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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

A Project Submitted

To

Governors State University

By

Kasarala Vikram Reddy

Masters in Science

December 2011

Governors State University

University Park, Illinois

Dedicated to my **Family and Friends**

Acknowledgements

I would be very grateful to Dr. Henne who had advised and guided me in carrying out this research by supplying me with all the necessary equipment's without which this project would be unsuccessful. His biochemistry class that I took was one of the main reasons to choose this project which was very motivating and included lot of challenge.

I also feel glad for the help provided by my friends Gopi Krishna, Kuldeep Reddy and my family who had supported me in every aspect of my life.

And lastly I would like to thank my professor Dr. Ramalingam who had aggravated my interest to study masters in Chemistry at one of the reputed institutions with necessary instrumentation for which I had to choose Governors state university.

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Abstract

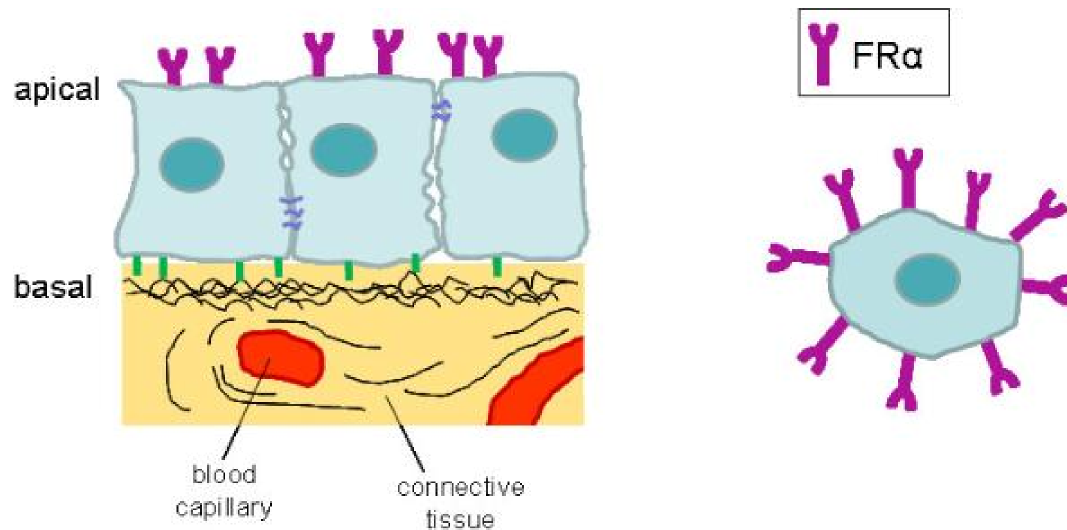
Folate is an essential component for cell growth and cell division, playing a major role in DNA synthesis. It is common sense that the rapidly dividing cells synthesize more DNA, including cancer cells. Also, it was reported that cancer cells over express Folate receptors [1] and this was the basis for our probe for cancer cells. We used long PEG biotin in conjugation with Folate. Previous studies in our group showed that the PEG could be used in the sensor to detect and capture cancer cells. The present study was aimed at evaluating the effect of Long PEG containing Folate based biotin probe in capturing cancer cells from solution, which will be the sensing portion in immune polymerase chain reactions (PCR) detection scheme. Here we report an extremely facile, cheap and rapid method of coupling long PEG with Folate and Biotin in comparison with those antibody based conjugation techniques. The synthesis of the probe was based on standard solid phase FMOC synthesis[2-3] using a conjugatable long PEG biotin scaffold a simple and a 3-4 step process using a simple syringe based method. The probe, after preparation, was purified and characterized using HPLC diode array analysis, and LC-mass spectrometry. Future studies will include high-resolution fluorescent imaging to show the probe's selective binding to cancerous cells like FR-alpha positive cells utilizing fluorescently labeled streptavidin. Biotin-avidin pair has the strongest known affinity $K_d = 10^{-15}$ almost equivalent to chemical bond [4]. Wherever appropriate, folic acid competition experiments and alternative cell lines devoid of FR alpha receptors will be used as controls to demonstrate selective and binding affinity. Ultimately, we intend to perform real-time PCR studies with FR-alpha protein standards. These latter aims will involve additional student research projects.

Introduction

Folate receptors

Glycosyl-phosphatidylinositol (GPI)-anchored folate receptors (FR) have physiologic and pharmacologic relevance in mediating cellular and transcellular folate/antifolate transport; three FR isoforms with differing relative affinities folates[5]. Functional isoforms of FRs are anchored to the membrane by a glycolipid anchor, the glycosylphosphatidylinositol (GPI) anchor [6]. Folate is necessary for DNA synthesis, repair, and methylation, and biological reactions involving folate require vitamins B6 and B12 as cofactors. Low concentrations of these vitamins can impair one-carbon metabolism pathways [7], leading to homocysteine accumulation, insufficient methyl groups for DNA methylation, or depletion of DNA synthesis and repair precursors, thus, potentially promoting carcinogenesis [8].Of the three isoforms of the folate receptor two, the most common ones are FR alpha and beta. FRα (also called folate receptor 1, or FOLR1) is overexpressed by epithelial tumors and FR beta (also called folate receptor 2 or FOLR2) is overexpressed by non-epithelial tumors FRα has a high affinity for folic acid (Kd ~ 1 nM). FRα cells occur mostly in non-cancer tissues and are exclusively expressed in tissues of epithelial origins, with limited distribution to the kidneys, lungs, choroid plexus, and placenta. The receptors in these tissues, except the placenta, are localized in the apical membrane facing away from the blood (toward urine, and airway) (Figure1). This makes FRα inaccessible to the Folate conjugates administered intravenously and intraperitoneally. However, epithelial cancer cells that express FRα lose this polarity, so FRα becomes expressed all over the cell surface, and at high levels, which make FRα accessible intravenously. All this makes FRα an ideal candidate for therapeutic targeting on cancer cells [9].

Figure 1



Long PEG

Long PEG is also called PEG 3350, with a molecular weight of PEG being 3350. Below 55°C, it is a free flowing white powder freely soluble in water. It is generally used as an osmatic agent that makes its use possible as an immobilizing agent. Polyethylene glycol is being widely used to bioconjugate with targeting agents like folate[10] and other drug delivery systems like liposomes[11] to be delivered to the brain. The main reason for PEG's wide application is because it's less toxic LD50; in mice it was > 4000mg/kg body weight [12].

Biotin-Avidin

Avidin is a tetrameric biotin-binding protein. The tetrameric protein contains four identical subunits (homotetramer), each of which can bind to biotin (Vitamin B7, vitamin H) with a high degree of affinity and specificity. The dissociation constant of avidin is measured to be KD ≈ 10−15 M, making it one of the strongest known non-covalent bonds

[4]. Hence, our strategy to probe cancer cells with fluorophore attached streptavidin using biotinylated probe was developed.

Why develop Folate PEG Biotin?

There are several methods reported to detect low levels of a free Folate receptor: QCM biosensors, SPR biosensors, radio-labeled binding studies and immuno-magnetic diffraction detection.[13][14][15]Although highly sensitive, these methods suffer from several major drawbacks including the cost, lack of commercially available high throughput systems, scale up problems and the use of antibodies[15] and radioisotopes which decrease the shelf life of the probes and diminish the potential for being incorporated into point of care operations. Hence the anti bodies were replaced by the folate which makes it specific as well as cost effective.

Materials

Biotin PEG nova teg resin was obtained from Novabiochem, with the loading of 0.2-0.5 mmole/gm.

Dimethylformamide (DMF) (99.9%) was obtained from Aldrich with Molecular Weight: 73.09gm/mol.

20% piperidine (99.9%) Molecular Weight: 85.15gm/mol.

Glutamic acid was obtained from Nova bio-chem ($C_{24}H_{27}NO_6$) Molecular Weight: 425.5gm/mol.

N, N -Diisopropylethylamine (DIPEA) [$(CH_3)_2CH]_2NC_2H_5$] (99.5%) was obtained from Aldrich, Molecular Weight:129.24gm/mol.

HATU ($C_{10}H_{15}N_6OPF_6$) was obtained from Aldrich with Molecular Weight: 380.3gm/mol.

N10 (trifluoroacetyl) ptreroic acid was obtained from Aldrich, Molecular Weight: 408.3gm/mol.

2%hydrazine was obtained from Aldrich (batch # 08504CJ) Molecular Weight: 32.05gm/mol.

Dichloromethane (DCM) (99.6%) was obtained from Aldrich Molecular Weight: 84.93gm/mol.

Trifluoroacetic acid (TFA) (99%) was obtained from Aldrich Molecular Weight: 114.02gm/mol.

Methods

Method for long PEG Biotin[15] (see figure 9 for complete synthetic scheme):

PEG biotin resin Swell PEG biotin resin with 20% piperdin /DMF for 20 min,

Add 20% piperdin /DMF 3 to 5 times, every step takes 5mim.

Wash with DMF for 3 to 5 times.

Add PEG (0.082) + HATU (0.062) +DIPA with DMF, Place for overnight reaction Wash with DMF for 3 to 5 times, Wash with 20% piperdin/DMF 3 to 5 times.

Add F-glutamic acid (0.045gm)+HATU (0.067gm)+DIPA (0.062ml), Wash with DMF for 3 to 5 times, Wash with DCM for 3 times, Wash with Methonal for 3 times.

Dry resin in nitrogen gas, wash with DMF 3 times. Swell in DMF for 20 min, Add 20% piperdin /DMF 3 times, every step takes 5min, Wash with DMF 3 times

Add Pteroic acid (0.0216gm) + HATU (0.067gm) + DIPEA (0.062ml), Place for over night reaction, Wash with DMF 3 times. Add 2%hydrizine, Wash with DMF 3 times, Wash with DCM 3 times, and Wash with Methonal 5 times

Dry with nitrogen gas for 1 hour dry under high vacuum

Analytical instrumentation

Rigel HPLC C18 column (sr#9-122A) 5um was used for this preparative HPLC. The dimensions were 10x250nm for purification and column with dimensions 3.5um, 4.6x150mm for separation zorbax and ammonia buffer with pH 7 which was made by adding 0.9 gm of ammonium carbonate into 1L of water. The HPLC used was Hewlett Packard, series 1050 with DAD.

Methods:

The method for HPLC:

- SOLVENTS a) 10mM NH_4HCO_3 b) Acetonitrile
- Flow rate: 1mL/min
- Run time: 60 min

No.	Time (min)	%B
1	0.0	1.0
2	40.0	30.0
3	50.0	60.0
4	55.0	80.0
5	56.0	99.0
6	65.0	99.0

The column used for LCMS was XDB C^{18}. The positive ion mode for mobile phase was 0.1% formic acid, ACN and water.

- Flow rate: 0.5mL/min
- Stop time: 10 min
- Scan range: 600- 1000m/z
- Sample : 20ul

Results and discussion

Analysis of Compounds by HPLC

The probe PEG-biotin-Folate was characterized by standard reverse phase chromatography and the probe was separated and eluted at a 32.6 minutes peak was sharp and was characteristic of Folates in the literature which was also compared and confirmed with the DAD (diode array detector) spectra for the Folate and that agreed very well.

The elution time Folate peg biotin was 32.6 minutes. Once we knew the elution time we collected the sample at that particular time, concentrated the sample and raised its purity enormously using the preparative column {Rigel HPLC column (5 um,10x 250mm)} with larger amounts for LCMS. The probe that was collected from the preparative HPLC was introduced into LC-MS as there were no major impurities and measured on the plate reader to determine the final concentration which was found to be about 100 ppm. DAD analysis of the same peak suggested that the purified probe showed absorbance maxima's at 284 & 350 nm confirming the presence of Folate in the probe.

Analysis of Compound by LC-MS

Identities of the relative structures obtained from the LC-MS data were predicted using chemdraw data (figure 6-10). From the mass spectroscopic data of positive ion modes yielded similar results i.e. 870 (M+1) respectively which were consistent with calculated values Folate short PEG Biotin. They agreed very well with literature value for the probe i.e.869 daltons. A second peak corresponding to a molecular weight of 892 & 890 (M±1+Na+) was noted. The occurrence of this band was possibly due to Na+ in the water supply or an impurity in any of the solvents Aside from this, there were no other contaminants in the system. Test for the additional impurities were performed this was done using TIC (total ion chromatography), which gave 2x106 ion counts that pretty well matched with the peak for the Folate. No additional peaks were found confirming absence of any cross-contamination.

Conclusions

The Short PEG-BIOTIN-FOLATE probe was successfully synthesized using FMOC synthesis in a simple rapid and cost effective method. The probe was successfully characterized using LC-MS and was confirmed using DAD analysis .The separation and purification of Folate Short-PEG-biotin was quick and easy when RP-C18 preparative column chromatography (reverse phased) was used. Analysis using the Photoplate reader (UV/VIS), apart from confirming the identity of folate made quantification also possible. LC-MS confirmed the molecular weight of the compound at positive ion mode at m/z 868 & 870 (actual 869) respectively with no cross contamination in the purified sample. Na+ adducts additionally confirmed the structural compound identity.

Long PEG-Biotin is unsuccessfully synthesized using FMOC Synthesis.

Funding Acknowledgements

This research was funded considerably by the Governor's State University and also, Dr. Walter Henne. The HPLC series 1050, preparative columns, micro pipettes, etc items were provided sufficiently by Walter Henne.

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Synthetic scheme and figures

Figure 2: Vortexer (Shaker) for synthesis (overnight reaction)

Figure 3: Sample after dried under high vacuum

Figure 4: Sample before purification

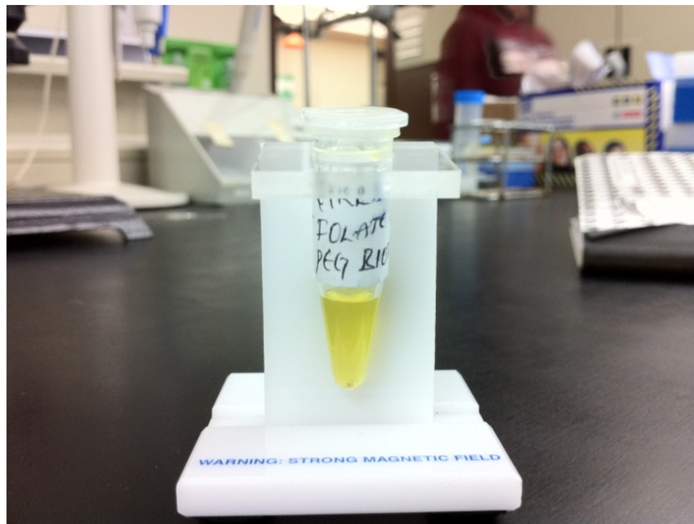


Figure 5: High peak at 32.6min on HPLC (purification)

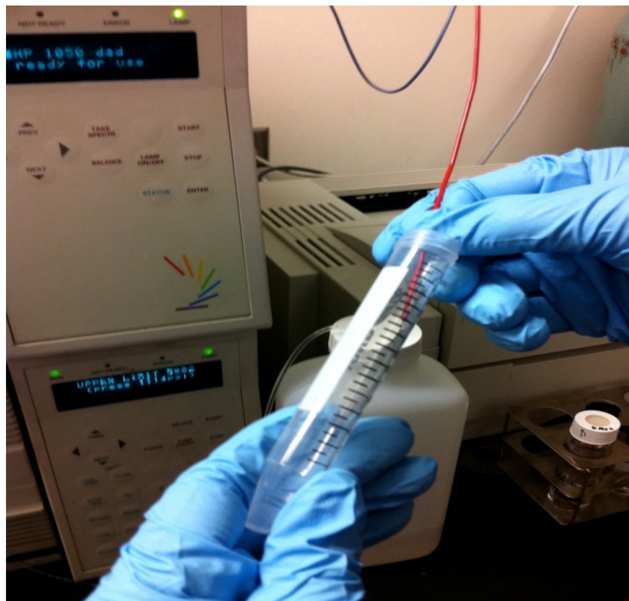


Figure 6: Folate spectra (DAD)

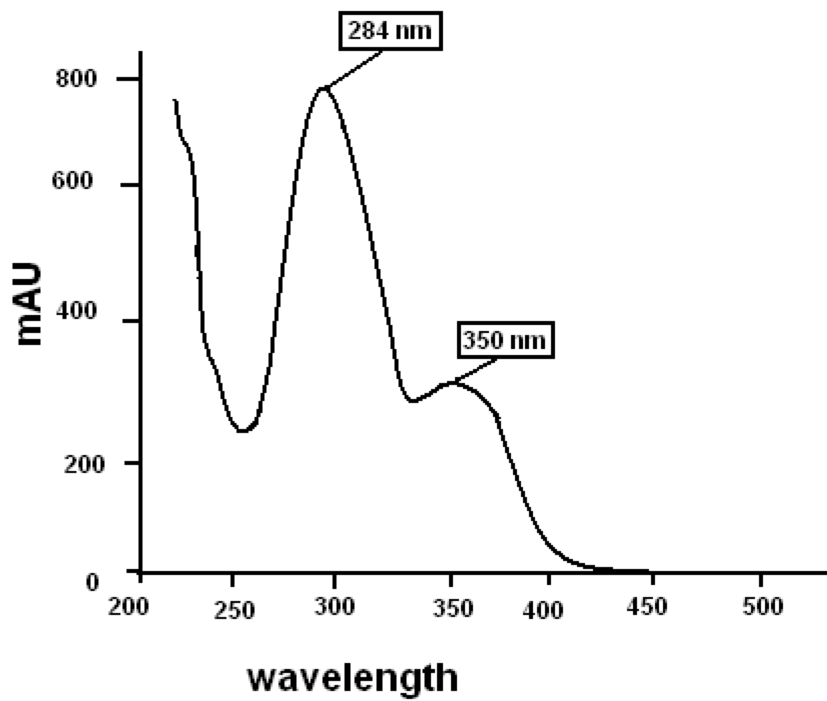


Figure 7: HPLC chromatogram of Folate Long PEG Biotin (Abs 280)

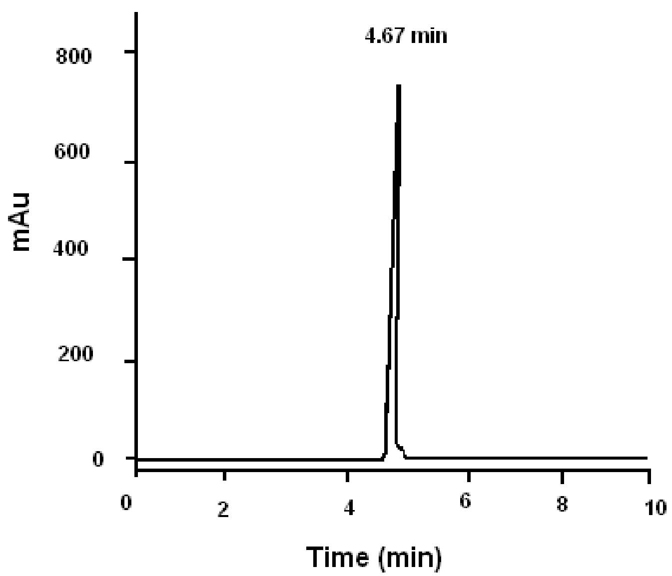


Figure 8: Mass spectrum of Folate Short PEG Biotin in positive ion mode

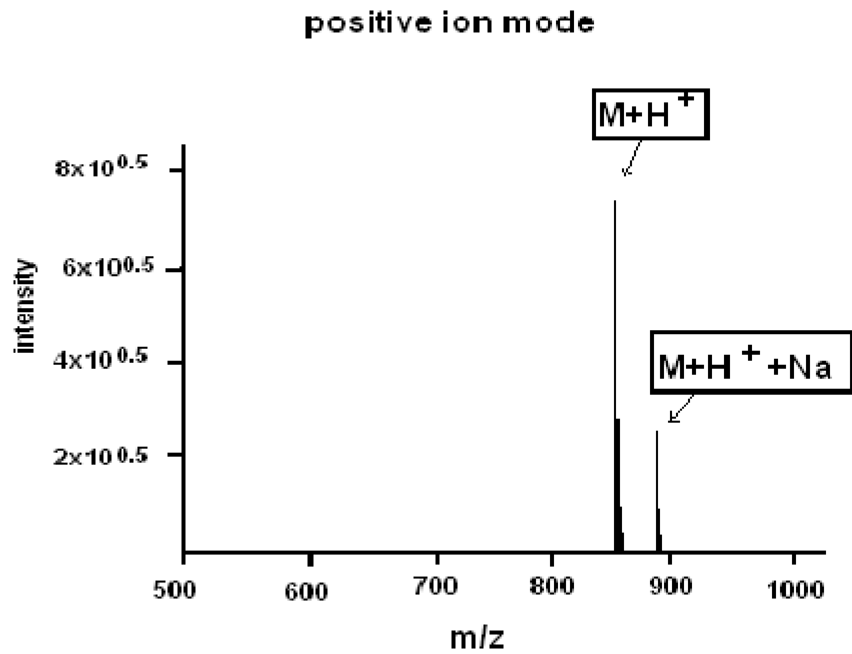
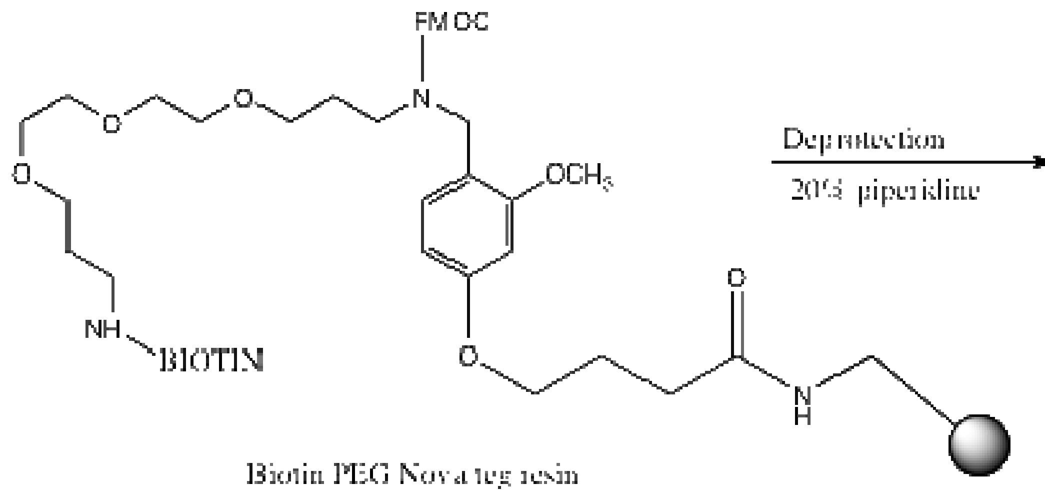
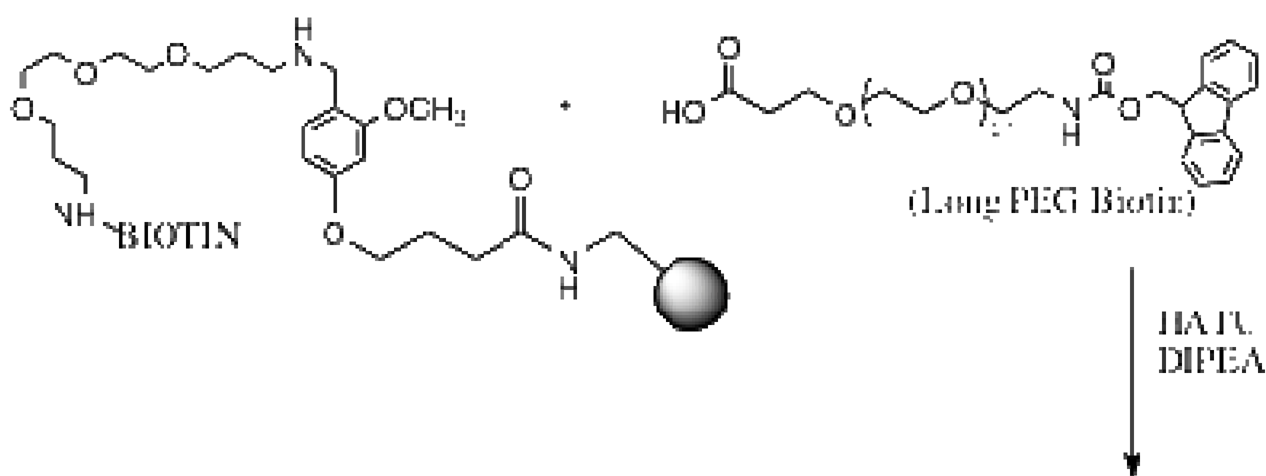
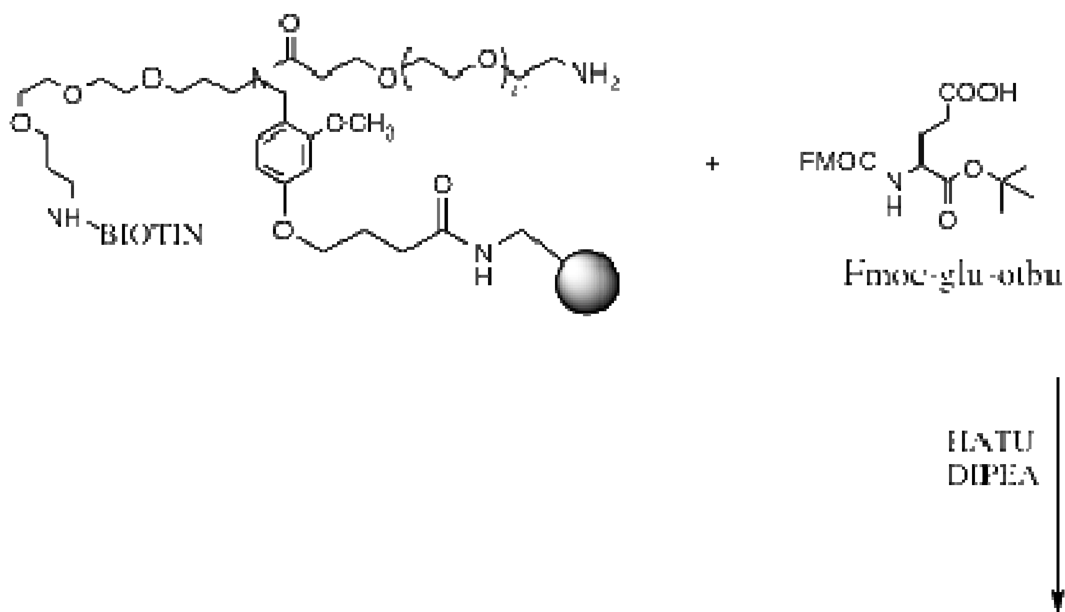
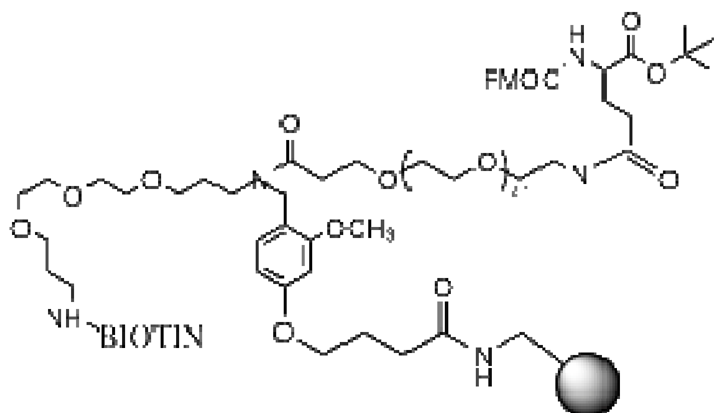


Figure 9: Synthesis scheme of Folate Long PEG Biotin

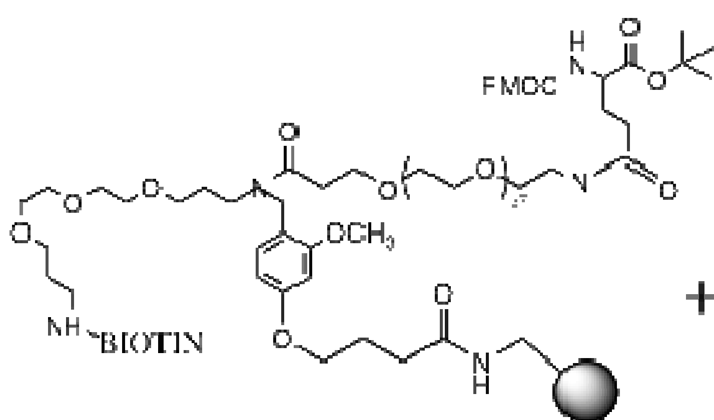



NH
BIOTIN
OCH3
HO
(Long PEG Biotin)
HATU
DIPEA

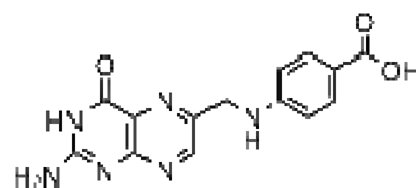

NH
BIOTIN
OCH3
NH2
COOH
FMOC
Fmoc-glu-otbu
HATU
DIPEA



FMOC Deprotection
20% piperidine

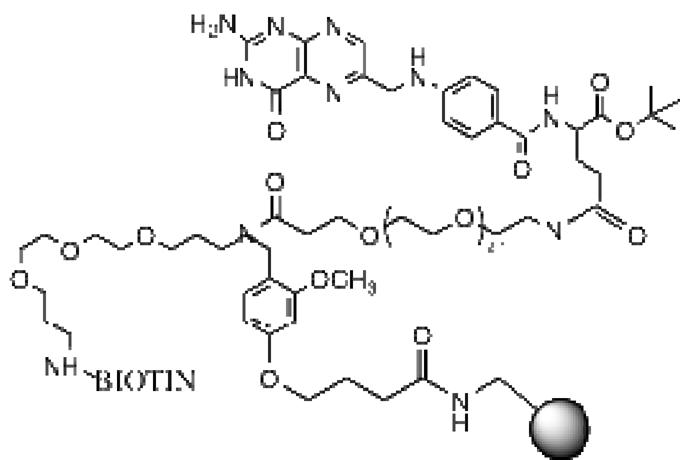


+



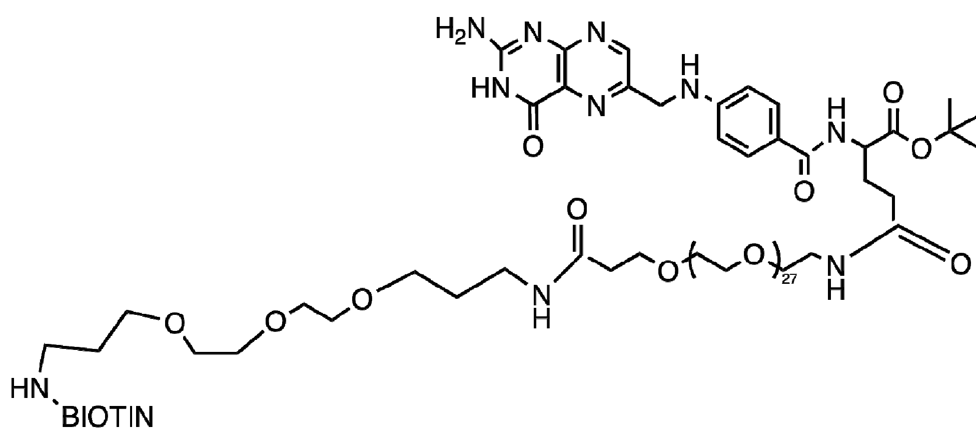
1) N^{10} (trifluoroacetyl) pteroic acid
HATU, DIPEA

2) 2% Hydrazine

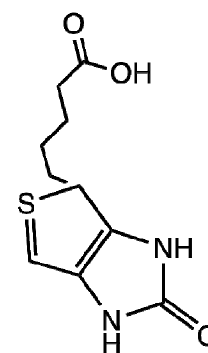


Cleavage

(92.5% TFA.2.5% H_2O. 5% TIPS)

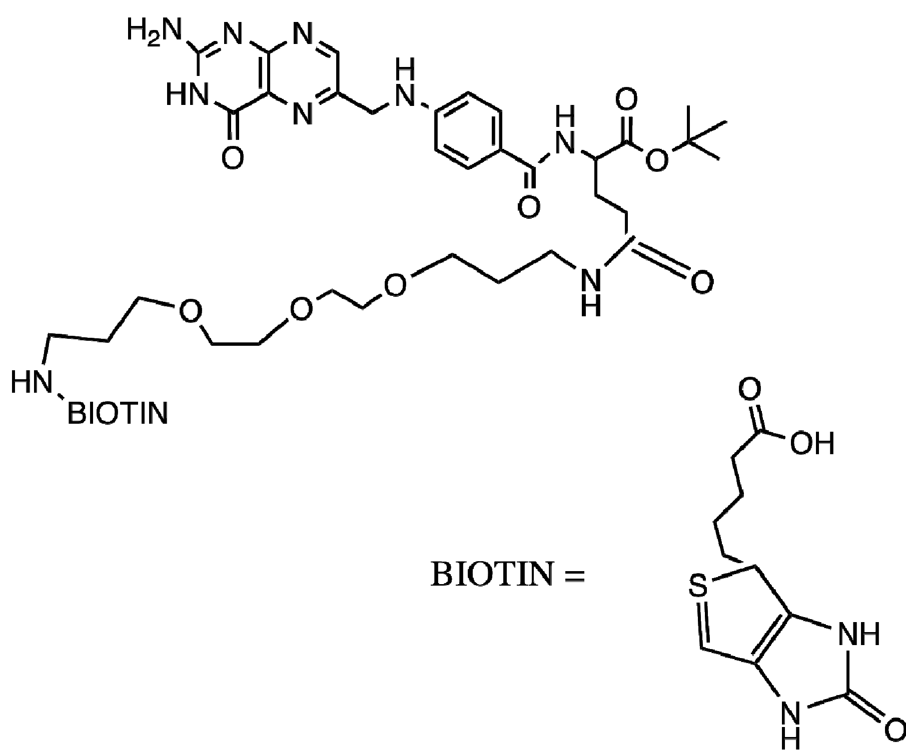


BIOTIN =



Folate Long PEG Biotin

Figure 10: Chemical structure (chem draw)



Folate Short PEG Biotin

Figure 11: Excel sheet- peptide calculator

		PEPTIDE	CALCULATOR				
	Resin Loading(mM/g)	Weigh used (g)	M. W. (g/mole)	Moles	Excess of moles	Weight for addition (g)	Volume for addition (ml)
PEG Biotin Resin	0.47	0.075		3.50E-05			
HATU			380.2	0.000176	5	0.067	
DIPEA			129.5	0.000353	10	0.0456	0.062
PEG27			1544.8	5.29E-05	1.5	0.0817	
Fmoc-Gul-aOtBu			425	0.000106	3	0.0449	
Pteroic Acid N10 TFA			408	5.29E-05	1.5	0.0216	