purified essentially the same as with SPDP coupling. Determination of folic acid on the protein was measured with UV plate at 363nm.[19]

Fluorescein Loading:

Flourescein loading of the Apoferritin cage was accomplished. First, 150µl of Apoferritin sample was taken and dilute it to 500µl with PBS. Then we washed it 3 times with PBS to get rid of glycerol. Then we lowered the pH of the solution to 2 by adding 83µl of 0.1HCl. Then we took 2mg of fluorescein and diluted it with 5ml PBS(0.1 mM). Then we lowered the pH to 2 by adding 48µl of 0.1M Hcl. Then add 50µl pH 2 fluorescein solution to Apoferritin solution and

allowed to stand for 20min and then we raised the pH of the solution to 8.5 by adding 92.5µl of 0.1M NaOH and then spinned it for 2hrs and then run the solution on PD10 column.[4,20]

1. • Take 150µl of Apoferritin sample and dilute it to 500µl with PBS
2. • wash it thrice with PBS
3. • Take 2mg of fluorescein and dilute it with 5ml PBS(0.1mM)
4. • Lower the pH of the fluorescein to 2 (added 48µl of 0.1M HCl)
5. • After the three washes, lower the Apoferritin sol pH~2 (added 83µl of 0.1M HCl)
6. • Add 50µl of pH~2 fluorescein solution to the Apoferritin sol
7. • Allow to stand for 20min
8. • then raise the pH of the sol to 8.5(added 92.5µl of 0.1M NaOH)
9. • Magnetic stirring for 2 hours
10. • run on PD 10 column
11. • Do the BCA Assay and UV-Vis with the collected fractions

Methylene Blue Loading:

The same procedure was followed as stated above with few modifications. Just that there is a slight change that the solution was made to be 7.5 instaed of 8.5 and rest of the procedure remained the same and we ran on a PD-10 column and the eluents were taken and we did the BCA Assay.[17]

BCA Assay:

BSA stock solution was taken and several dilutions were prepared as per the Thermo Scientific Pierce BCA Protein Assay Instructions. The following were the different concentrations that we prepared- 1500µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 25 µg/ml and 0 µg/ml(blank). We used a microtitre plate and we pipetted 10 µl of each std as well as our eluents(unknown) duplicates in the wells. Then we added 200 µl of the working reagent (10ml of BCA solution and 200 µl of Cu(II) sulphate solution and mixed it in a tube).[21] Then incubated the microtitre plate for half hour and then found the concentrations by using the BCA Assay Gen 5.0 software.

Results and Discussion

BCA Assay:

The Bicinchonic acid method employs the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium. The combination of BCA and Cu^{+1} creates a purple colored product that absorbs at 562nm. The amount of product formed is nearly linear with increasing protein concentrations over a broad working range(0 - 1500µg/ml).

Upon final purification using the spin filter method, the compound was analyzed for protein concentration using BCA Assay. Based on the BCA Assay, we determined the concentration to be 2191mg/ml.

Folate labelling:

Based on the molar extinction coefficient of folic acid at pH 7.5 we determined the degree of folate labeling which was approx. 100. This is consistent with the work of [18] which found that 4 exposed lysine units were found on the Apoferritin cage surface which means there are approximately 96 (4*24) residues that are conjugatable.[18]

Therefore we could label 96 sites. Thus our 100 value is consistent with this data and indicates we were getting full folate labeling.

Fluorescein loading:

Attempts to load the cage with fluorescein and with methylene blue have been undertaken but at this stage we did not get any results in fluorescence spectroscopy due to low volume.

We tried to do it by increasing the concentrations of fluorescein but the fluorescein spectroscopy studies are yet to be done.

Conclusions:

Conjugated folate to the protein cage and determined the degree of labelling to the protein. Purified folate protein cage using spin filters. Attempted to load the protein with fluorescent dye and determined the degree of dye incorporation. Tried to trap the fluorescein with different concentrations of it into the apoferritin cage. Loaded the protein with methylene blue dye and determined the degree of dye incorporation.

Future Studies:

Purify folate protein cage via size exclusion chromatography rather than spin filters and determine the degree of dye incorporation by doing fluorescence studies. Establish and maintain FR+ cancer cells and and do the cell testing using the Apoferritin protein cages that are prepared.

Funding

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20 PD-10 Desalting Columns, Trap, GE Healthcare

21 Pierce BCA Protein Assay Kit, Thermoscientific from www.thermo.com/pierce

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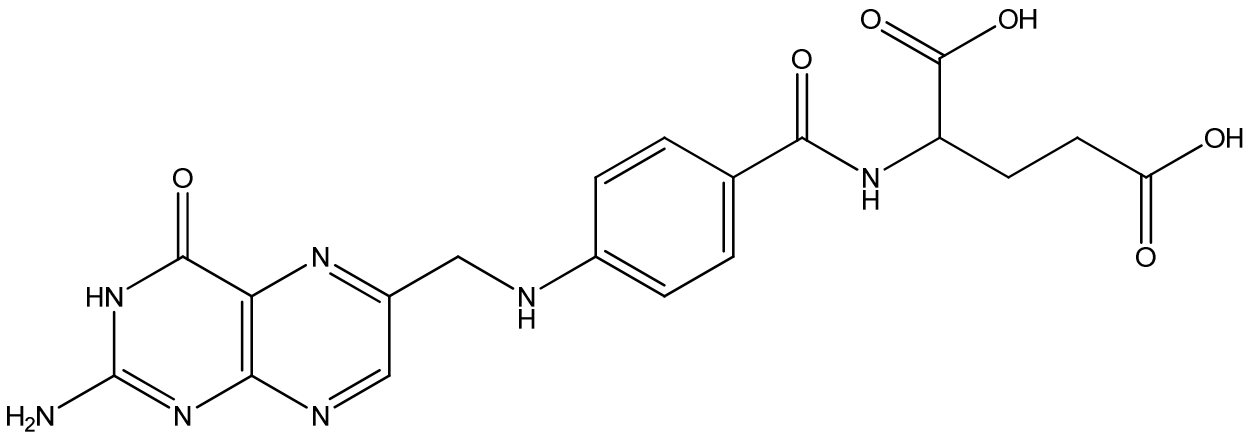


folic acid

Figure-1: Structure of folic acid

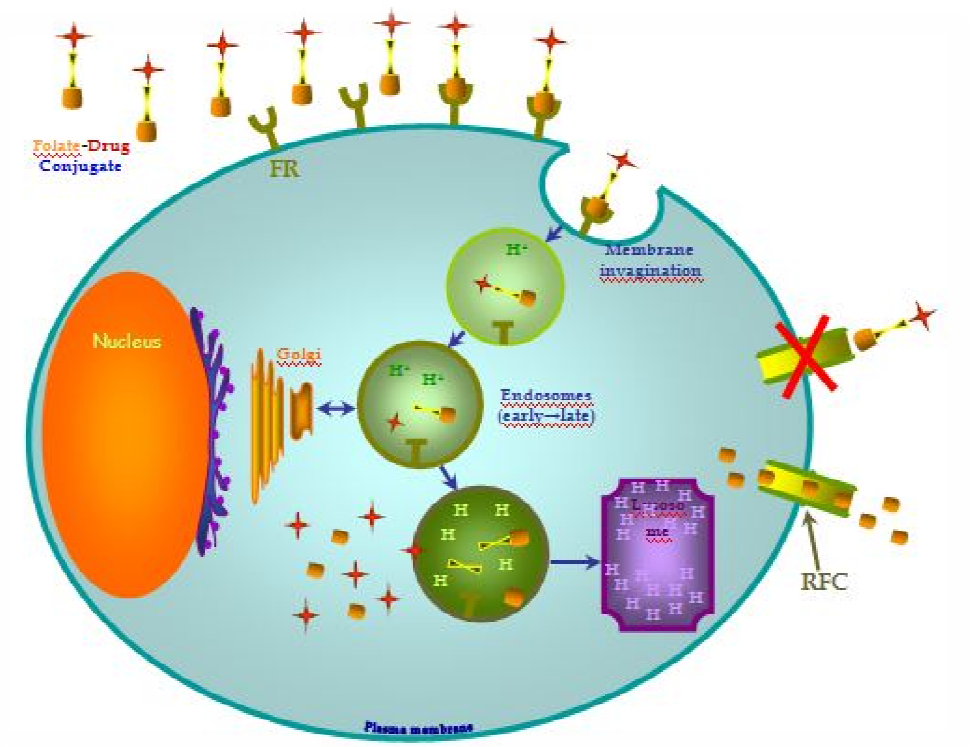
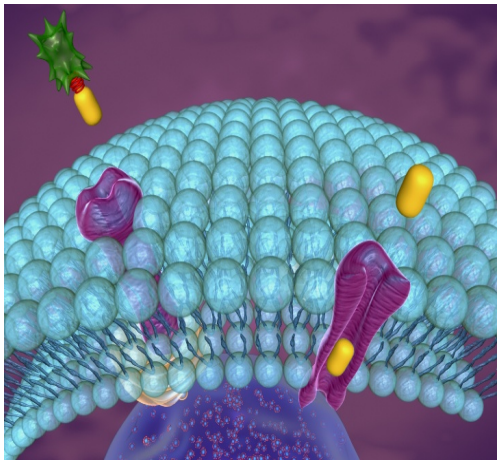


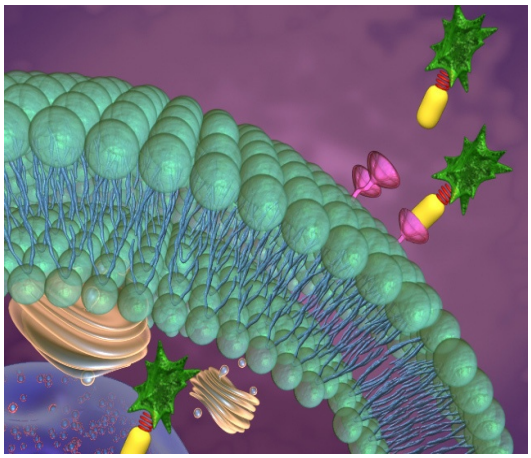
Figure-2: uptake of folate conjugates (Low, Henne, Accounts of Chemical Research. Vol. 41, No.1, Jan 2008)

Normal Cells
Reduced folate carrier



Reduced folate carrier
(Kd=10^{-10} M)

Malignant Cells
Folate receptor(alpha-FR)



Folate receptor
(Kd=10^{-5} M)

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

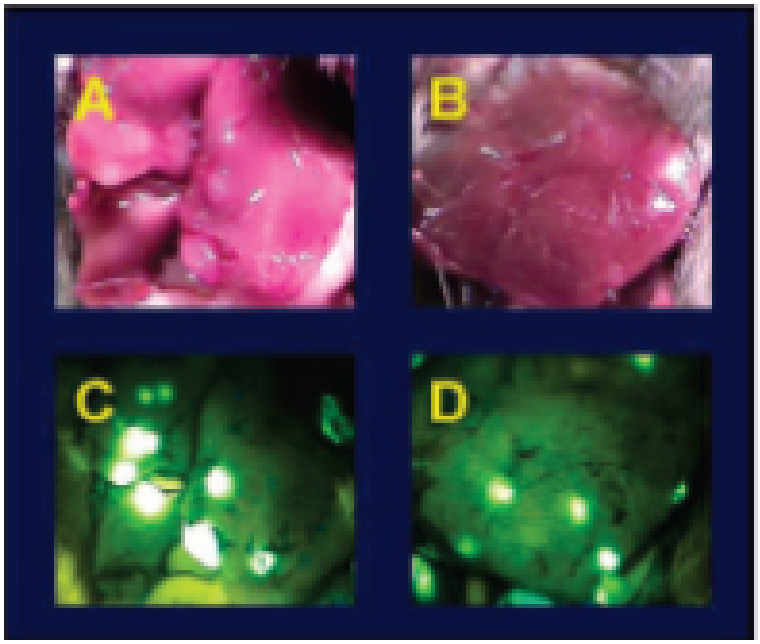


Figure-4: Panels A and B: Bright field image of liver tissue Panels C and D**:** Laser-illuminated liver tissue Kennedy, et al., J.Biomedical Optics 2003[1]

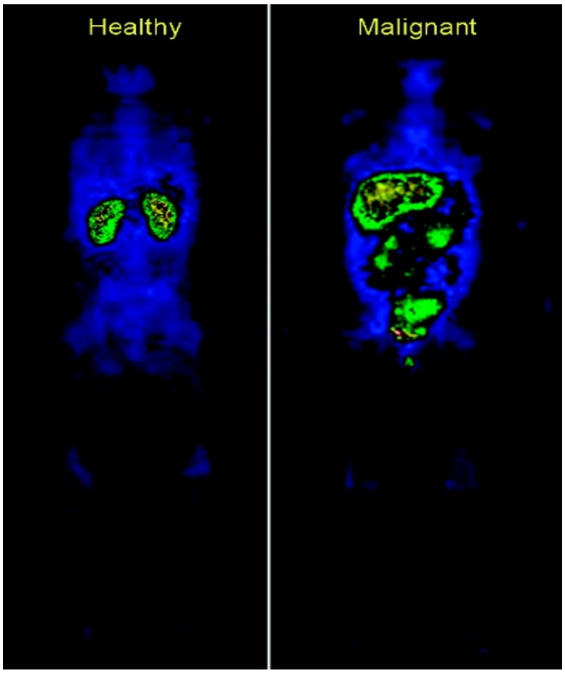


Figure-5: Whole body scintigraphic images of a healthy volunteer and an ovarian cancer patient. Uptake in the cancer patient is seen in both the malignant tissue and kidneys, whereas only kidney uptake is observed in the healthy individual. Image reproduced with permission from Endocyte, Inc.[1]

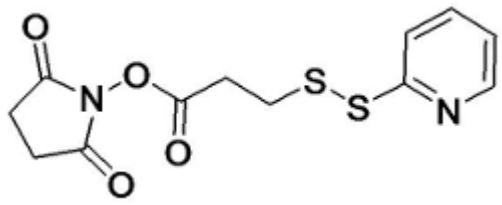


Figure-6: SPDP Structure.

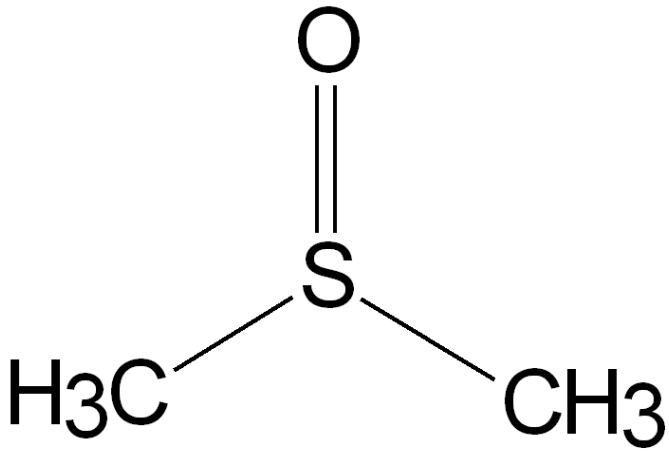


Figure-7: DMSO(Dimethyl Sulfoxide)

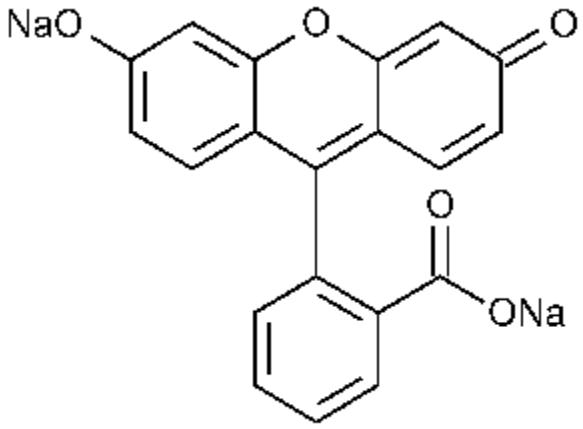


Figure-8: Fluorescein Sodium Salt(C20H10Na2O5)

Figure-9: Centrifuge: Beckman CS-15R Centrifuge at 8898 RPM for 12 mins.

Figure-10: pH meter

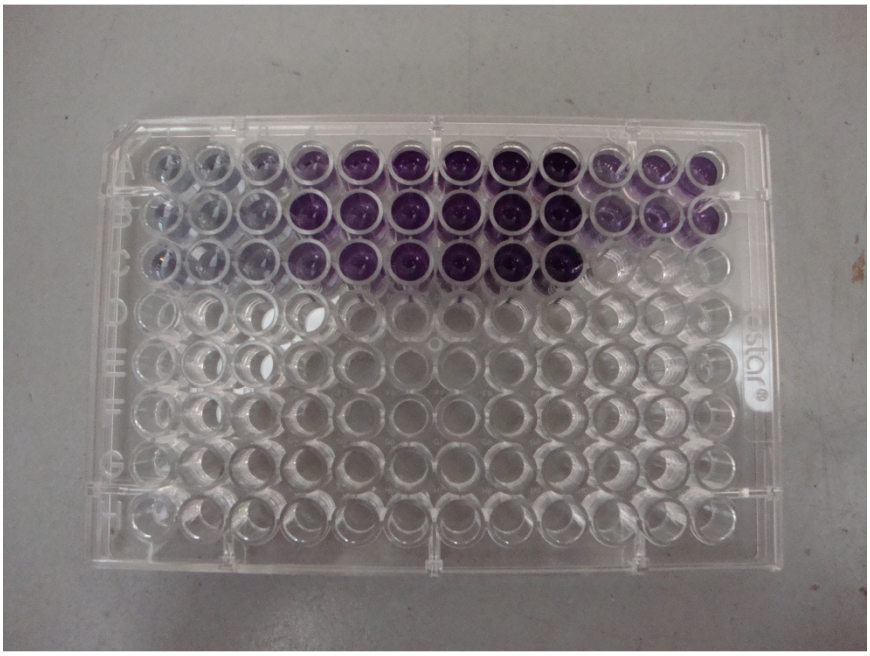


Figure-11: Generic Bio-One, microplate, 96well flat bottom, Lot No: E091006L;

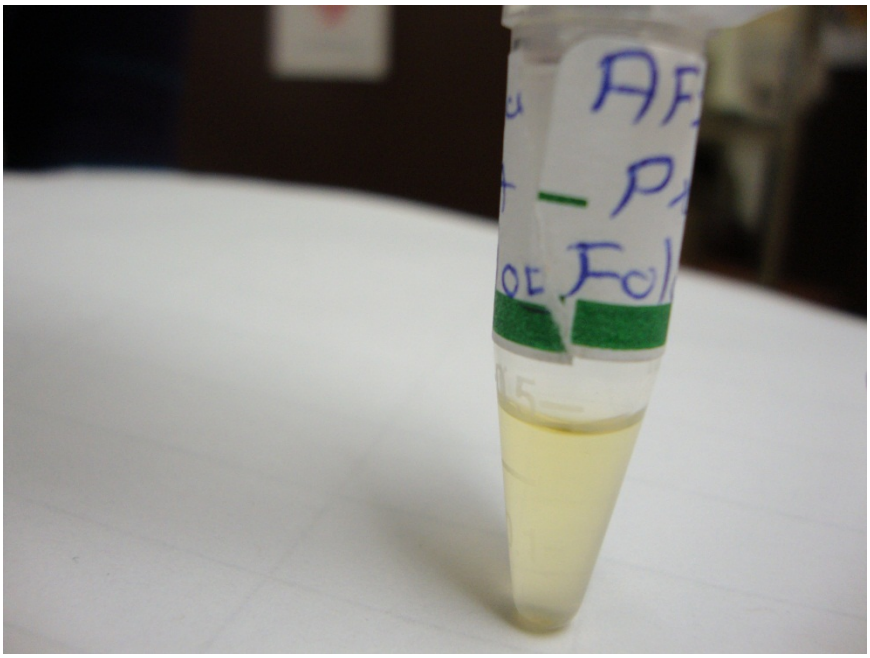


Figure-12: Folate labeling to Apoferritin.

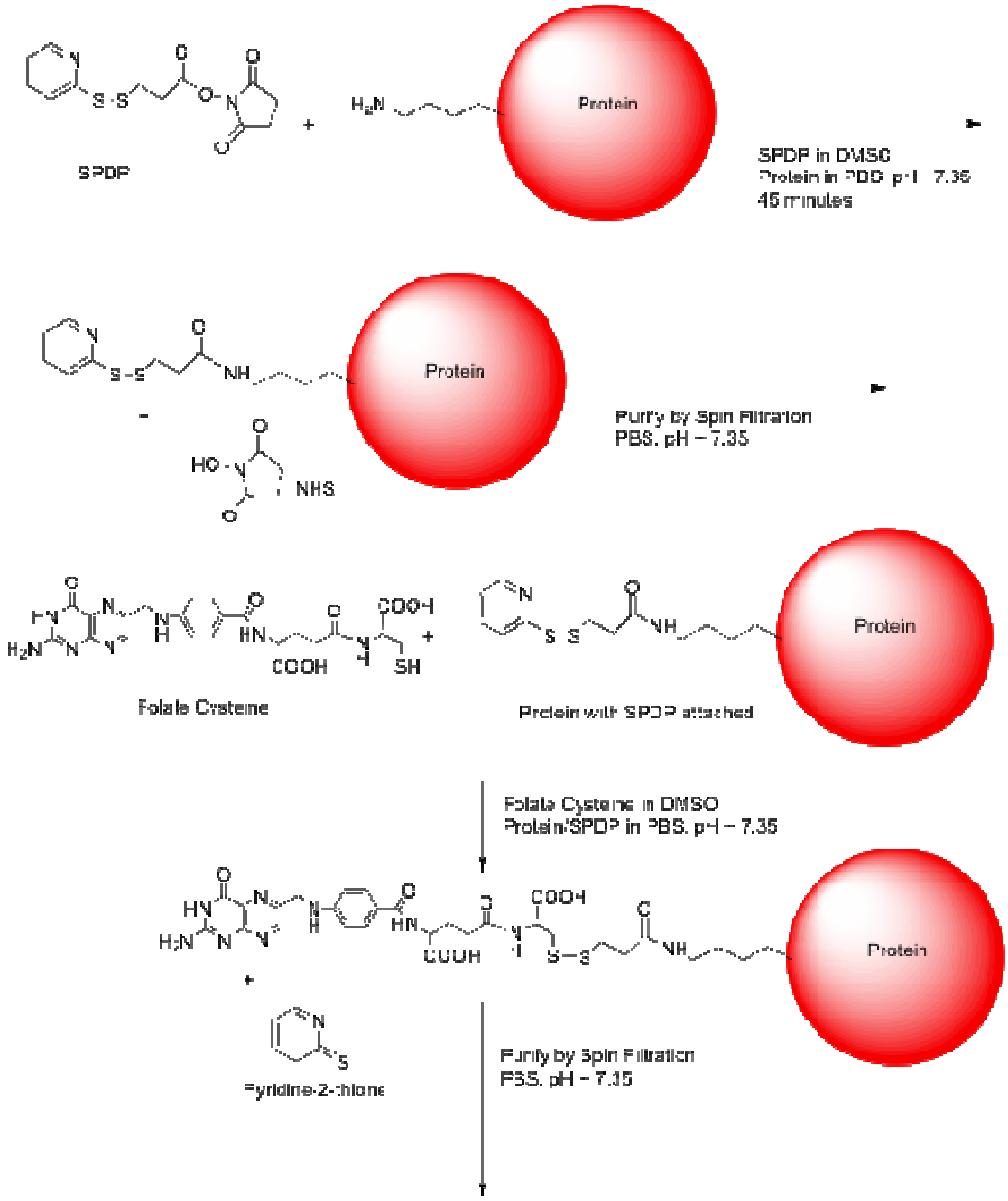


Figure-13: SPDP Labelling.

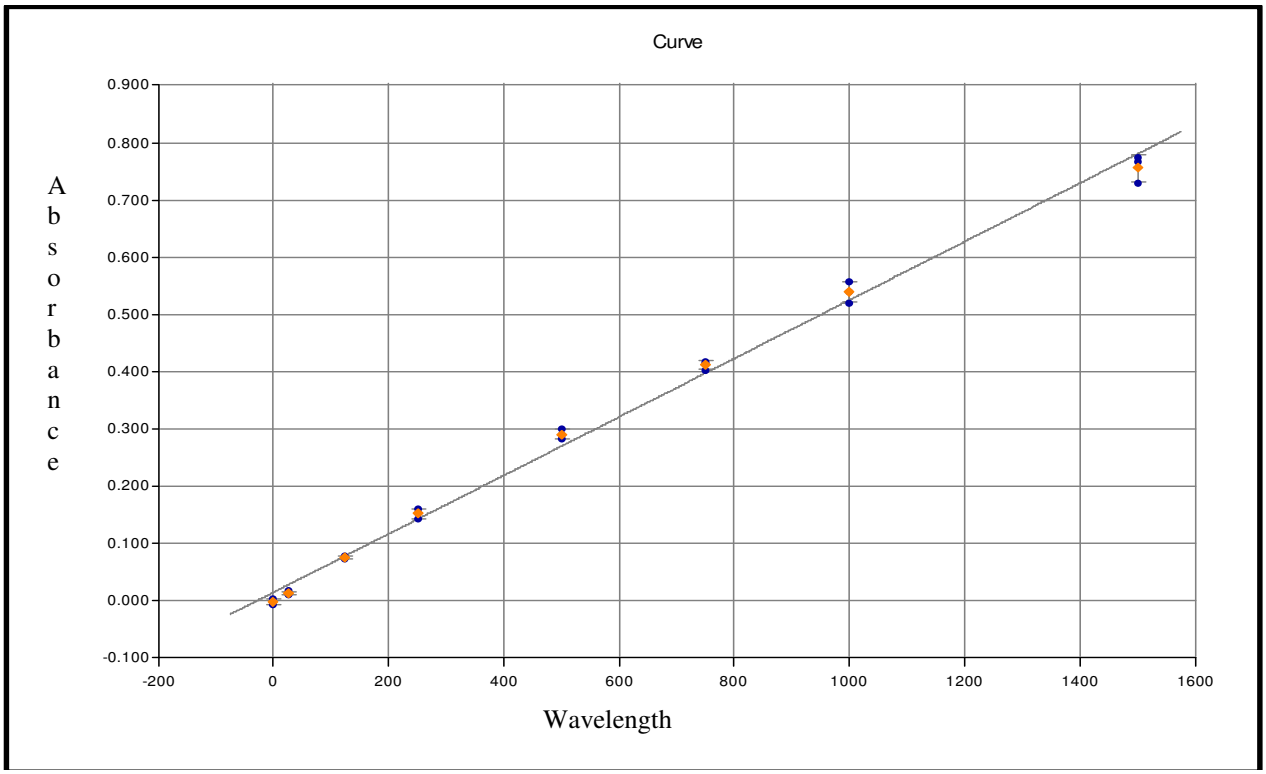


Figure-14: BCA Graph.

Figure-15: UV-Vis Spectrum of Apoferritin

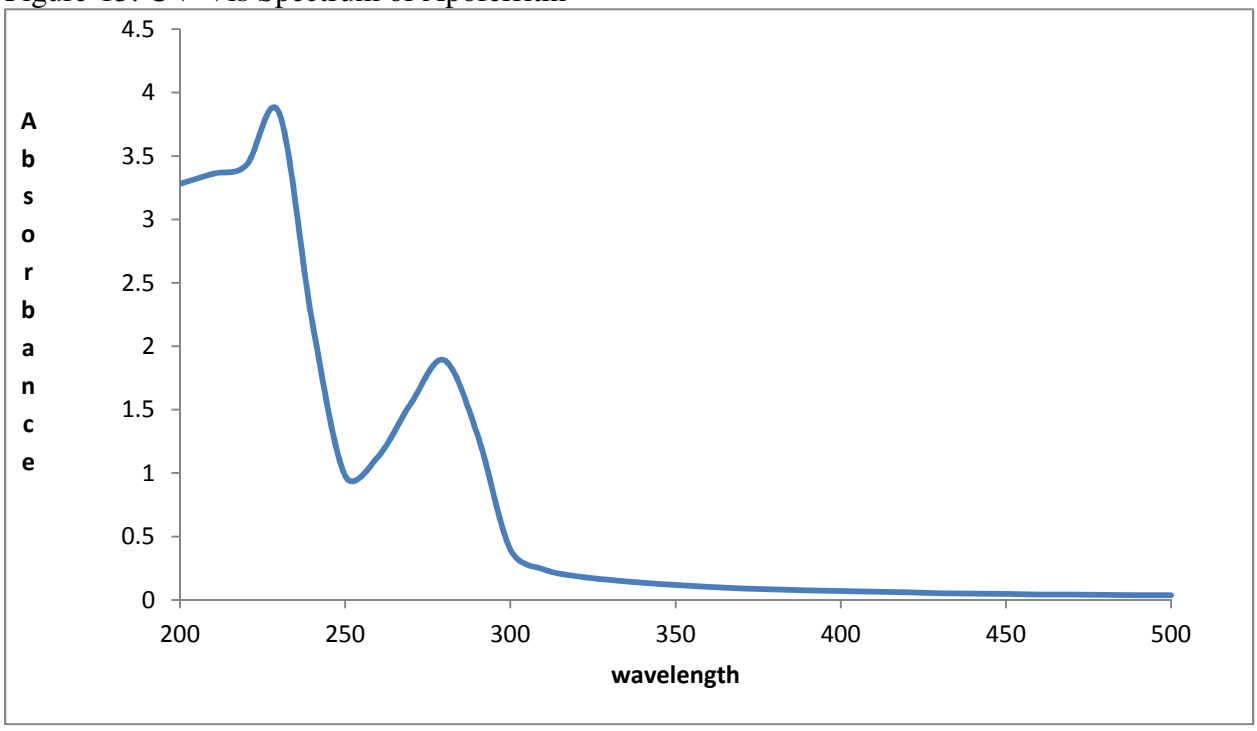


Figure-16: UV-Vis Spectrum of Folate

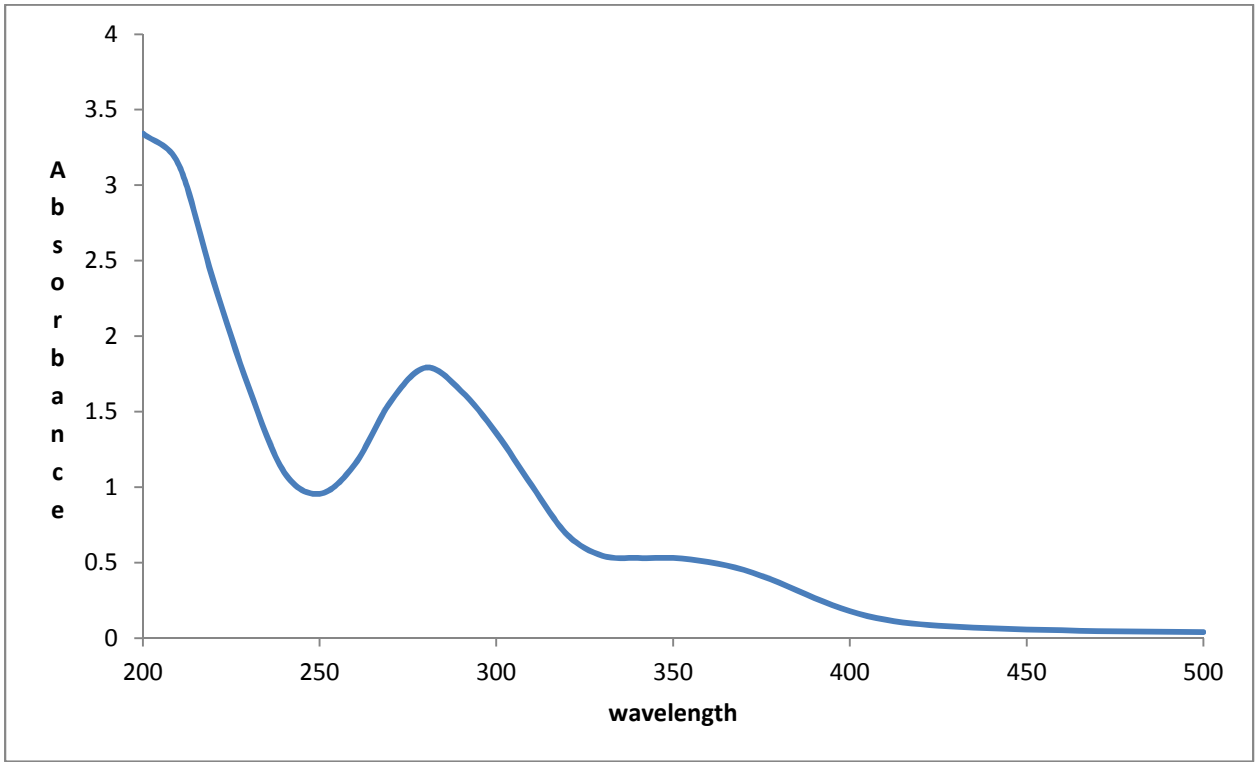


Figure-17: UV-Vis Spectrum of Apoferritin + Folate