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Folate-PEG-Histidine for Use in Detection, Capture, and Analysis of FR+ Cells

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Folate-PEG-Histidine for use in the Detection, Capture and Analysis of FR+ Cells

A Project Submitted to:

Governors State University

By: Sean Dwyer

In partial fulfillment of the requirement for the Masters of Science in

Analytical Chemistry

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This paper is dedicated to my professors, family, friends, and to all of those who supported me through my academic journey.

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Abstract

Folate receptor alpha is a membrane-bound protein displaying high affinity for folic acid. This receptor serves as a receptor-mediated transport system of folic acid into cancer and cells associated with inflammation (e.g., macrophages). Folate is a basic component of cell metabolism in both the synthesis of DNA and proteins, which is hypothesized to be necessary for maintaining adequate processes during increased metabolic demand. Interestingly, most normal cells in the body have little or no high affinity folate receptor alpha. Based on these attributes, folate based drug delivery, imaging systems, and diagnostic systems are in several stages of development worldwide. To the best of our knowledge, this is the first time a folate 5-His conjugate has been used in this fashion.

In this project, analytical analysis of a folate polyethylene glycol (PEG) 5-histidine conjugate was undertaken. This conjugate is capable of binding to nitrilotriacetic acid (NTA) nickel in the classic 6-histidine (his) capture systems including NTA-Ni magnetic beads and Nano composites. The resulting compound was successfully synthesized and proven with the diode array detector during liquid chromatography separation with a maximal absorption at 280 nm with a shoulder peak at 363 nm at 15.277 minutes. This peak was isolated and then directly injected into the electrospray ionization ion trap mass spectrometer (Agilent technologies) which produced a doubly charged and triply charged anions at m/z 's of 665.0 and 443.7 respectively, which fall within the expected range for the protonated Folate-PEG-5-His molecule.

Introduction

Folic Acid

Folic acid is a necessary compound for humans to consume in their average diets, as it is a water soluble B vitamin with a fairly low molecular weight of 441.4 Da. (1-3) It is typically found within the kidneys and livers of animals, it is also found in plants, mushrooms, and algae.

(1) Folate is an essential component in metabolic pathways, specifically in the carbon transfer reactions in purine and pyrimidine biosynthesis as well as amino acid interconversions. (1, 2, 4)

Low folic acid intake can cause several debilitating and serious illnesses and birth defects. For instance low folate levels during pregnancy can cause defects in neural tube development. (1, 5, 6) It also has been associated with various disorders such as Alzheimer's, coronary artery disease, leukemia, and ectopic pregnancies. (1, 3, 6) Folate is also a known antimicrobial agent. (7) Folate's importance is due to the fact all cells require folic acid, but mammalian cells are unable to synthesize the compound by themselves. (8) Due to this unique characteristic mammalian cells contain at least one folate internalization pathway. (8) These are generally associated with a folate receptor, or a channel protein found within the cell membrane. (9)

Folate receptor (FR) is a glycosylphosphatidylinositol-anchored glycoprotein (GPI) with a high affinity for folic acid and the reduced vitamin N⁵-methyltetrahydrofolate. (10-13) GPI plays a crucial role in both the transport and targeting of the protein to both the plasma membrane and as well as its function. (14-16) The GPI anchor is made up of a lipid moiety and a glycan backbone; they follow from the endoplasmic reticulum (ER) to the golgi being modified until their final destination at the plasma membrane. (14-16) When reaching its location within the plasma membrane, the GPI protein begins vital roles in physiology, signal transduction, cell-cell

interactions, cell adhesion, and biosynthesis. (16) The folate receptor has several isoform variants, but FR- α and FR- β are of particular interest when it comes to disease. (6, 17, 18) In healthy adult tissues, FR is virtually nonexistent, with folate uptake using alternative pathways. (9) The FR functions to concentrate folic acid and its derivatives into the cytosol by endocytosis. (19, 20) Overexpression of FR in cancer cells appears to give growth advantages in comparison to normal tissue, which may explain how highly metastatic cancers with FR spread more rapidly than other cancer types. (20)

FR- α and FR- β isoforms are found in several cancers, and activated macrophages. (17) FR- α specifically is linked to several carcinomas in humans such as endometrial, kidney, lung, mesothelioma, breast, and myeloid leukemia. (7, 8, 9, 17, 19) FR- β receptors are associated with activated macrophages which are thought to be the cause of inflammatory diseases. (17) Some FR directed antifolates have been shown to help in treating diseases thought to be caused by FR- β diseases like rheumatoid arthritis, psoriasis, Crohn's disease, systemic lupus, atherosclerosis, diabetes, ulcerative colitis, osteoarthritis, glomerulonephritis, and sarcoidosis. (17) Current research is pouring into new and novel drug therapies to specifically target these diseases that express these two folate receptors. FR treatments work by utilizing a tumor-specific ligand that is covalently attached to the therapeutic agent. (17) Examples of this are folate linked chemotherapy agents, nanoparticles, drug-loaded liposomes, and oligonucleotides. (1, 18)

Poly-Histidine Affinity Chromatography

Affinity tags are exogenous amino acid sequences with a high affinity for a specific biological or chemical ligand. These tags are utilized as they have a high yield during purification of over 90% and offer a viable money/time-saving method that also reduces the

number of chromatography steps. (21-23) One of the most highly used affinity based tags are polyhistidine tags. (23-24) This is usually accomplished by putting a histidine peptide chain which binds its ligand to a solid support. (23) Purification can be accomplished by using immobilized chelated metals that are attached to the imidazole side chain. (22-24)

In this experiment, this will be accomplished with 5-histidine chain attached to polyethylene glycol conjugate. The purpose of this unique conformation is to bind to immobilized metals (22, 23) specifically NTA-nickel magnetic beads or nano particles which allow for the capture of FR positive cells that can be rapidly identified via quartz crystal microbalance (QCM) biosensors, surface plasmon resonance (SPR) biosensors, and immunomagnetic diffraction detection. This tagging system should allow for identification of FR positive cells in very low quantities due to the high affinity this unique molecule has for the receptor. (22-24)

Methodology

Preliminary Synthesis

The preparation of PEG/folate conjugate was made in accordance to solid phase peptide synthesis. (25) The 6-histidine addition was made via the following several steps. To begin, 727 mg of Fluorenylmethoxycarbonyl-histidine (Fmoc his) and 447 mg of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU) were weighed out. Both were then placed in 10 ml. of Dimethylformamide (DMF) with 400 µl of *N,N*-diisopropylethylamine (DIPEA). Next, 34.2 mg. of PEG/Folate conjugate were weighed out and placed within a syringe. This was finally swelled

with 1 ml of DMF for 25-30 minutes. After these initial preparatory steps the following reaction steps to add the six histidines required via a stepwise procedure.

One ml of the His/HATU+DIPEA was added to the conjugate in the syringe. The mixture was allowed to react for 30 minutes while being mixed on a Thermo-Fisher Digital Vortex mixer. Afterwards, 5-6 washes with 1 ml. of DMF were made within the syringe. Following this, three, five minute washes with 20% piperidine were made and allowed to shake on the vortex mixer. Following this step, another 5-6 washes of DMF occurred within the syringe. This entire process was repeated five times to produce the molecule of interest.

HPLC purification

A 30 µl injection was made into the reverse phase HPLC column (Agilent technology 1260 Infinity) The diode array detector was set to 280 nm to locate the folate characteristic peak. The solvents used for purification were a 10 mmol ammonium bicarbonate/water mixture for solvent A and acetonitrile for solvent B. The methodology for the solvent gradient is listed in Table 1 below. Though the overall method lasted 50 minutes, the peaks that were needed for mass spec were recovered within 15-20 minutes after the run started. The purified samples were recovered and placed into centrifuge tubes, for later direct injection into the electrospray ionization ion trap mass spectrometer (Agilent technology 1100 LC/MSD trap).

Table 1

Time (min)	A (% 10mmol ammonia bicarbonate/water)	B(% acetonitrile)	Flow (mL/min)	Max Pressure Limit (bar)
0	99	1	1	250
30	50	50	1	250
50	5	95	1	250

(Table 1) Methodology utilized for solvent gradient in the Agilent 1260 Infinity HPLC machine.

Direct Injection into Agilent 1100 LC/MSD XCT trap

The purified samples that were recovered from Agilent 1260 were then directly injected into the MSD XCT trap. The parameters utilized for this step of the methodology is summarized in Table 2 below.

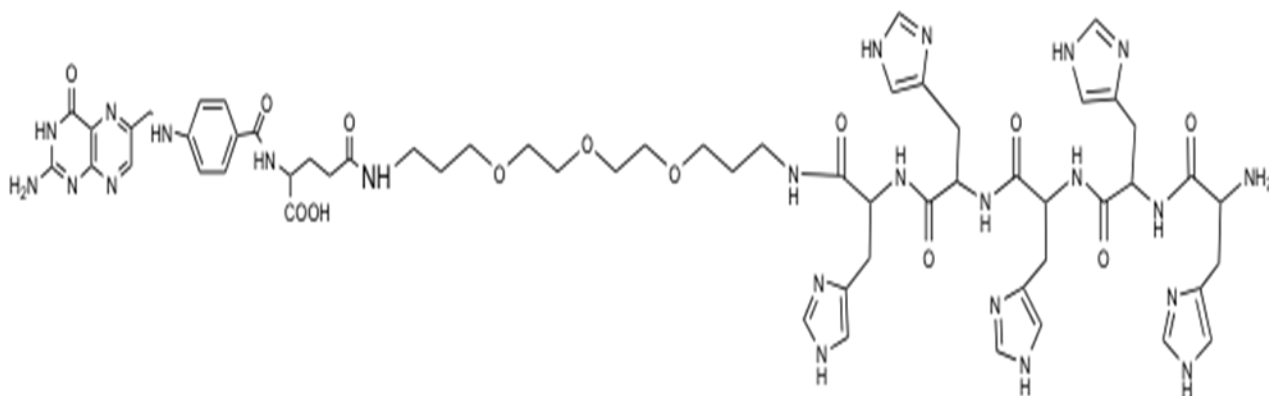
Table 2

Nebulizer (psi)	Dry Gas (l/min)	Dry Temp (°C)
40	8	300

(Table 2) Nebulizer pressure, Drying Gas flow rate, and temperature for the MSD XCT trap for mass spectral analysis.

The mass spectrometer was run in the negative mode to detect anions. The mass of the prepared molecule was 1329 Da. A total ion chromatogram was generated, which was utilized to find possible multiple charged species.

Results



Chemical Formula: $C_{59}H_{76}N_{24}O_{13}$

Exact Mass: 1328.60

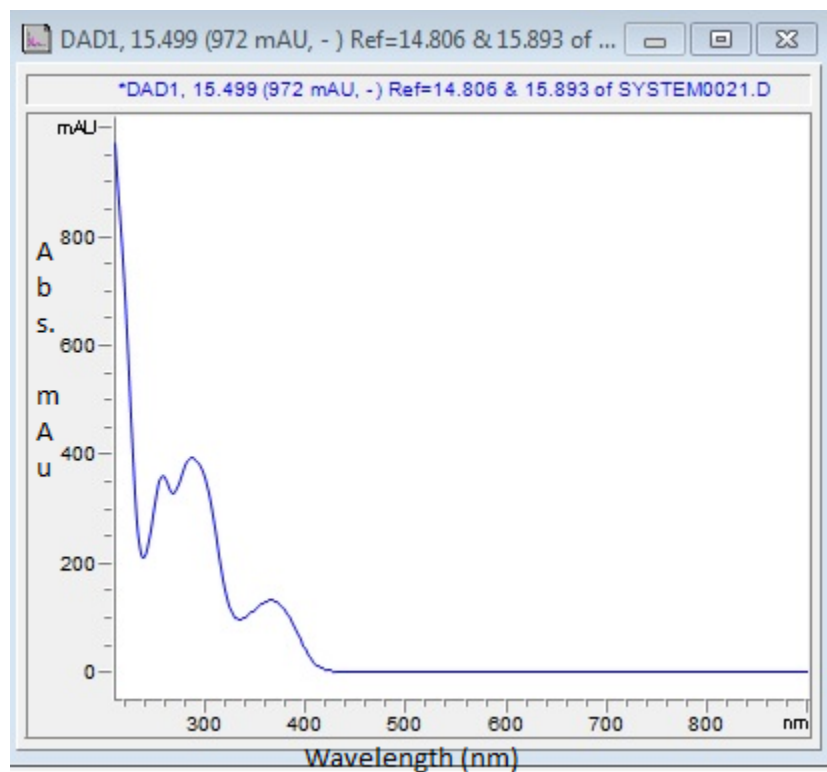
Molecular Weight: 1329.39

m/z: 1328.60 (100.0%), 1329.61 (65.2%), 1330.61 (23.6%), 1329.60 (8.9%), 1331.61 (7.8%), 1330.60 (6.1%), 1332.61 (1.1%)

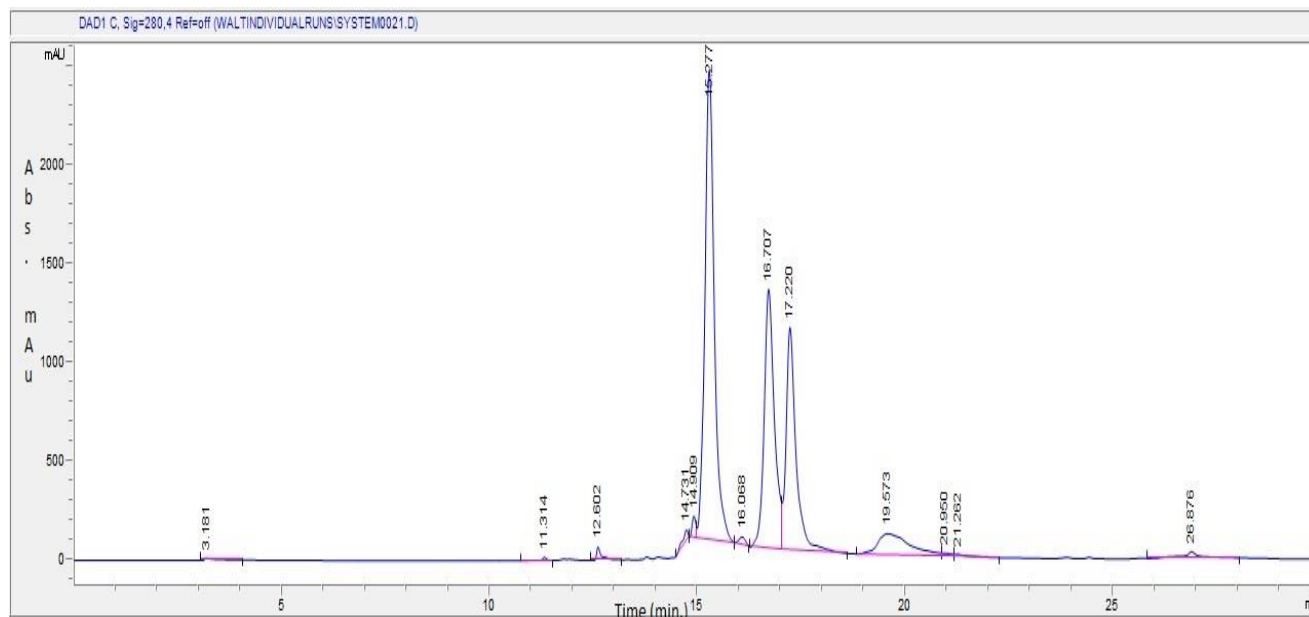
Elemental Analysis: C, 53.31; H, 5.76; N, 25.29; O, 15.65

(Figure 1) Hypothetical synthesized compound with molecular formula, mass, and weight along with expected protonation values.

The compound synthesized via the process discussed previously was a folate-peg-5 histidine molecule. To verify this, HPLC and direct injection into the mass spectrometer were performed, with the results shown in figure 2, and 3.

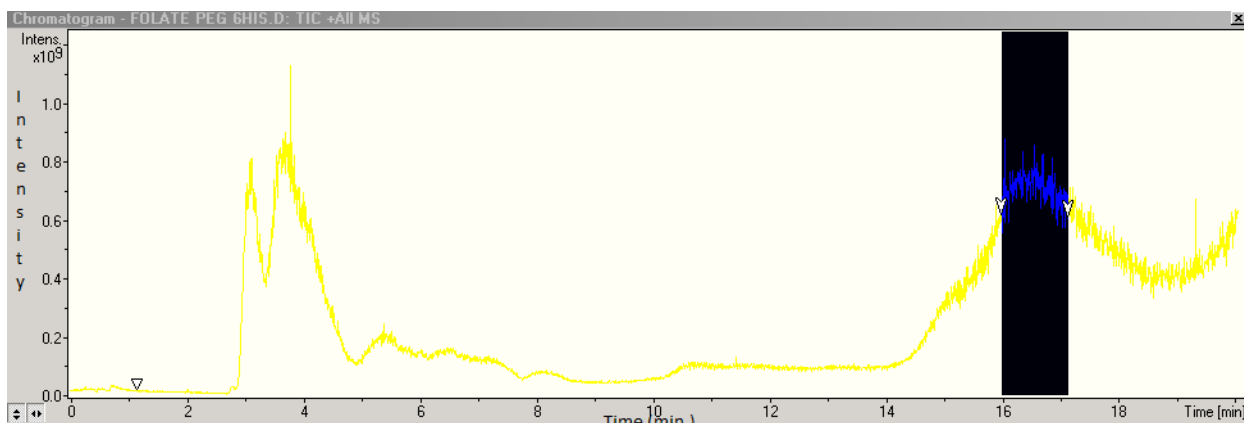


(Figure 2) Peak 1 Diode array detector results with folate character showing maximal absorption at 280 nm, with a side peak at 363 nm. (26)

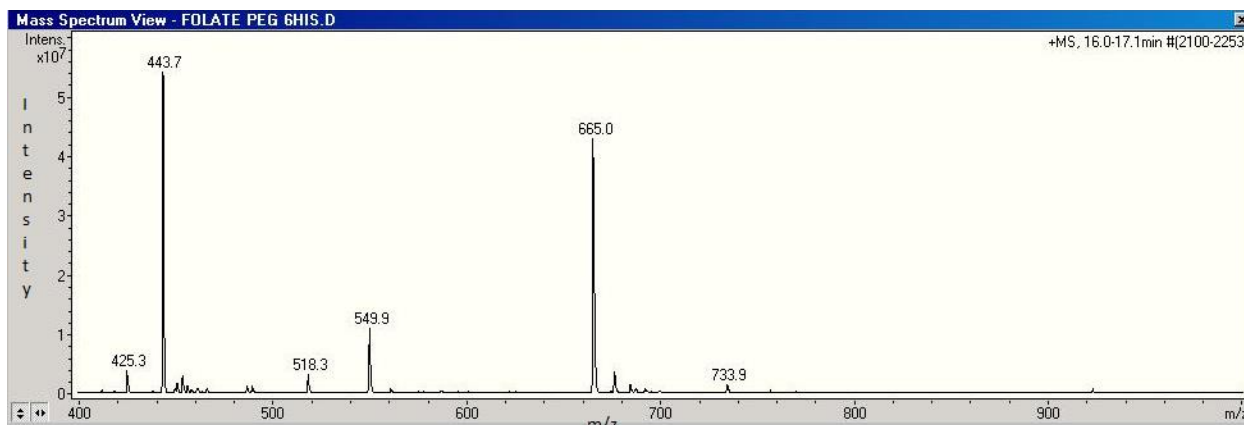


(Figure 3) Chromatogram from HPLC purification of the Folate-PEG-5-His compound the principle peak at 15.277 minutes was isolated and utilized for direct injection into the Ion Trap Mass Spec.

Figure 2 shows the folate character with maximal absorption occurring at 280 nm with a lesser band occurring at 363 nm. The major peaks from the resulting purification occurred at 15.277, 16.707, and 17.220 minutes as shown in Figure 3. (26) All three peaks demonstrated major folate character within them, and the sample eluted at 15.277 mins was used for direct injection.



(Figure 4) Total ion chromatogram of direct injection done in negative mode for the principle peak isolated in HPLC.



(Figure 5) MS of TIC at 16.0-16.5 minutes with resulting doubly charged occurring with an m/z of 665.0 and triply charged species with an m/z of 443.7 being present in the chromatogram.

The total ion chromatogram shown in Figure 5 showed double and triple charged ions that corresponded to the synthesized molecule. The base molecule did not show up in the results, which was expected given the molecule has multiple possible protonation sites.

Table 3

$M^* - 2H^+(m/z)$	$M^* - 3H^+(m/z)$
664.5	443

(Table 3) hypothetical m/z values for the doubly and triply charged species of the target molecule.

The doubly charged anion was observed with an m/z of 665.0 which was 0.5 Da away from the hypothetical value, and the triply charged molecule occurred at m/z of 443.7 again only 0.7 Da away from the theoretical value as shown in Table 3, providing evidence that the desired synthesized molecule was successfully synthesized. These values are consistent with the mass accuracy of the mass spectrometer use.

Discussion

HPLC purification of the molecule proceeded accordingly with near perfect separation of the compound from contamination. The desired peaks had near ideal separation with very little modification to the overall methodology. Further, the folate character demonstrated on the diode array detector was consistent with the 280 nm. and 363 nm. doublet consistent with folic acid, again demonstrating the desired molecule was most likely present within the peaks. (26) Mass spectrometry analysis of the folate-peg-5 histidine molecule proved quite challenging, because the molecule has a tendency to bind to metal ions due to its high metal affinity.

Conclusion

Folate polyethylene glycol 5 histidine molecule was successfully synthesized and analyzed via HPLC and mass spectrometry. Multiply charged anions of the synthesized molecule were readily observed. These peaks indicated successful production of folate-peg-5 histidine. Future synthesized species will incorporate a fluorescein indicator for dual affinity based capture and detection. It is hoped that these molecules will work well with QCM biosensors, SPR biosensors, and immuno-magnetic diffraction detection for the detection of FR positive cells.

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