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Evaluation and In Vitro Studies of Folate PEG Biotin and other PEG Agents

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Evaluation and in vitro studies of folate PEG Biotin and other PEG agents

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Prepared by

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[redacted]

Dedicated to my Family, Friends, Professors, and all others who have encouraged my academic pursuits

Acknowledgements

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Abstract

Folate is a member of vitamin B family and plays an essential role in cell survival by participating in the biosynthesis of nucleic and amino acids. Receptors folic acid are frequently over expressed on epithelial cancer cells. These receptors are believed to serve as a receptor-mediated transport system of folic acid into cancer cells and cells associated with inflammation. Interesting, most normal cells in the body have lower frequency of these receptors. Based on these attributes, folate based drug delivery, imaging systems, and diagnostic systems are in several stages of development worldwide.

In this current project, we have characterized the affinity of the proprietary folate PEG-biotin-fluorescein- (FPBF) conjugate synthesized by Dr. Walter Henne. We have successfully purified and characterized FPBF capture ligand using LC/MS. Further, affinity studies of FPFB towards Streptavidin coated Dynabeads was carried out utilizing fluorescein microscopy. It was demonstrated that the conjugate has site specific interaction toward the Streptavidin coated Dynabeads, a much important characteristic for the effective cancer cell capture. The inexpensive and previously produced folate probe may be substituted for the more costly and cumbersome antibody based ligands, which are typically used for this method of drug delivery system to treat cancer. This method significantly reduces false positive events associated with non-specific binding and capture of non-targeted cells (a problem associated with the aforementioned affinity capture protocols).

Introduction

Folate receptor-targeted anti-cancer agent delivery:

Despite the fact that there have been significant developments in anti-cancer technology, such as radiotherapy, chemotherapy and hormone therapy, cancer still remains as the second leading cause of death following heart disease in the United States (1). Most often, the main cancer treatment is chemotherapy utilizing highly potent drugs, which include mitomycin, paclitaxel and camptothecin. In many cases, these chemotherapeutic agents show a doseresponsive effect, and cell kill is proportional to drug exposure (2). Highly aggressive style of dosing is thus necessary to eradicate neoplasms; however, high-dose chemotherapy is hindered by poor selectivity for cancer cells and severe toxicity to normal cells (3).

Clearly, this lack of tumor-specific treatment is one of the many hurdles that needs to be overcome by current chemotherapy. An ideal solution to current chemotherapy limitations would be to deliver a biologically effective concentration of anti-cancer agents to the tumor tissues with very high specificity. In order to reach this ultimate goal, tremendous amount of efforts were undertaken to develop tumor-selective drugs by conjugating anti-cancer drugs to hormones, antibodies and vitamin derivatives (4). Among them, one low molecular weight vitamin compound, folic acid, shows a great deal of promise as a tumor-homing agent.

Folate (Figure 1) is a member of vitamin B family and plays an essential role in cell survival by participating in the biosynthesis of nucleic and amino acids (5). This essential vitamin is also a high affinity ligand that enhances the differential specificity of conjugated anti-

cancer drugs by targeting folate receptor (FR)-positive cancer cells (6). The FR, a tumor associated glycosylphosphatidylinositol anchored protein, can actively internalize bound folates and folate conjugated compounds via receptor-mediated endocytosis (7, 8). It has been found that FR is up-regulated in more than 90% of non-mucinous ovarian carcinomas. It is also found at high to moderate levels in kidney, brain, lung, and breast carcinomas while it occurs at very low levelsin most normal tissues (9). The FR density also appears to increase as the stage of the cancer increases (10).

Exploiting the above-mentioned facts, it is hypothesized that folate conjugation to anti-cancer drugs will improve drug selectivity and decrease negative side effects. Based on the previous research that folate conjugation allows a drug molecule to target and become endocytosed into FR-positive cancer cells, numerous types of anti-cancer drugs were conjugated and evaluated for their biological activity (11). Particularly, folate-mitomycin C conjugates, EC72 and EC118, were found to be highly cytotoxic and outstandingly selective for FR-positive M109 cells (12, 13). In addition, EC72 and EC118 significantly extended lifespan of nu/nu mice with human KB xenografts without evidence of toxicity to major organs or delayed cumulative myelosuppression, the most common negative side effect of mitomycinC (14). Furthermore, combination therapy with paclitaxel produced a synergistic anti-tumor effect without any apparent adverse effects, suggesting a possibility of adjuvant use of folate conjugated drugs. Overall, performance of EC72 and EC118, both in vitro and in vivo, proves that folate conjugation enhances drug specificity thereby reducing lethal toxicity

Streptavidin- Biotin affinity Capture

Streptavidin is a tetrameric protein that binds biotin with an affinity that is among the highest displayed for noncovalent interactions between a ligand and protein (Ka 1013 M-1) (15). The x-ray crystallographic studies of Streptavidin by Weber et al. (16, 17) and Hendrickson et al.(18,19) have provided considerable insight into the structural origins of the high affinity of the biotin-Streptavidin system. The structure-function origins of this unusually high-affinity interaction, however, have yet to be elucidated. Streptavidin displays a number of commonly observed molecular recognition motifs in the interaction with biotin: these include hydrophobic and Vander Waals dispersive interactions that are largely mediated by the aromatic side chains of Trp residues (20-21), hydrogen bonding networks mediated by donor/acceptor side chains (22-23), and disorder-to-order transitions mediated by the ordering of surface polypeptide loops upon ligand binding (24-25).

Dynabeads Ligand Affinity Capture

The strength and specificity of the Streptavidin and biotin interaction has led it to be one of the most widely used affinity pairs in molecular, immunological, and cellular assays. Traditional methodologies for cell separation involve various forms of centrifugation and/or filtration. These techniques, while useful, have many drawbacks including low specificity without costly instrumentation development(26), high levels of sheer force leading to decreased number of active cells post separation, costly consumables, and multiple time consuming steps when dealing with complex matrices, such as whole blood(27). Modern techniques have been developed which utilize paramagnetic properties of metals. These techniques rely on the cells of

interest containing either intrinsic magnetic properties or are tagged with a magnetic label for separation (for a thorough review of magnetic cell separation techniques see reference 28). Of the two techniques, cell types containing intrinsic magnetic properties are extremely limited with red blood cells (erythrocytes) and magnetotactic bacteria being the only cell types existing in nature. Magnetic labeling, however, is limited only by the existence of an appropriate cell surface target and some form of affinity ligand with bound paramagnetic particle. After successful attachment of paramagnetic particles, cells can then be separated using either flow-through or batch magnetic separators.

Dynabeads® M-280 Streptavidin (Invitrogen™)

These uniform and super paramagnetic beads are 2.8 µm in diameter, with a monolayer – not a multilayer - of recombinant Streptavidin covalently coupled to the surface and further blocked with BSA. The monolayer of Streptavidin leaves the vast majority of the biotin binding sites sterically available for binding, not only of free biotin, but also for binding of biotinylated ligands/targets. They show rapid liquid-phase reaction kinetics. Their specific and defined surface allow for efficient capture, separation and downstream handling. The Streptavidin monolayer ensures negligible leakage, and the lack of excess adsorbed Streptavidin ensures batch consistency and reproducibility. The reduced particle variability would give reliable and reproducible results on which studies, diagnostic assays and therapeutic protocols may be established.

In this report, we describe the development of a folate PEG biotin fluorescein (FFBF), Figure 3, its purification using Preparative HPLC, its affirmation by LCMS and its site-specific affinity behavior towards Dynabeads. The fluorescein fluorophore in the folate PEG biotin chain was exploited for assessment and confirmation of the site-specific nature of the ligand and beads interaction via a secondary detection scheme (i.e. fluorescence microscopy). This principle can be extended for isolation and detection of folate receptor positive cancer cells. This method significantly reduces false positive events associated with non-specific binding and capture of non-targeted cells (a problem associated with affinity capture protocols).

Materials & Methods

Preparative HPLC of Folate-PEG-Biotin Fluorescein

The proprietary Folate- -PEG -Biotin Fluorescein molecule prepared by Dr. Walter Henne was dissolved in DMSO and centrifuged for 10 minutes. The preparative HPLC was then carried out with a Hewlett Packard, series 1050 HPLC equipped with a Diode array detector and ChemStation software.

The following instrument and method parameters were utilized:

Column	Rigel 5µm C18 10X250mm from Stellar Phases Inc. (cat.#: 5C18-10-250)
Solvent A	10 mM Ammonium Bicarbonate Buffer (NH_4HCO_3)
Solvent B	Acetonitrile (ACN)
Flow Rate	1ml/min
Run Time	100 min

- Gradient:

Time (min)	**NH_4HCO_3 (%)**	**ACN (%)**
0	99	1
30	70	30
35	40	60
40	10	90
45	10	90
60	99	1

LC/MS Analysis

The purified FPBF sample was then analyzed using an Agilent 1100 series LC/MSD Trap for identity and purity confirmation. The instrument uses Agilent ChemStation and Bruker software.

The following instrument and method parameters were utilized:

Column	Eclipse XDB C18
Solvent	Methanol and Water
Injection volume	5µl
Flow Rate	0.5ml
Scan Range	500-1500 m/z
Mode	Negative ion

Gradient:

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

Lyophilization:

The purified compound was lyophilized to get rid of the solvent (DMSO) used for preparative HPLC. The vial with FPBF was frozen in liquid Nitrogen tank. With a porous cap, it was then subjected to high vacuum conditions in High vacuum refrigerated conditioning trap (SAVANT). The setup was kept undisturbed overnight and the solvent was sublimed.

Affinity studies of FPBF towards Streptavidin Coated Magnetic Beads

- Dynabeads® M-280 Streptavidin manufactured by Invitrogen™ -One mg of Dynabeads® M-280 Streptavidin typically binds 650-900 pmoles free biotin.
- Phosphate Buffered Saline: 1X from Cellgro (Mediatech, Inc) without Calcium and Magnesium, Cat. No. 21-040-CV

Purified Sample of FPBF was dissolved in PBS to produce 12µM concentration of ligand. To 10µL of this, 30µL Dynabeads were added and the final volume was made up to 280µL using PBS. The mixture was homogenized using HulaMixer™ for 10 minutes for uniform binding and allowed to stand in a magnetic stand for 10 minutes. After washing the beads and captured FPBF with PBS three times, small amount of sample was spread on the cover slide and observed under Fluorescence Microscope.

A control slide was also prepared with a competent Folate PEG biotin (96µM) incubated with Dynabeads prior to addition of FPBF sample. Similar steps of incubation, mixing and washing were performed. The sample and control were prepared at the same time to maintain accuracy with respect to time and working conditions.

Results and Discussion

The chromatogram(figure 4) obtained from the Preparative HPLC (Hewlett Packard series 1050 HPLC) showed a sharp and intense peak at 29.40 minutes monitored at 365nm wavelength. The other small and irregular peaks at 27.5, 28.0, 29.0,30.5,31.0 and 32.0 minutes are from impurities and the intense, sharp peak at 29.40 minutes is due to pure FPBF, which was later verified by diode array and LCMS.

Further, the peak at 29.40 minutes was coordinated and the purified FPBF was collected to be analyzed by Agilent Technologies, 1100 series LC/MSD Trap for identity confirmation. The diode array response (LC chromatogram- figure 5) showed a prominent peak approximately at 5.40 minutes monitored at 280nm. The presence of only one peak was confirmation of the purity of the compound.

A diode array spectrum was scanned at retention of 5.40 minutes to confirm folate peak. The diode array spectrum gave rise to a typical double event with maximum absorbance at approximately 280 and 360 nm, which is indicative of folate (Figure 6). In addition, a typical peak associated with fluorescein was observed at approximately 480-490 nm (Figure 6). Combining data from HPLC and DAD leads to confirmation of a relatively pure compound.

Total ion chromatogram showed a peak at 5.3-5.70 minutes (Figure 7). Mass spectrum of this retention time showed a single molecular ion peak of FPBF at m/z = 1384.8 and doubly charged ionic peak at m/z = 692 (Figure 8). The calculated molecular mass of FPBF is 1386.6

Da. Since the run was in negative ion mode, the observed peaks in the mass spectrum correspond to $M^{*} - H^{+}$ and $M^{**} - H^{+}$ ion species.

Affinity studies of FPBF with the Dynabeads showed a colony of well-distributed beads (Figures 8A, 10A) under Light microscopy. However, the fluorescence Microscopy showed the fluorescence of the beads in the sample but not in the control (figures 9B,10B). The affinity of the FPBF towards the Dynabeads can be confirmed as site specific as there is no fluorescence in control, which was incubated with FPB competitor prior to addition of FPBF (figures 9B, 10B).

Conclusion

Folate PEG biotin fluorescein was purified by Preparative HPLC and characterization of the proprietary FPBF yielded a high purity compound with confirmed components of folate and fluorescein accompanied by structural confirmation via mass spectrometry. The affinity studies of Dynabeads and purified Folate PEG biotin fluorescein aided by Fluorescence microscopy, successfully demonstrated that the conjugate has site specific interaction toward the Streptavidin coated Dynabeads, a much important characteristic for the effective cancer cell capture. The inexpensive and previously produced folate probe may be substituted for the more costly and cumbersome antibody based ligands, which are typically used for this method of drug delivery system to treat cancer

Future Studies

Further study of FFPB in the realm of characterization should include additional structural confirmation utilizing 1H-NMR, 13C-NMR, X-ray diffraction, positive ion-mode mass spectrometry, differential scanning calorimetry, and thermogravimetric analysis. Due to the success of FPBF and its affinity characteristics, cell capture studies can be performed which might replace the expensive antibody mediated cell capture.

Funding

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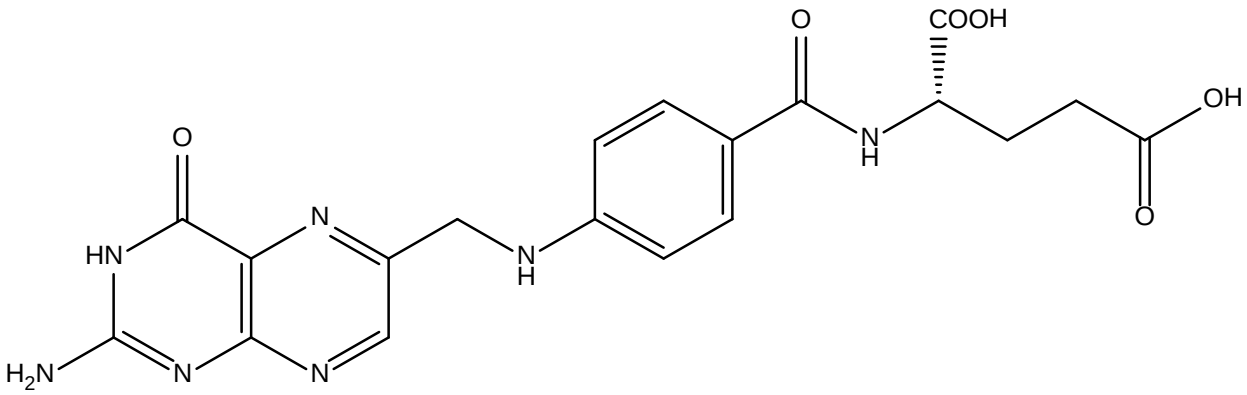


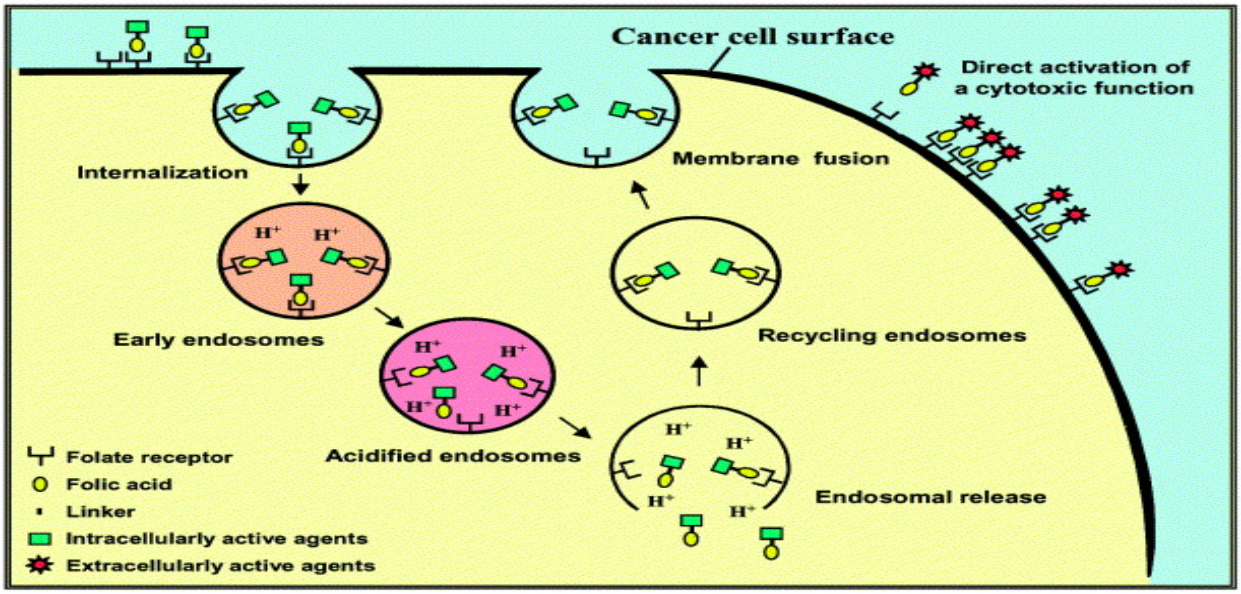
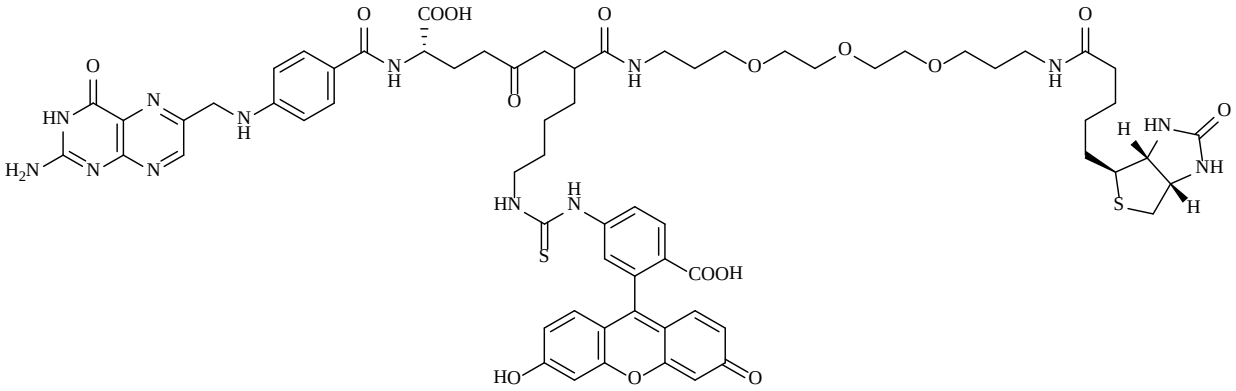


Figure 3: Folate-Fluorescein-PEG-Biotin Bridging Ligand Structure (drawn using ChemBioDraw Ultra v. 11.0.1)



Chemical Formula: $C_{67}H_{79}N_{13}O_{16}S_2$
Molecular Weight: 1386.6

Figure 4: Preparative LC chromatogram for Folate- PEG-Biotin- Fluorescein at 280 nm

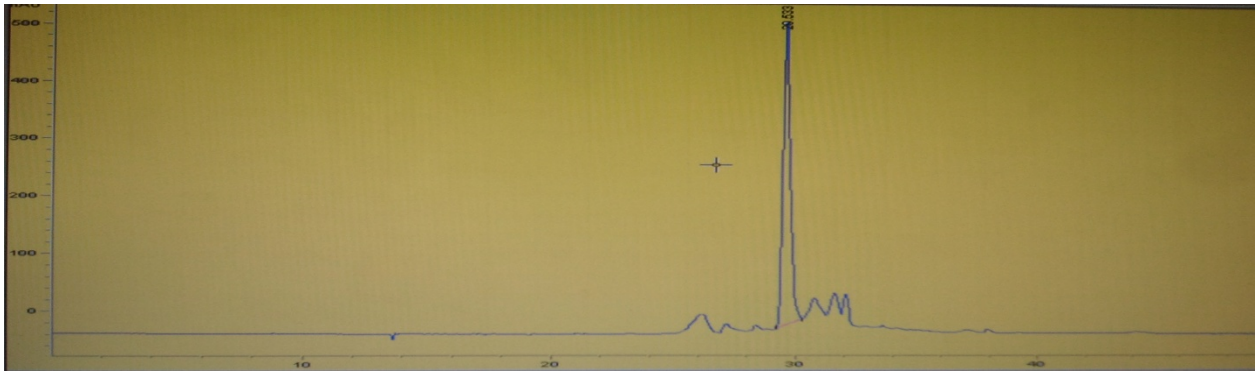


Figure 5: LCMS analysis (LC chromatogram at 280nm)

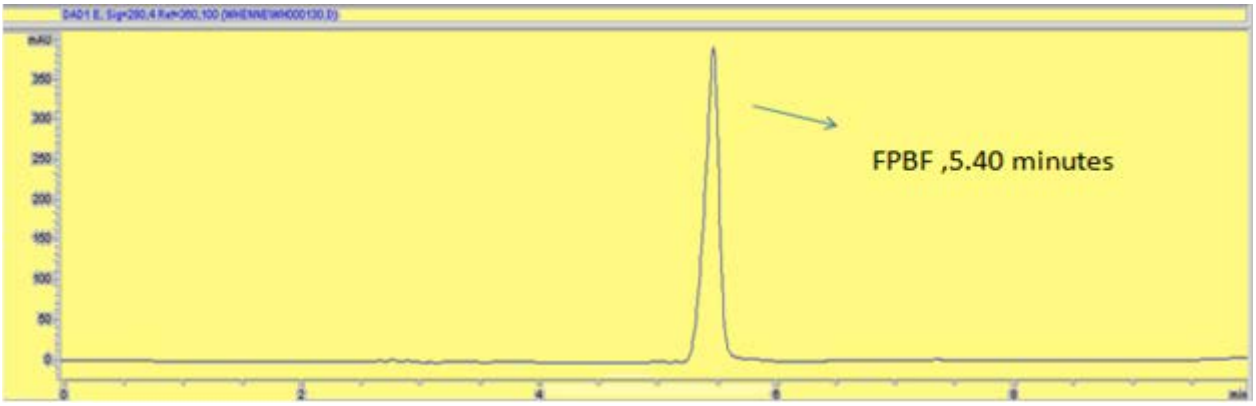


Figure 6: Diode Array Spectrum at 5.40 minute Retention time

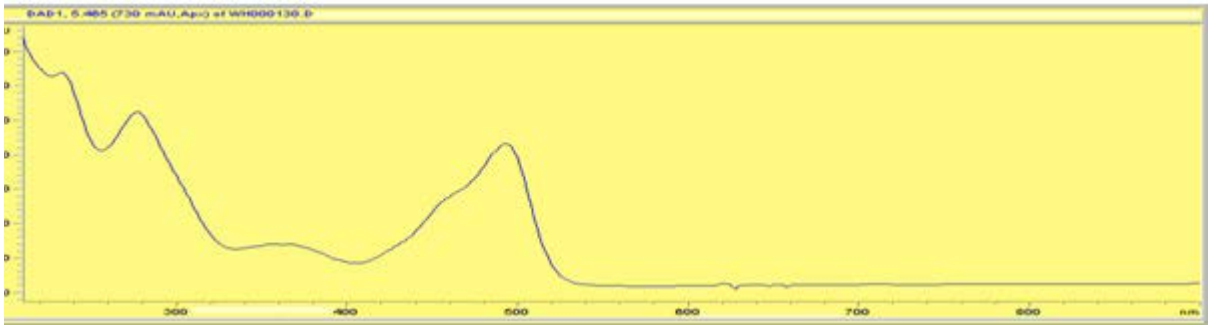


Figure 7: LCMS analysis (Total ion chromatogram)

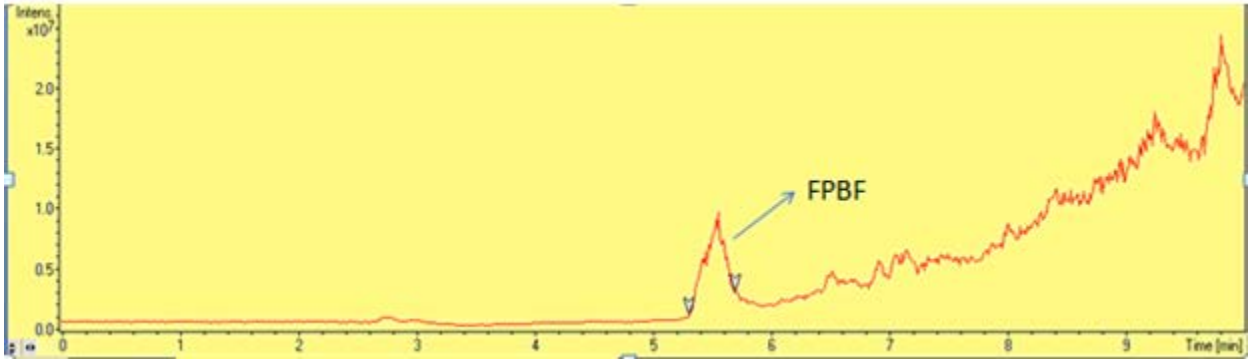
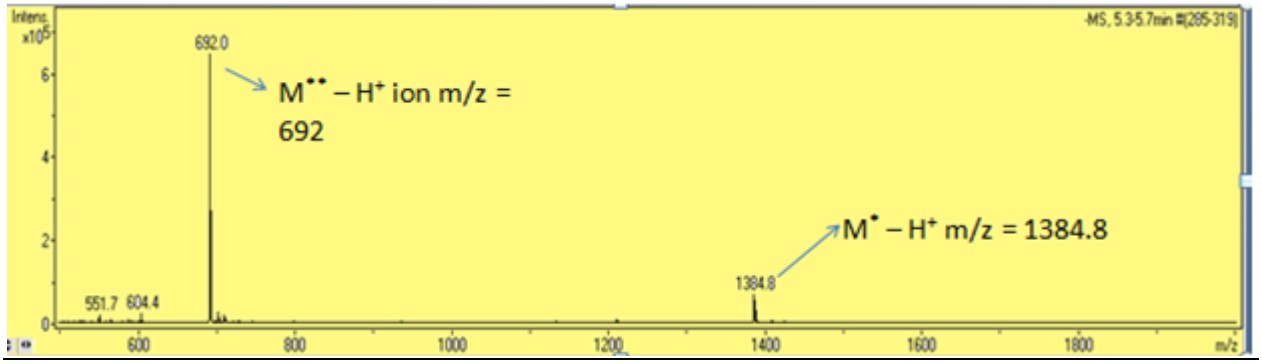
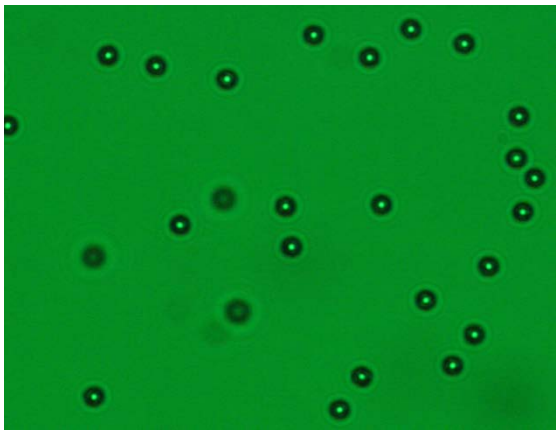


Figure 8: Mass Spectrum for Folate-Fluorescein-PEG-Biotin (Negative Ion mode)

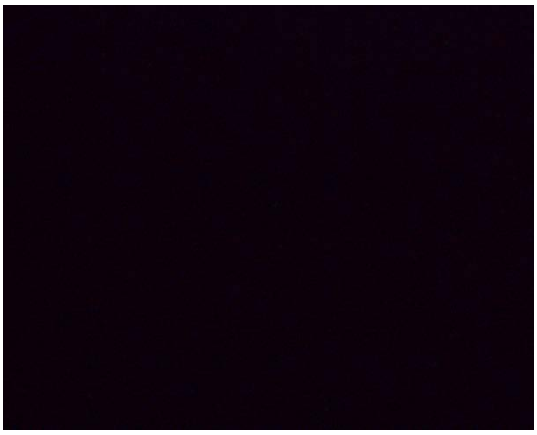


Figures 9 and 10: Dynabeads Affinity Comparison of FPBF without and with Folate- PEG – Biotin(control)

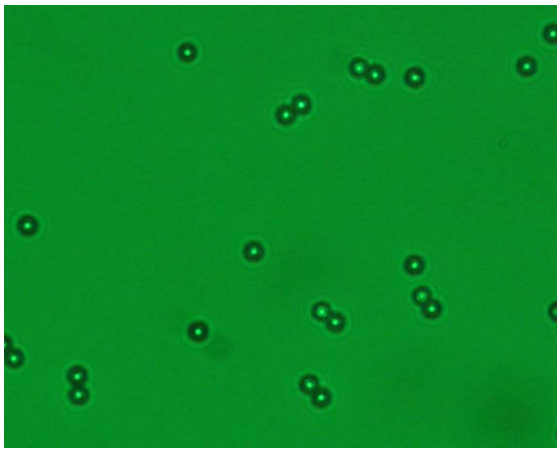
9A) Light Microscopy (control)



9B) Fluorescence Microscopy (control)



10A) Light Microscopy (sample)



10B) Fluorecence Microscopy (sample)