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Evaluation and In Vitro Studies of Folate-PEG-Biotin

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Evaluation and In-vitro Studies of Folate-PEG-Biotin

A Project

Submitted

To

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By

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Dedicated to my **Family, Friends and Teachers**

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Table of Contents

Abstract

Folate receptor is a membrane bound protein which is highly expressed by cancer cells and macrophages. Folic acid is required for the normal growth of cells and it has been found that rapidly growing cancer cells require high levels of folic acid. Folic acid binds to folate receptors and through receptor mediated transport folate enters cells. This property has been used to synthesize a probe using folate as ligand through polyethylene glycol-Biotin linker. This can be used to attach a drug which is ultimately delivered at the site of infectious cells. The study involved here was to determine how folate and magnetic beads can be used to develop imaging systems and transport drugs to identify and treat pathologies. Folate-PEG-Biotin probe (already synthesized from Dr. Walter Henne and Mr. Rohan Patel) was purified by HPLC and LC/MS which was then used to perform cell binding studies with streptavidin coated magnetic beads on L1210 type cancer cells. Biotin which is a B-complex vitamin is also required for the growth of cells. This biotin has the highest non-covalent affinity to streptavidin in nature and hence was used to link streptavidin coated magnetic beads to folate. Polyethylene glycol was used as a spacer. This streptavidin protein in the form of magnetic beads captures cells from solution when used with the Folate-PEG-Biotin probe. Cells were observed under a high resolution light microscope and the images were taken with a high resolution camera available from Dr. Tim Gsell's laboratory. Cells were also studied by just adding streptavidin beads alone to observe if they were captured. To another control, Folate-PEG-Biotin/Streptavidin coated magnetic beads were added along with 1000 fold excess of folic acid.

This method is economical and can be used to replace other cancer cell detection techniques. Future studies include the conjugation of folate-peg-biotin probe with a proprietary drug conjugate to determine the drugs activity on cancer cells which will be carried out at Purdue University.

Introduction

Folic Acid: Folic acid (Figure 1) is vitamin B_9, and naturally occurs as folate. Folic acid shows its activity in the form of its tetrahydrofolate derivative[9]. Folic acid is required for the synthesis of DNA and its repair. It is also required as a cofactor in some biological reactions. It is required in the body for cell division and growth of cells. Lack of folic acid in the body leads to anemia as folate is required for the synthesis of red blood cells. Recent studies provide evidence that high levels of folic acid assist in growth of some cancer cells. It has been found that cancer cells require folate in order to divide and maintain its functions.

Folate Receptor: Folate receptor alpha (Figure 3) is a glycophophatidylinositol (GPI) membrane bound protein which is encoded by the FOLR1 gene[1]. These kinds of proteins have a high affinity towards folic acid and its derivatives and aid in receptor-mediated transport of folic acid into the cancer cells. It has been found that tumors of epithelial origin express high FR-alpha levels than normal cells[2-3]. FR- α receptors are prominently observed in cancers of kidney, ovary, lung, colon, lung, brain etc[4]. Studies have shown the presence of folate receptors on many different cancerous tissues[5-6]. This enabled researchers to identify the potential of folate receptors in targeted drug delivery and diagnosis methods[7-8].

Streptavidin: It is a protein of the tetramer[10-11] kind obtained from *Streptomyces avidinii*. It has the high non-covalent binding towards biotin[11]. Its resistance towards organic solvents, enzymes, high temperature and pH proves useful for research studies[12].

Here, we used streptavidin coated magnetic beads to isolate cancer cells[13]. Magnetic beads are made of iron. Magnetic property of the beads pulls the constructed ligand from the solution and also the cells when introduced.

Biotin: It is a B-complex vitamin which is required for cell growth. Biotin is generally used to isolate proteins by linking it to proteins, a process called biotinylation[14]. Biotin has high affinity and binds specifically to a protein named streptavidin[15]. This affinity between biotin and streptavidin has been used to isolate cancer cells through folate-PEG-biotin.

Polyethylene Glycol (PEG): It is a polymer of ethylene oxide and in this study, it has been used as a spacer between streptavidin and biotin that reduces aggregation by decreasing steric hindrance and increases solubility and also reduces toxic and immunological effects.

Materials

a) Magnabind™ Streptavidin from Thermo Scientific**:** Capacity- 2µg Biotin/ml resin

Lot no: KH1223234

Prod # 21344

b) **Biotin-PEG Resin**- from Novabiochem

c) **Cancer Cells**- L1210 from Purdue University.

d) **RPMI Medium 1640** from Gibco/Invitrogen

[+]L-Glutamine

[+]Phenol Red

[-]Folic acid

Reference no: 27016-021

Lot no: 714204

e) **Phosphated Buffered Saline**, 1X from Cellgro (Mediatech, Inc)

Without Calcium and Magnesium

Cat. No. 21-040-CV

Lot. No. 21040174

Exp- 07/2011

Method

Preparative HPLC of Folate-PEG-Biotin

The synthesized sample was then purified on a HPLC from Hewlett Packard, series 1050 equipped with a Diode array detector.

The following method and parameters were adopted:

Column: Rigel C18

Solvent A: Ammonium Bicarbonate Buffer

Solvent B: Acetonitrile

Flow Rate: 1ml/min

Run Time: 60 min.

No.	Time (min)	% B
1	0	1
2	5	1
3	35	30
4	45	50
5	55	60
6	60	1

LC/MS Analysis

Sample from the HPLC run was collected. This sample was then analyzed with LC/MS to confirm the identity and purity of the sample through its mass spectra under negative ion mode.

Column: Eclipse XDB C18

Solvent for Negative Ion Mode: Methanol and Water.

Sample Size: 30µl

Flow Rate: 0.5ml

Run Time: 10 min

Scan Range: 600-1000 m/z.

Time in min	**Methanol**(%)
0	30
1	50
2	70
3	90

Capture of FR-α Cells With Streptavidin Coated Magnetic Beads

To 1ml cancer cells, 10µl of purified Folate-PEG-Biotin sample was added. This was stirred for 15 minutes at 37degrees (angle) on a mechanical shaker. The sample was then centrifuged for a minute. Upon centrifugation, unbound cells separated out at the top and were removed with a pipette. To the obtained sample 500µl of PBS was added. This mixture was shaken for 15 minutes and then centrifuged for a minute. Clear liquid that separates out at the top is removed. This wash with PBS was repeated twice. To the washed sample, 500µl of PBS and 50µl of streptavidin coated magnetic beads were added. It was then stirred for 15 minutes and allowed to stand for 3 minutes on a magnetic stand. Any unbound cells were pipetted out. The resulting sample was then washed with PBS, stirred for 15 minutes and centrifuged twice to remove any unbound cells.

Another 1 ml of the same cancer cell type was taken as control. To this, streptavidin coated magnetic beads alone were added. To another control, Folate-PEG-Biotin and streptavidin coated magnetic beads were added along with 1000-fold excess of folic acid.

Both the controls and cells under study for capture were observed under a high resolution light microscope.

Results and Discussion

The chromatogram described in figure 4 is that of Folate-PEG-Biotin. The large intense peak at 37 minutes was proved by the DAD spectrum (figure 5) which shows maximum absorbance at 284 and 350nm. Minor peaks adjacent to the main peak are due to some impurities present in the sample. To confirm Folate-PEG-Biotin identity, the sample was collected and subjected to LC/MS analysis.

A Purified sample from preparative run from HPLC was collected and analyzed in LC/MS. Figure 6 shows an intense peak at 5.2 minutes for LC-DAD. The peak (molecular ion peak) at m/z 868 (figure 7) for the negative ion mode matches the calculated value of 869 for Folate-PEG-Biotin. This confirms the identity of the purified sample. Peaks at m/z 891 may be due to sodium adducts present in glassware or instruments.

Cells under study were then observed under a light microscope and images were captured with a camera. Images obtained confirm that only streptavidin (coated onto magnetic beads) conjugated to Folate-PEG-Biotin could capture cells. Cells that were added with streptavidin alone (control) could not penetrate the cell membrane. For control-2, excess of folic acid is taken up by the cancer cells and hence streptavidin coated magnetic beads could not capture the cancer cells.

Conclusion

Folate can be used as a ligand for isolation and detection of cells. In this work we were able to use magnetic beads to isolate FR+ cancer cells. Since cancer cells express high folate receptors and required large amount of folate for metabolism, folate can be used to transport drugs into cells by developing folate based ligands for imaging and therapeutic purposes. This

way, folate can be used as a ligand to selectively deliver chemotherapeutic agents. This ensures that only impaired cells will have the effects of the drug and normal cells would be left untouched. This property of folate can be utilized to develop many more therapeutics agents for the cure of various cell related abnormalities and diseases.

Magnetic beads can be used to isolate cells through linkage to ligands for isolating cells. We could easily visualize the cells coated with beads. Thus, we were able to capture and detect with the same system. This magnetic property can be used for future studies for developing photo-magnetic imaging systems. It is a simple process and economical. This may be combined with other down-stream applications besides microscopy such as flow cytometry to count cells captured.

Future Studies

Further study may involve how folate can be used as a ligand to develop imaging and therapeutic agents and delivering drugs specifically at the target. Folate receptor targeted therapy is just not limited to cancer but also to large number of diseases. This is mainly because of its high expression in tumors and macrophages. Advantages like low cost, ease of handling, suitability for use with various agents, "lack of immunogenicity and specificity to pathologic cells" makes it a useful agent in studying and developing folate based imaging systems and constructing chemotherapeutic agents with ligands for therapeutic purposes.

Funding

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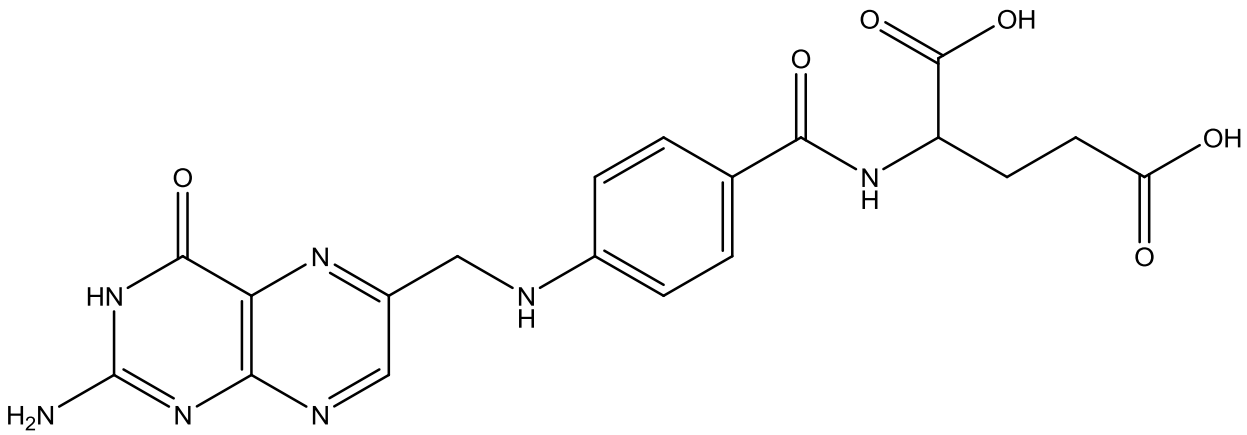
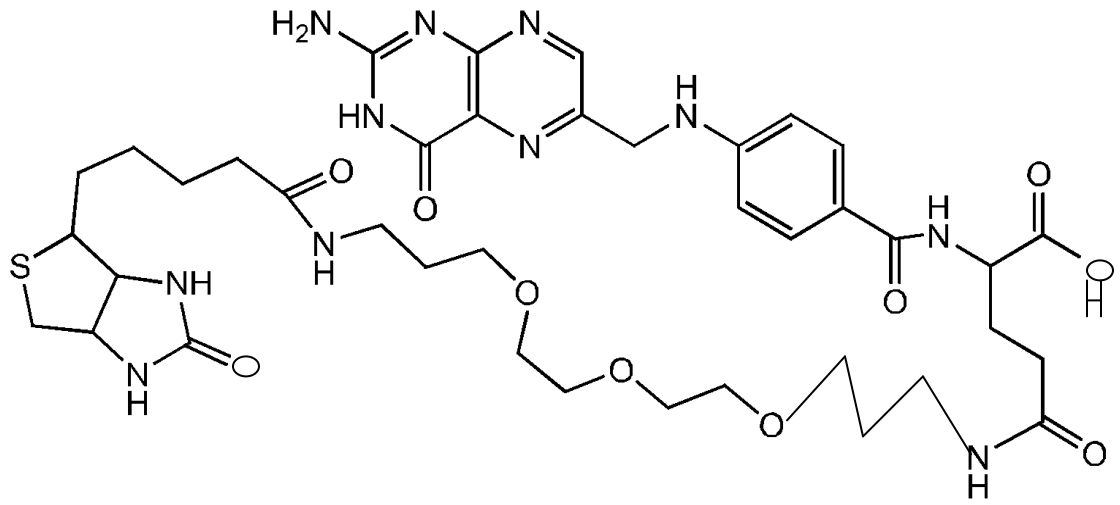
List of Figures

Figure 1: Structure of Folic Acid



folic acid

Figure 2: Structure of Folate-PEG-Biotin



Molecular Weight: 869.99

Figure 3: Folate Uptake Pathway

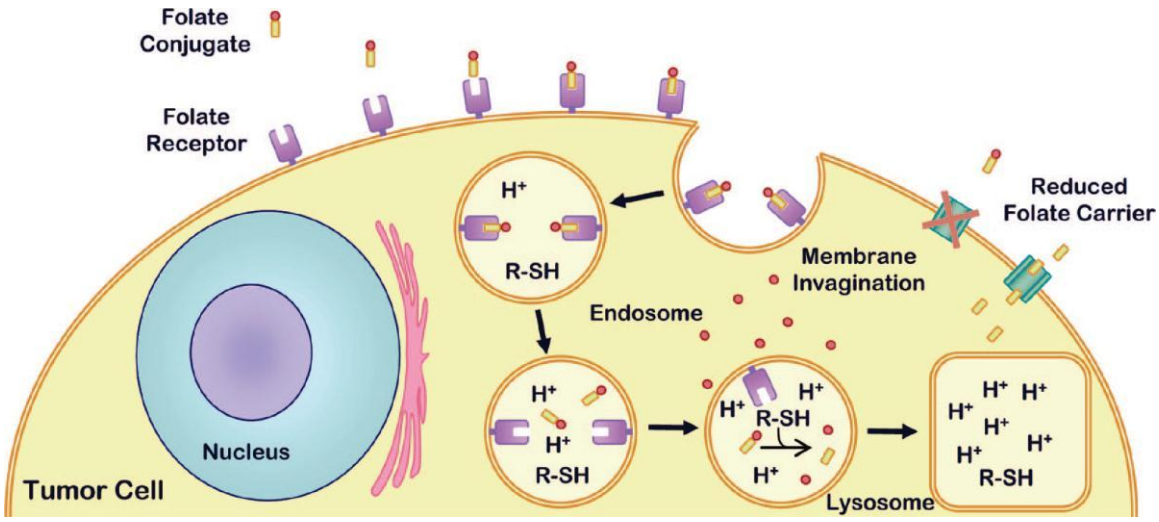


Figure 4: Chromatogram for Folate-PEG-Biotin

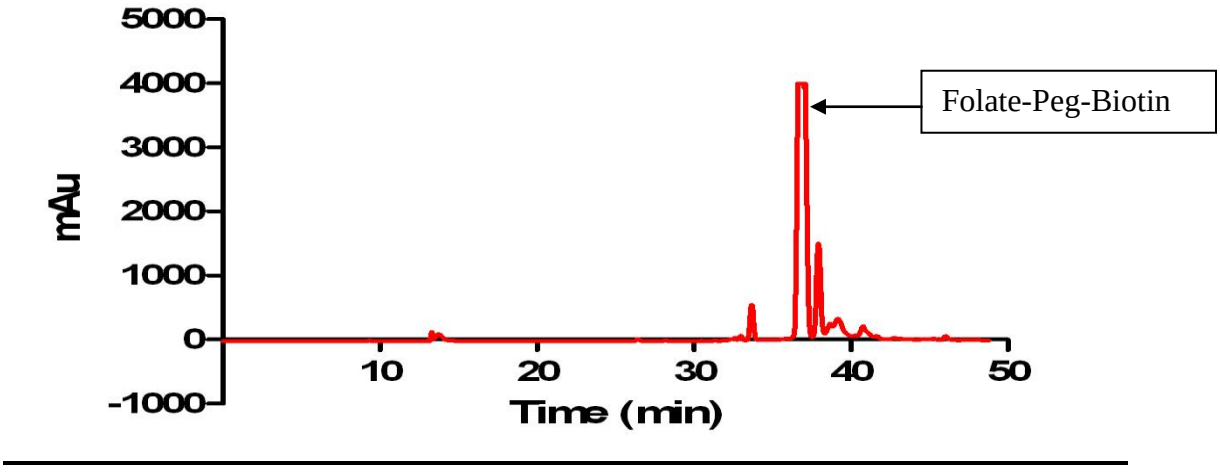


Figure 5: DAD Spectra of Folate-PEG-Biotin

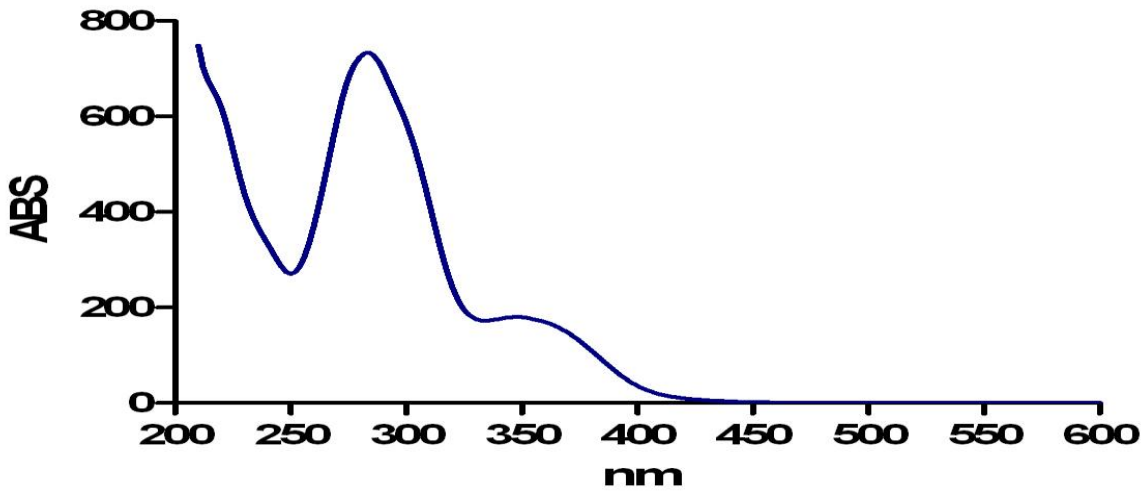


Figure 6: LC/MS Folate-PEG-Biotin

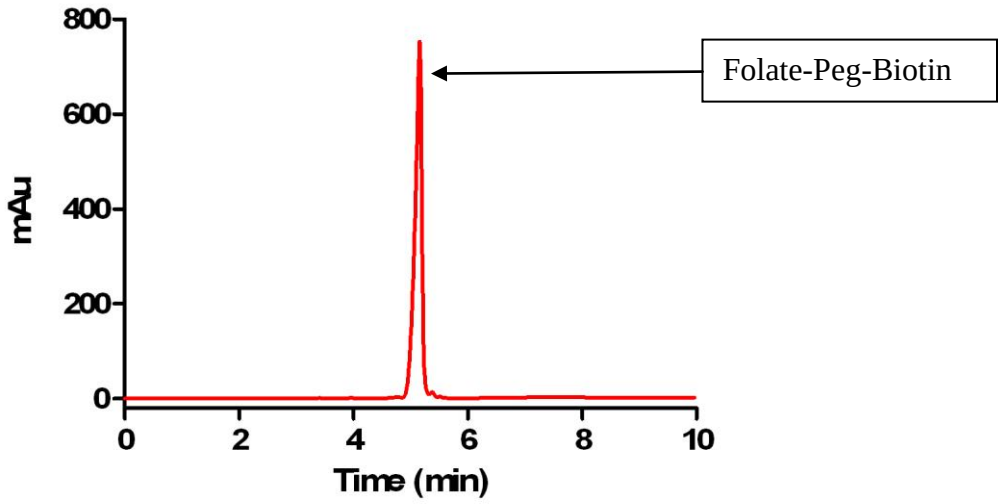


Figure 7: Folate-PEG-Biotin (Negative Ion mode)

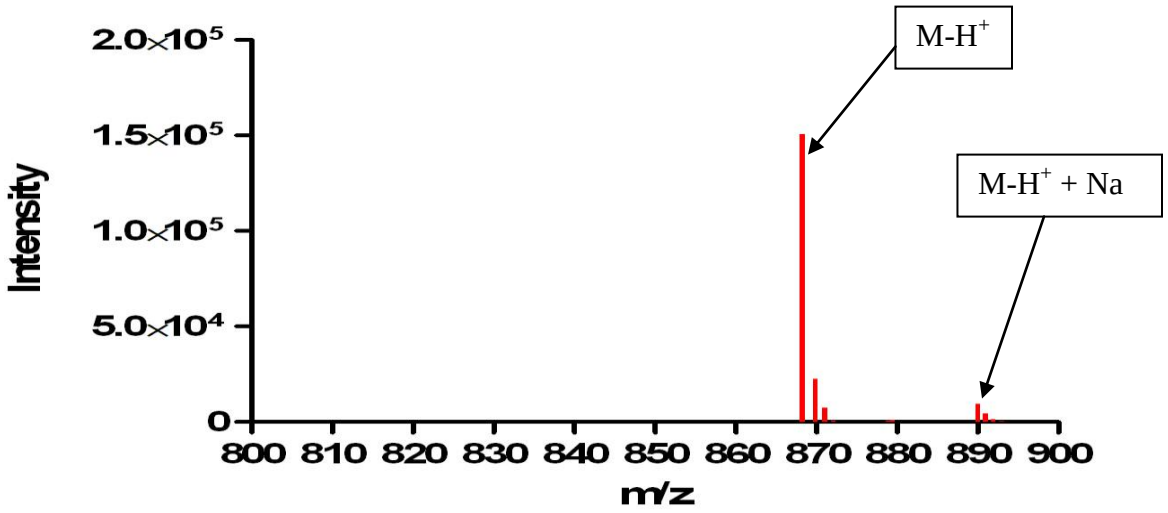


Fig 8: Diagrammatic Representation of Folate Based Cell Capture

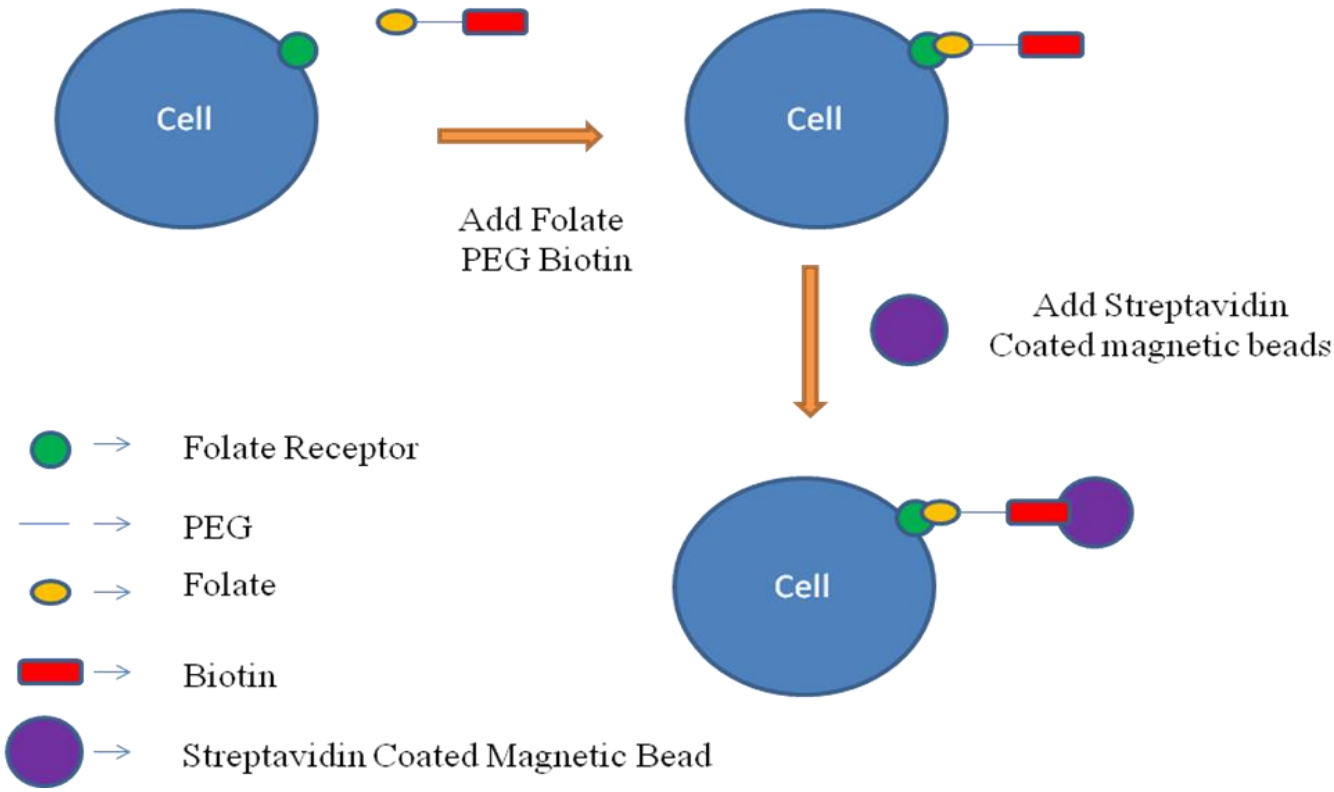
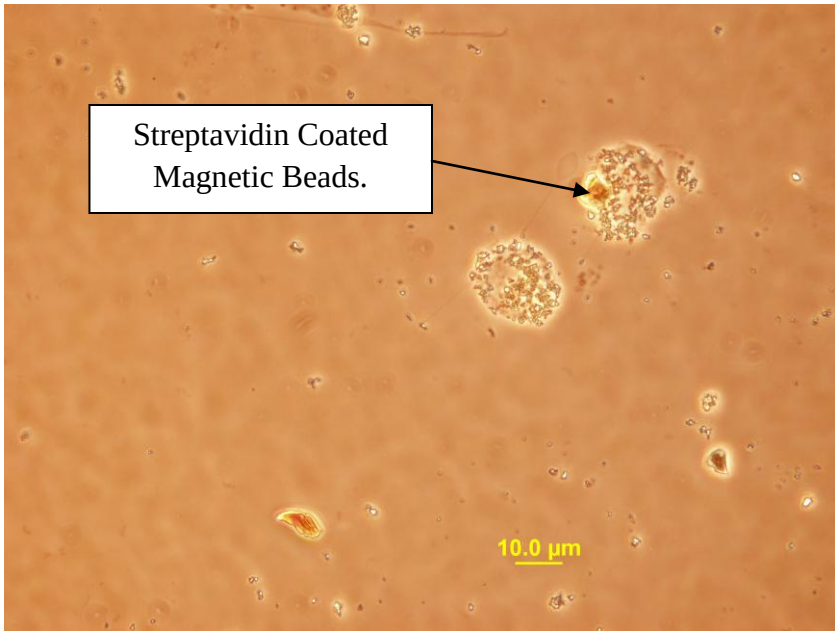


Figure 9: Cells Isolated by Folate-PEG-Biotin/Streptavidin Coated Magnetic Beads

a)



b)

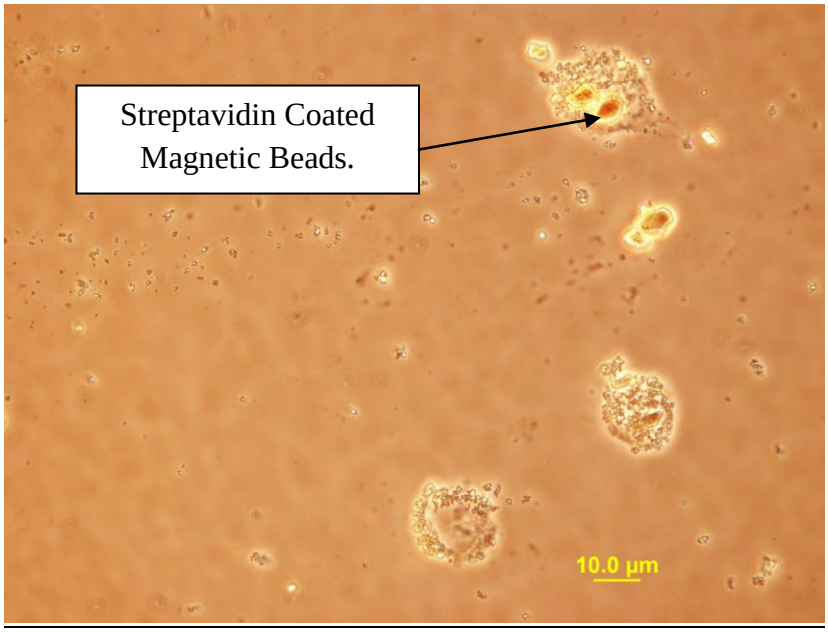


Figure 10: (Control) Cells with Streptavidin Coated Magnetic Beads Alone

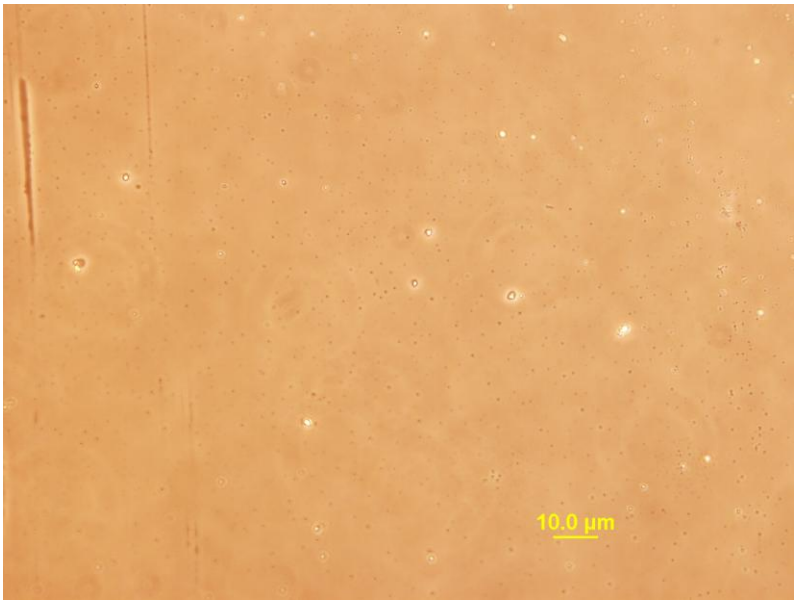


Figure 11: (Control-2) Cells Added with Folate-PEG-Biotin + Streptavidin Coated Magnetic Beads + 1000 fold Excess of Folic Acid