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Entitled

Analysis of Folate Binding Protein and Associated N-Glycans by Mass Spectrometry and Light Microscopy

By
Nidhi Jaiswal

Submitted to the Graduate Faculty as partial fulfillment of the requirements for
The Masters of Science degree in Chemistry

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The University of Toledo

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An Abstract of

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Folate binding protein (FBP), also known as folate receptor (FR), is a glycoprotein which binds vitamin folic acid and its analogues. The FBP contains multiple N-glycosylation sites and is overexpressed in human cancers including ovarian, lung, kidney, and breast cancer. However, the structure and the composition of N-glycans bound to the FBP are still unknown.

We performed structural characterization of FBP N-linked glycans originating from bovine and human milk. The N-linked glycans were enzymatically released from FBP, purified, and permethylated. The glycans were analyzed by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), while tandem MS (MS/MS) was used for their further structural characterization. In addition, deglycosylated FBP was purified by solid phase extraction and analyzed by MALDI-MS. It was found that FBP from human milk contains putative structures that have composition consistent with high-mannose (Hex$_{5-6}$HexNAc$_2$) as well as hybrid and complex N-linked glycans (NeuAc$_{0-1}$Fuc$_{0-3}$Hex$_{3-6}$HexNAc$_{3-5}$). The FBP from bovine milk contains putative structures corresponding to high-mannose (Hex$_{4-9}$HexNAc$_2$) as well as
hybrid and complex N-linked glycans (Hex$_{3,6}$HexNAc$_{3,7}$), but these glycans mostly do not contain fucose and sialic acid.

To image the FBP in live and fixed cells, KB cells were incubated with folic acid conjugated with fluorescein isothiocyanate (FITC) and FITC alone. Cells labeled with conjugated folic acid showed higher fluorescence intensity than cells labeled with FITC. In addition, KB cells were cultured in order to isolate FBP from cell lysate and cell culture media using an epoxy-activated folate-sepharose column.

In summary, glycomic characterization of FBP provided a valuable insight into the structure of this cancer-relevant glycoprotein, and may be beneficial for a glycomic analysis of FBP originating from diseased cells and tissues. Imaging of FBP in cells can help to locate folate-based anti-cancer drugs and to follow their effect on cancer cells.
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<th>Full Form</th>
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<tr>
<td>FBP</td>
<td>Folate Binding Protein</td>
</tr>
<tr>
<td>FR</td>
<td>Folate Receptor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray Ionization-Mass Spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>LID</td>
<td>Laser induced dissociation</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
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<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<tr>
<td>PNGase F</td>
<td>Peptide-N-glycosidase F</td>
</tr>
<tr>
<td>F-FRPMI</td>
<td>Folate-Free Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle’s Medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>LC-MS</td>
<td>High Performance Liquid Chromatography-Mass Spectrometry</td>
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Chapter 1: Introduction

Despite the tremendous growth in medicine in last few decades, cancer is still the second largest cause of death in The United States of America following heart disease.\textsuperscript{1} For example, ovarian cancer in females is one of the concerns worldwide. It is the eighth most leading cause of cancer death among US women. The cancerous tumor spreads to other parts of the body in later stages which makes the treatment more complicated.\textsuperscript{1}

Consequently, survival rate of patients suffering from cancer at stages I and II is highly dependant on the growth of cancer cells whereas the survival rate of the patients suffering from cancer at stages III and IV is low to negligible.\textsuperscript{2} The mortality rate of all women suffering from gynecologic malignancies would decrease with early cancer diagnosis. Hence, there is a need to detect and treat the abnormal cells at the early stage.\textsuperscript{2}

Therefore, early diagnosis, cure, and prevention of cancer is very much an anxiety and interest of biochemists and chemists throughout the world. Efforts are underway to detect cancer at an early stage, which can be performed by observing changes in cell profiles with time after intimately observing a biomolecule known as a biomarker.

The aim of cancer biomarker discovery is to detect cancer at an early stage using simple tests. Glycosylation patterns are altered with development of cancer due to changes in the internal and external environment of cells. Therefore, glycoproteins can be sensitive indicators of changes in cell function and can play a significant role as cancer biomarker. Glycosylation changes that occur in cancer cells can take a variety of forms
such as increase in expression of certain glycans, disappearance of glycan structures, altered branching patterns, changes in amount of sialic acid, and in some cases, appearance of novel glycan structures.\(^3\)

Alterations in glycosylation are therefore becoming widely used as markers for early diagnosis and to follow the chemotherapy. Several publications have produced promising results with different glycoproteins as cancer biomarkers, but not many have been validated for clinical applications.\(^4\) For instance, CA-125 is present at a prominent level in highly developed ovarian cancer patients and can be used to monitor progress of the chemotherapy. However, CA-125 is not useful for early cancer detection.\(^5\) Glycoprotein folate binding protein (FBP) is present in very low levels in most normal tissues, but as the stage of cancer increases, the level of FBP increases.\(^2,6\) As a result, FBP, which was discovered in 1991 could be used to detect cancer in early stage and has the potential to serve as cancer biomarker.\(^6\)

1.1. Folate Binding Protein

Folate Binding Protein is overexpressed in several epithelial malignancies including lung cancer, kidney cancer, brain cancer, breast cancer and was explored for the diagnosis of ovarian cancer.\(^7\) The membrane associated folate binding protein (FBP), is highly clustered on cell surface and is responsible for the uptake of folate into the cells through the process of endocytosis. It is a glycoprotein that binds the vitamin folic acid in a stoichiometry of 1:1.\(^8\) FBP has high affinity for folate with \(K_d\) approximately \(10^{-10}\) M.\(^6,8\)

It is present in several isoforms (\(\alpha, \beta, \gamma, \gamma'\)) in humans, bovine, and murine.\(^8,9\) Human FBP \(\alpha\) and \(\beta\) isoforms, anchored to a membrane by a glycosylphosphatidylinositol (GPI) anchor tail, contain two and three N-linked glycosylation sites respectively. FBP \(\gamma\)
isoform is a soluble glycoprotein and γ’ isoform is a truncated form of soluble protein. FBP α and β isoforms are more hydrophobic than other forms of FBP. FBP α isoform can be also shed from the cell surface into the neighboring medium and is responsible for the DNA synthesis and cell growth. FBP has been identified by a $[^3]$H]folic acid binding assay in several human cell lines such as HeLa, KB, L1210, M109, and Molt 4. It was found that FBP has the highest membrane or particulate folate binding capacity in KB cell line, which is derived from human epidermoid carcinoma and is found as contamination of HeLa. In addition, soluble FBP is also shaded in the culture media. Milk, plasma, and serum have been identified as a potential source for soluble folate binding protein, as well as membrane folate binding protein.

In prior studies, amino terminal sequence, amino acid composition and associated glycan composition of both soluble and membrane human FBP have been determined. Bovine FBP is formed of 220-237 amino acids, and the molecular weight of deglycosylated FBP from bovine milk was found to be approximately 26.5 kDa. Previous studies have proposed that FBP from bovine milk consists of low to negligible amounts of sialic acid, which is one of the monosaccharides present in glycans, when compared to human milk. FBP in bovine milk can serve the various biological functions and is secreted by the mammary gland. It has also been found to serve as a regulatory protein which regulates the availability of milk folate to the suckling animals.

Human folate binding protein isolated from KB cells has a major form with molecular weight of 160 kDa, whereas the minor form has a molecular weight of 40 kDa. Soluble folate binding protein has molecular weight of 40 kDa.
1.2. Glycans and Glycoproteins

Carbohydrate is defined as an organic compound consisting of polyhydroxyaldehyde or polyhydroxyketone. Carbohydrates can be further divided into four groups: monosaccharides, disaccharides, oligosaccharides and polysaccharides. Oligosaccharides are carbohydrates formed by a linear or branched chain of monosaccharides linked by glycosidic bonds. Proteins can be modified with oligosaccharides during post-translation. This is the most common form of post translational modifications also known as glycosylation. Glycoproteins constitute more than 70% of the known proteins. Glycosylation of proteins by oligosaccharides is very important and serve a number of specific biological functions as regulatory switches. Other forms of post translational modifications include sulfation, phosphorylation, methylation, O-acetylation, etc.

Glycosylation which occurs on the cell surface is an enzymatic process where oligosaccharides are attached to the protein or lipid. If oligosaccharides are covalently bonded to the protein via N- or O-linkages, the resulting protein is known as a glycoprotein. Glycans are oligosaccharides found in glycoproteins. Proteins attached to the glycans are also present in the nucleus and cytoplasm. As shown previously, glycan structures have different biological activities and functions even if they are located on the same glycoprotein. Glycans are classified according to the nature of the core linked to the protein. There are two different types of glycans:

a) O-linked glycans that are bound to the hydroxyl side chain of serine or threonine.
b) N-linked glycans that are bound to the amide side chain of aspargine by N-glycosidic bond within the peptide sequence: Asn-X-Ser/Thr where X is any amino acid but proline.
N-linked Glycans are branched structures composed of several monosaccharides including N-acetyl glucosamine, mannose, galactose, sialic acid, and fucose. N-linked glycans are further divided into three types: high mannose, complex, and hybrid. High mannose glycan structure consists of variable number of mannose but only two N-acetyl glucosamines. Complex N-linked glycan structures contain variable numbers of mannose, galactose, sialic acid, fucose and N-acetyl glucosamine whereas the characteristic feature of both high mannose and complex N-linked glycans combines to form hybrid type N-linked glycans as shown in Figure 1.

Figure 1. Prototypic structures of N-linked glycans. Examples of the three classes of carbohydrate chains.
The common core of all N-linked glycan structures is $\text{H}_3\text{N}_2$ (Figure 2).\textsuperscript{18} Multiple glycosylation patterns are possible at a single asparagine residue in a protein, which makes analysis of N-linked glycans more complex. Heterogeneity of glycans varies from glycosylation site to glycosylation site, from protein to protein, and also from cell type to cell type adding to overall complexity of glycan structures.\textsuperscript{3} For example, more than 30 different N-glycan structures corresponding to high mannose,\textsuperscript{16} complex,\textsuperscript{19} and hybrid N-glycans has been found on a single N-glycosylation site at 292\textsuperscript{nd} asparagine residue in a 385 amino acid glycoprotein, ovalbumin.\textsuperscript{16} Apart from the role of glycans as cancer biomarkers for early cancer detection, glycans can be used to follow the effect of drugs or toxins on a disease process.\textsuperscript{20} In a previous study Ratnam et al have shown that N-glycosylation is important for sorting,\textsuperscript{21} proper folding, intracellular trafficking, and functional expression of folate binding protein.\textsuperscript{22} N-linked glycans are also important in various cellular functions such as cell development and cell-cell interactions.\textsuperscript{3} About 70%
of glycan composition of human milk is due to sialic acid milk contains 0.3-1.5 mg/mL of sialic acid) which plays a major role in physiological functions during infant stage.\textsuperscript{14}

Glycobiology is the branch of science which includes the study of different glycan structures and its chemical and biological synthesis. Glycans from protein are first isolated by most common method known as enzymatic cleavage. Study of glycans is much more tedious than study of protein because they possess complex (linear or branched) structure, which makes analysis and validation of glycan structure and its sequencing difficult.\textsuperscript{3} The development of new analytical technologies has made analysis easier for chemists and biochemists for determination and identification of the glycan structures. Due to the fact that glycosylation changes with diseases, it has been interesting to investigate glycans in many diseases including cancer.\textsuperscript{17}

1.3. Mass spectrometry

Mass spectrometric techniques have emerged recently for the analysis of disease markers,\textsuperscript{17} glycopeptides\textsuperscript{21} and oligosaccharides.\textsuperscript{24} There is a huge interest in the MS analysis of glycans and glycoproteins present in milk and disease-related tissues. Besides nutritional and immunological importance of glycans found in milk, it was shown that aberrant glycoprotein expression and altered glycosylation are associated with human diseases such as cancer.\textsuperscript{14} Moreover, most of currently used biomarkers of cancer are glycoproteins and their glycosylation was studied by MS.\textsuperscript{4} For example, glycosylation studies of biomarkers of prostate (prostate specific antigen, PSA), ovarian (CA125), and pancreatic (CA19-9) cancer were accomplished by mass spectrometry.\textsuperscript{4} MS has been also extensively used to analyze N- and O-linked glycans isolated from serum of cancer patients, and has helped assigning putative glycan biomarkers of cancer. For example,
Morelle et al used the mass spectrometric approach for screening of total serum N-glycome in human serum. Also, in prior studies it was shown that changes in N-linked glycosylation on the whole glycoproteome due to cancer can be studied by combining separation techniques (e.g., ion mobility) with mass spectrometric analysis. Since FBP is overexpressed in various types of cancer and can transfer folate based drugs into a cancer cell, it has been extensively explored as both a putative biomarker of cancer and target of anticancer drugs. Therefore, the knowledge of the glycomic structure of FBP would be both biologically and biomedically relevant.

Mass spectrometric analysis provides the molecular weight of glycans present in the sample which can allow determination of compositions of glycans as most oligosaccharides are comprised of relatively few different monosaccharides with the unique residue masses such as fucose (Fuc, m/z 146 Da), hexose (Gal, Man, m/z 162 Da), N-acetylhexosamine (GlcNAc, m/z 203 Da) and sialic acid (NeuAc, m/z 291 Da). Sample preparation for detection of glycans using mass spectrometric techniques is essential because it enhances sensitivity and reproducibility of the analysis.

Most common mass spectrometers used to analyze glycan samples include electrospray ionization mass spectrometer (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS). Advantages of ESI and MALDI coupled to a time-of-flight (TOF) mass analyzer to analyze glycans include: high sensitivity (50-100 femtomoles), high resolution, high mass accuracy and the ability in some cases to analyze underivatized glycans. DHB is the most common matrix used for the analysis of glycans by MALDI-MS. However, sialylated oligosaccharides lose sialic acid when analyzed by MALDI which accounts for poorer detection limits
Therefore, there is a need to derivatize glycans in some cases to improve ionization efficiency and stability. Derivatization increases stability of sialic acid which is the most abundant species in human glycans.\textsuperscript{17} Mass spectrometry is implemented to analyze both native and derivatized glycans. Permethylation, methylation, and pyridylamination are the chemical modifications of glycans.\textsuperscript{17} Permethylation of N-glycans increases ease to determination of branching patterns through a detailed structural analysis. It also helps to resolve the presence of configurational and conformational isomers.\textsuperscript{29}

However, structural analysis is difficult based on MS data alone. Therefore, tandem mass spectrometry (MS/MS) is often used for the structural characterization and determination of composition of glycans.\textsuperscript{30}

1.3.1. Structural analysis and sequencing of N-linked glycans

For structural analysis of peptides, proteins and glycans, high performance tandem mass spectrometry is an essential technique. Structure of each compound can be identified by using its mass to charge ratio (m/z) for parent ions and its fragment ions. Tandem mass spectrometry is advantageous for heterogeneous samples where structure of each component can be identified. In the MS/MS experiment, a particular parent ion to be fragmented is selected in the first mass analyzer at a resolution of one mass unit. Collision induced dissociation (CID) is a well established fragmentation method for the analysis of biomolecules. Selected parent ion collides in a collision cell with an inert gas such as argon to produce daughter ions and the collision products are transmitted to a second mass analyzer at a resolution of one mass unit.\textsuperscript{31} Nomenclature pertaining to
fragmentation of carbohydrates was introduced by Domon and Costello.\textsuperscript{31}

Two types of cleavages take place in glycans: glycosidic cleavages and cross-ring cleavages. In glycosidic cleavage, there is a cleavage of a bond between two sugar residues which reveals information mainly about determination of sequence and branching. Cross-ring cleavages involve breaking of two bonds on the same sugar residue. Cross-ring cleavages give additional information on the linkages. High energy CID usually provides information on cross-ring cleavages. According to this nomenclature, cleavage of glycosidic bond from the reducing end leads to assignment of $Y_j$ and $Z_j$ ions. Subscripts for $Y$ and $Z$ represent the number of inter glycosidic bonds cleaved and counted from the reducing end terminal. Cleavage of glycosidic bonds from the non-reducing end leads to assignment of $B_i$ and $C_i$ ions. Subscripts for $B$ and $C$ represent the number of glycosidic bonds cleaved and counted from the non-reducing end. Cross-ring cleavage at the reducing end leads to assignment of $X_j$ and cross-ring cleavage from the non-reducing end leads to assignment of $A_i$. Subscripts for $X$ represent the number of sugar residues cleaved and counted from the reducing end terminal. Subscripts for $A$ represents the number of sugar residues cleaved and counted from the non-reducing end. Superscripts for $A$ and $X$ represent the position of the cleavages within carbohydrate rings. If more than one cleavage takes place for the ions produced during fragmentation, they are represented with the help of a slash between the sites of cleavages e.g., $(B_3^{0,1}A_4)$.\textsuperscript{30,31} Analysis of glycan structures is more complicated because the core structure $H_3N_2$ itself is a branched structure. In case of branched glycan structures, each branch is labeled with a greek letter $\alpha$, $\beta$, and $\gamma$. Branches are also known as antennae. Numbering of the glycan ions begins in the core unit and continues to the branches where
it is represented by a greek letter also and vice versa as shown in Figure 3.

**Figure 3.** Designations for fragment ions from a hypothetical glycan according to Domon and Costello.\(^{31}\)

In our studies, we used commercial samples of FBP originating from bovine and human milk for structural characterization of N-linked glycans by mass spectrometry. Our goal is to identify and characterize novel glycans present in folate binding glycoprotein and to look for their potential role as cancer biomarkers. N-linked glycans were enzymatically released from FBP by enzyme peptide-N-glycosidase F (PNGase F), purified, and permethylated. Native and permethylated N-glycans were further analyzed by MALDI-MS and ESI-MS, while tandem mass spectrometry (MS/MS) was used for their structural characterization. Also, deglycosylated folate binding protein was purified
by solid phase extraction and analyzed by MALDI-MS.

Additionally, we were interested in imaging and isolation of FBP from cancer cells. Fluorescent probes, which can be used as powerful detection and treatment tools has aroused much interest in recent years. These probes allow detection of biomolecules such as DNA, RNA, and proteins via fluorescence microscopy. Some dyes such as Thiazole orange (TO) show 1,000 to 3000 times enhancement depending on the bound partner.\textsuperscript{32} These dyes have higher affinity toward tumors than to normal cells, a characteristic property which can be used for early-stage imaging, labeling, and detection of cancer. FBP retains its high affinity for folic acid even after covalent modification of the latter with a tracer. This feature makes folic acid an important carrier for anti-cancer drugs and fluorescent dyes providing cancer treatment and imaging of cancer cells, respectively. In prior studies, FBP has been imaged using folic acid-conjugates \textit{in vitro} and \textit{in vivo}, and analyzed for allergic reactions against folic acid-FITC conjugate.\textsuperscript{33,34}

Here, we described the imaging of FBP in live and fixed cells using a folic acid fluorescent tag in order to see its abundance in cancer cells. We labeled the folic acid with FITC, which can specifically bind to FBP. We analyzed the fluorescence images that depended on the degree of folate binding protein expression \textit{in vivo}. Imaging of FBP can enable localization of the drugs and other compounds.\textsuperscript{7} We also tried to isolate and purify folate binding protein from a human cancer cell line KB in order to perform structural characterization of FBP N-linked glycans.
Chapter 2: Materials and Methods

2.1. Materials

KB cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). DMEM Media, Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Mediatech Inc. (Manassas, VA). F-FRPMI-1640 medium was purchased from Gibco Laboratories (Gaithersburg, MD). Trypsin was purchased from HyClone Laboratories, Inc (Logan, UT). Petri plates, Petri Dish, 1X PBS, HPLC grade water, methanol, chloroform, ethanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). [³H] folic acid was purchased from Moravek Biochemicals Inc (Brea, CA). Activated charcoal, folic acid, FITC, Triton X-100, epoxy activated sepharose 6B, paraformaldehyde, n-propyl gallate, poly-L-lysine stock solution, bovine folate binding protein (purity: 20-40% by Warburg-Christian assay), NaOH beads, PNGase F, trifluoroacetic acid (TFA), formic acid and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich (St. Louis, MO). Quartz cuvette was purchased from World Precision Instruments, Inc. (Sarasota, FL). Human folate binding protein (purity: nominally > 95% by SDS-PAGE) was purchased from Sunny Lab (SCIPAC Ltd, Sittingbourne, UK). C18-Sep-Pak solid phase extraction cartridges were purchased from Waters (Franklin, MA), and macro spin activated charcoal columns and empty spin columns were purchased from Harvard Apparatus (Holliston, MA). SDS PAGE reagents and silver staining reagents were purchased from Bio-Rad Laboratories, Inc. (Richmond,
CA). HeLa cells, primary and secondary antibodies were courtesy gifts from Dr. Manohar Ratnam.

2.2. Electrophoretic and Western blot analysis

2.2.1. Preparation of 12% SDS gel: 12% SDS gel was prepared fresh.

2.2.1.1. Preparation of solutions for gel: Following solutions were prepared:

1) 1.5 M Tris-HCl was prepared by dissolving 27.23 g of Tris Base in 80 mL of deionized water. pH was adjusted to 8.8 with 6 N HCl and deionized water was added to final volume of 150 mL. The prepared solution was stored at 4 ºC until further use.

2) 10% Ammonium persulphate (APS) was prepared by dissolving 100 mg of APS in 1 mL of deionized water. The prepared solution was stored at 4 ºC until further use.

3) 0.5 M Tris HCl was prepared by dissolving 6 g of Tris Base in 60 mL of deionized water. pH was adjusted to 6.8 with 6 N HCl and deionized water was added to final volume of 100 mL. The prepared solution was stored at 4 ºC until further use.

2.2.1.2. Preparation of resolving gel:

12 mL of 30% acrylamide bis, 7.5 mL of 1.5 M Tris-HCl, 300 µL of 10% SDS, and 10.2 mL of H₂O were mixed together and the reaction mixture was degassed for 15 minutes. To the degassed reaction mixture, 300 µL of 10% APS and 15 µL of tetramethylethylenediamine (TEMED) were added. The solutions were mixed well. Casting stand and frame were assembled and resolving gel solution was poured. Gel was formed in ~30-45 minutes.

2.2.1.3. Preparation of stacking gel:

2.64 mL of 30% acrylamide bis, 5.04 mL of 0.5 M Tris-HCl, 200 µL of 10% SDS, and 12.0 mL of H₂O were mixed and the reaction mixture was degassed for 15
minutes. To the degassed reaction mixture, 100 µL of 10% APS and 20 µL of TEMED were added. Stacking gel solution was poured till the top of the glass plates. The comb was placed and left for 30 minutes until the gel was formed. The 12% SDS-PAGE gel was stored at 4 °C until further use.

2.2.2. SDS-PAGE Analysis:

2.2.2.1. Preparation of samples:

1) 2 µL of human folate binding glycoprotein was added to 47.5 µL of SDS sample buffer and 2.5 µL of 2-mercaptoethanol.

2) 1 mg of FBP from bovine milk was dissolved in 1 mL of 20 mM NH₄HCO₃. 50 µL of prepared sample was added to 47.5 µL of sample buffer and 2.5 µL of 2-mercaptoethanol.

2.2.2.2. SDS-PAGE:

All the samples were heated at 90 °C for 10 minutes. Samples were allowed to cool down. Prepared 12% SDS gel was placed into the instrument. Prepared 1X SDS buffer was added in the gel tank (Preparation of 1X SDS Buffer: 100 mL of 10X SDS PAGE buffer was added to 900 mL of deionized water). 5 µL of standard marker and 20 µL of each sample were loaded on the gel. Voltage applied was 200 V to run the gel. After 45 minutes, the instrument was stopped and the gel was removed from the gel tank. Gel was then placed in the gel box and washed with deionized water for 3 times, 5 minutes each. The gel was left in coomassie blue for an hour with continuous shaking. After 1 hour, the gel was again washed with deionized water and the image was taken using gel scanner.
2.2.3. Western immunoblotting analysis:

2.2.3.1. Preparation of reagents:

Following solutions were prepared fresh prior to the experiment.

Transfer Buffer: 16 liters of transfer buffer solution was prepared by adding 48.48 g of Tris base, 230.56 g of glycine, and 3.2 L of methanol was added to 12.8 L of deionized water.

Nonfat milk: 0.4 mL of Tween was added to the 20 g of milk powder. Deionized water was added to make the final volume 400 mL. The solution was stirred for 15 minutes and pH was then adjusted to 7.5 with 10 N NaOH.

TBST: The solution was prepared by addition of 5 mL of 2 M Tris HCl, 5.84 g of NaCl and 0.5 mL of Tween. The solution was stirred and distilled water was added to 1000 mL.

2.2.3.2. Preparation of 12% SDS gel:

12% SDS Gel was prepared fresh as described above in the section 2.2.1.

2.2.3.3. Preparation of samples:

1) 1.5 µg of folate binding glycoprotein from bovine milk was dissolved in 100 µL of 20 mM NH₄HCO₃. From the prepared solution, 6.6 µL of sample was taken and added to 3 µL of SDS sample buffer.

2) 1.2 µL of human folate binding glycoprotein was taken and to this sample 2 µL of SDS sample buffer was added.

2.2.3.4. SDS-PAGE:

Prepared 12% SDS gel was placed into the instrument. Prepared 1X SDS buffer
was added in the gel tank. 5 µL of standard markers and each of the prepared samples were loaded on the gel. Voltage applied was 100 V to run the gel. After 90 minutes, the instrument was stopped.

2.2.3.5. Transfer of gel:

Prepared transfer buffer was added in a tray. A sponge, a filter paper, and a cassette were soaked in the tray containing transfer buffer. In another tray, methanol was added and a PVDF membrane was soaked carefully to this tray. Gel was then removed from the gel tank. With the help of a gel scrapper, stacking gel was scrapped. The resolving gel was placed in the transfer buffer. Gel was then placed in the cassette containing a sponge and a filter paper. The membrane was then placed followed by filter papers and a sponge. The set up was closed and placed in the gel tank. Transfer buffer was added to the top of the tank. The tank was closed and placed in the cold room with constant stirring. The Voltage applied was 100 V for 1 hour. After 1 hour, the membrane was removed from the cassette and placed in a box containing non-fat milk for 1 hour with continuous stirring. Non-fat milk was discarded after 1 hour. Affinity purified rabbit antibody was added as the primary antibody in the ratio 1:3000 to non-fat milk. Prepared antibody solution was added to the membrane. It was placed on a rocker and left overnight in the cold room. Washing of the membrane was done with the prepared TBST solution at room temperature three times for 10 minutes each. The alkaline phosphatase conjugated goat anti-rabbit antibody was added in the ratio 1:3000 to non-fat milk. Prepared secondary antibody solution was added to the membrane and placed on the rocker and left for 2 hours at room temperature. The solution was discarded after 2 hours. Washing of the membrane was done with the prepared TBST solution at room
temperature three times for 10 minutes each. Membrane was incubated for 1 minute with the chemiluminescence reagent in the dark room. Membrane was placed in the autoradiography cassette. Exposure was done for 1 second with the help of X-ray film.

### 2.3. Mass spectrometric analysis of biomolecules

#### 2.3.1. Analysis of N-linked Glycans from bovine folate binding protein using mass spectrometry:

##### 2.3.1.1. Enzymatic release of N-linked glycans from folate binding protein originating from bovine milk: ¹⁶

1) 1 mg of folate binding glycoprotein was dissolved in 1 mL of 20 mM NH₄HCO₃. Sample was incubated at 90 °C for 5 minutes and allowed to cool down.

2) After the sample was cooled to room temperature, 1 µL of the enzyme PNGase F was added. Reaction mixture was incubated at 37 °C for 22-24 hours.

##### 2.3.1.2. Solid phase extraction of N-linked glycans: ¹⁶

C18 Sep-Pak cartridge was used for solid phase extraction to remove salts and extract glycans.

1) C18 Sep-Pak cartridge was washed with 1 mL of ethanol and this was repeated 2 more times.

2) The cartridge was further washed 3 times with 1 mL of water.

3) Sample was then applied 5 times on the cartridge, and eluted glycans were saved as eluent 1.

4) The cartridge was washed with 1 mL of water and eluted glycans were saved as eluent 2.

5) Deglycosylated folate binding protein was eluted by washing the cartridge with 1 mL
of acetonitrile.

6) Deglycosylated folate binding protein was dried using vacuum evaporator. The dried sample was stored at -20 °C until further use.

2.3.1.3. Purification of N-linked glycans:16

1) An activated charcoal cartridge was used to purify glycans. The cartridge was spun down for 2 minutes at 2000 rpm.

2) 1 mL of ethanol was loaded on the cartridge, which was spun down for 2 minutes at 2000 rpm.

3) 1 mL of water was loaded on the cartridge and spun down for 2 minutes at 2000 rpm.

4) 1 mL of 1 M NaOH was loaded on the cartridge and spun down for 2 minutes at 2000 rpm.

5) 1 mL of water was loaded on the cartridge and spun down for 2 minutes at 2000 rpm twice.

6) 1 mL of 30% acetic acid was loaded on the cartridge and spun down for 2 minutes at 2000 rpm.

7) 1 mL of water was loaded on the cartridge and spun down for 2 minutes at 2000 rpm.

8) 1 mL of 0.1% TFA: 50% ACN (v:v) solution was loaded on the cartridge and spun down for 2 minutes at 2000 rpm.

9) 1 mL of 0.1% TFA: 5% ACN (v:v) solution was loaded on cartridge and spun down for 2 minutes at 2000 rpm twice.

10) Eluent 1 (saved during C18 Sep-Pak Cartridge Extraction, step 3) was loaded on the cartridge and spun down at 2000 rpm for 2 min.

11) Eluent 2 from step 4 was loaded on the cartridge and spun down at 2000 rpm for 2
12) The cartridge was washed with 1 mL of water for 2 minutes at 2000 rpm.
13) The cartridge was further washed with 1 mL of 0.1% TFA: 5% ACN.
14) Samples were eluted with 0.50 mL of 0.1% TFA: 50% ACN in eppendorf tube and repeated one more time.
15) Aliquots in 100 µL were taken from both the tubes in a single tube to run samples for MALDI-MS and ESI-MS as native N-glycans.
16) All samples were dried in vacuum evaporator. The dried samples of native N-glycans were stored at -20 °C until further use.

2.3.1.4. Permethylation of N-linked glycans:

1) To the 2 mL tube, a micro spin column was placed. With the help of 1 mL syringe, ACN was added to the micro spin column.
2) Micro spin column was loaded with NaOH beads using a 1 mL syringe. ACN was filled to the top of the column.
3) The column was spun down at 5 krpm for 30 seconds.
4) 200 µL of DMSO was added to the column and allowed to spun down for 30 seconds.
5) To the micro spin column, 200 µL of DMSO was added and sealed with the parafilm to prevent NaOH beads from getting dried.
6) The dried glycan samples obtained from activated charcoal purification were dissolved in 100 µL of DMSO, 3 µL of H2O, and 84 µL of iodomethane.
7) Parafilm was removed from micro spin column and spun down at 5 krpm for 30 seconds.
8) Micro spin column was moved to a new 2 mL eppendorf tube. Dissolved N-glycans
were loaded in micro spin column and centrifuged at 0.8 krpm for 30 second.

9) N-glycans were loaded again and the same procedure was repeated 7 times.

10) Then, the column was spun down at 8 k rpm for a short time to pull down everything inside the column.

11) The column was eluted with 50 µL of ACN at 0.8 k rpm for 30 seconds and spun down at 10 k rpm to pull down everything. The permethylated N glycans were extracted by liquid phase extraction.

12) To the permethylated eluate, 300 µL of chloroform and 300 µL of water were added.

13) Eluate was shaked and centrifuged at 2000 rpm for 2 minutes. Upper aqueous layer was discarded.

14) The chloroform layer was washed 3 more times with water.

15) The organic phase was dried in vacuum evaporator and then stored at -20 °C until further use.

2.3.1.5. MALDI-TOF and tandem mass spectrometric analysis of native and permethylated N-linked glycans:

The 2,5-dihydroxybenzoic acid (DHB) matrix was prepared by suspending 10 mg of DHB in 1 mL of 1 mM sodium acetate to produce 10 mg/mL matrix solution. The dried native and permethylated N-glycan sample was dissolved in 50:50 (v:v) methanol : water. 0.5 µL of analyte and 0.5 µL of freshly prepared DHB matrix were mixed on MALDI sample plate. The mixture was dried in a vacuum desiccator. All ions observed in the spectra were sodiated. MALDI data on folate binding protein N-glycans were obtained using positive ionization in reflectron mode.

A MALDI TOF-TOF instrument UltrafleXtreme (Bruker Daltonics) was utilized
for the analysis of glycans. This instrument is equipped with a smartbeam-II™ laser emitting at 355 nm with 1 kHz firing rate. MALDI-MS Profiling of both native and permethylated glycans was performed in reflectron positive ion mode. MALDI-TOF/TOF-MS analysis for structural characterization of glycans was performed by using both laser induced dissociation (LID) and collisionally induced dissociation (CID). MS-MS spectra were searched using software SimGlycan (PremierBiosoft). The structures of glycans were predicted using SimGlycan. This software matches experimental MS/MS data generated by mass spectrometry with its own database of the theoretical fragments.

The study of permethylated N-linked glycans was also performed on Omniflex MALDI-MS (Bruker Daltonics) at Bowling Green State University.

2.3.1.6. ESI-Q-TOF and tandem mass spectrometric analysis of permethylated N-linked glycans:

The permethylated N-glycan samples were dissolved in 50:50 (v:v) methanol : water containing 1 mM sodium acetate. The dissolved sample was infused at the flow rate of 150 µL/h into an ESI-Q-TOF mass spectrometer (Q-TOF Micro, Waters, Milford, MA), using nebulizer gas flow rate of 500 L/h and desolvation temperature of 200 °C. Collision gas used was argon. The cone voltage was set at 50 V and scan range from 600-1800 m/z was used. Tandem mass spectrometry experiments were performed for characterization of glycan structures using collision energy in the range between 30 and 40 eV depending on the size of carbohydrate.

2.3.1.7. ESI-ion trap mass spectrometric analysis of permethylated N-linked glycans:

The permethylated sample was dissolved in 50:50 (v:v) methanol:water containing 1 mM sodium acetate. The dissolved sample was infused at the flow rate 90
µL/h into an ESI ion trap instrument (Bruker, Esquire). The following conditions were used for analysis: scan range from 600-2000 m/z, nebulizer flow rate of 3.00 psi, dry gas flow rate of 8.0 L/min, and drying temperature of 305 °C.

2.3.2. *MALDI-TOF mass spectrometric analysis of deglycosylated folate binding protein from bovine milk:*

This study of deglycosylated protein was performed on Omniflex MALDI-MS (Bruker Daltonics) at Bowling Green State University. The dried protein sample was dissolved in 10 µL of TA (0.1% TFA in water:acetonitrile (1:2)). The sinnapinic acid (SA) matrix was prepared by suspending 20 mg in 1 mL of TA. 1 µL of analyte and 1 µL of freshly prepared SA matrix were mixed in eppendorf tube to give a ratio of 1:1. 1 µL of prepared mixture was spotted on a MALDI target plate. The mixture was allowed to air dry. MALDI data on deglycosylated folate binding protein were obtained using positive ionization in linear mode.

2.3.3. *Sample preparation for analysis of N-linked glycans from human folate binding protein using Mass Spectrometry:*

2.3.3.1. *Enzymatic release of N-linked glycans from folate binding protein originating from human milk:*

1) 15 µL of folate binding glycoprotein was added to 985 µL of 20 mM NH₄HCO₃. Sample was incubated at 90 °C for 5 minutes and allowed to cool down.

2) After the sample was cooled to room temperature, 1 µL of the enzyme PNGase F was added. Reaction mixture was incubated at 37 °C for 22-24 hours.
2.3.3.2. **Solid phase extraction of N-linked glycans:**

Solid phase extraction was performed as described in section 2.3.1.2.

2.3.3.3. **Purification of N-linked glycans:**

Purification using activated charcoal column was performed as described in section 2.3.1.3.

2.3.3.4. **Permethylation of N-linked glycans:**

Permethylation and liquid phase extraction were performed as described in section 2.3.1.4.

2.3.4. **MALDI-TOF and tandem mass spectrometric analysis of native and permethylated N-linked glycans:**

The dried native and permethylated N-Glycan sample were dissolved in 50:50 (v:v) methanol:water. 0.5 µL of analyte and 0.5 µL of freshly prepared 2,5-dihydroxybenzoic acid (DHB) matrix were mixed on a MALDI sample plate to give a ratio of 1:1. The mixture was allowed to dry in a vacuum desiccator. All ions observed in the spectra were sodiated. MALDI data on folate binding protein N-glycans were obtained using positive ionization in reflectron mode.

The conditions used for MALDI-MS were similar as discussed in section 2.3.1.5.

2.4. **Imaging of cells**

Purchased KB cell lines were stored in liquid nitrogen until further use.

2.4.1. **Preparation of cell culture media:**

Fetal bovine serum (heat–inactivated), 5,000 [I.U.]/mL penicillin, and 5,000 µg/mL streptomycin were added to DMEM media to give final concentration of 10% FBS, 1% penicillin-streptomycin. Prepared cell culture DMEM media were stored at 4°C.
until further use.

Fetal bovine serum (heat–inactivated), 5,000 [I.U.]/mL penicillin, and 5,000 µg/mL streptomycin, and L-glutamine were added to folate-free RPMI media to give a final concentration of 10% FBS, 1% penicillin-streptomycin, and 2 mM L-glutamine. Prepared F-FRPMI media were stored at 4 °C until further use.

2.4.2. Cell culture:

HeLa cells and KB cells were thawed in a water bath at 37 °C for 1 minute. To the cells, 1-2 mL of prepared cell culture media (DMEM) was added followed by thorough mixing. The mixed solution was transferred in 10 mL tubes. The solution was centrifuged at 500 rpm for 2 minutes and supernatant was discarded. 3 mL of cell culture media was added to the cell pellets and they were further mixed well. 0.5 mL of the mixed solution was added to 10 cm² cell-culture treated Petri dish and 0.1 mL of the mixed solution was added to each well of cell-culture treated 6-well plate. 10 mL of the prepared cell culture media (DMEM) was added to Petri dish and 1 mL of the DMEM media was added to each well of 6-well plate. KB cells were cultured at 37 °C containing 5% CO₂. Cells on 75% confluence were sub-cultured in F-FRPMI media. Media were replaced twice a week and the cell culture media (F-FRPMI) for KB cells were collected. Cells at 95% confluency were split and harvested.

2.4.2.1. Harvesting of cultured cells:

KB cells were scratched with a scraper and collected in a vial. After scrapping, Petri dish was washed twice with 1X PBS to collect as many cells as possible. Cell pellets were stored at -80 °C until further use.
2.4.2.2. Splitting and collection of cells:

HeLa and KB cells at 95% confluence were washed with 1X PBS. After addition of trypsin, the Petri dish was left in the incubator for 5 minutes to detach cell from the surface. 1X PBS was added to Petri dish and the solution was mixed well. 0.5 mL of the mixed solution was added to a new 10 cm² cell culture treated Petri dish and the cells were further grown to culture more cells whereas the remaining mixed solution was transferred to a 10 mL tube. The solution was centrifuged at 500 rpm for 2 minutes. The supernatant was discarded and 1 mL of 1X PBS was added and the solution was centrifuged at 500 rpm for 2 minutes and repeated one more time. After washing the cells with 1X PBS, cell pellets were stored at -80 °C until further use.

2.4.3. \[^{3}H\] folic acid binding assay:

Assay was performed to confirm that enough FBP is present in KB cells. Cells grown in 6-well plate at 95% confluency were left on ice for 10-15 minutes. Cells in each well were washed with 1 mL of an acidic buffer (1 liter of acidic buffer was prepared containing 10 mM of sodium acetate, 150 mM of NaCl, and pH was adjusted to 3.5 with glacial acetic acid) for 1 minute. Cells were further washed with 1 mL of ice cold 1X PBS for 1 minute in each well. 1 mL of freshly prepared cold folate (10 µM of folic acid in ice cold PBS) was added in 3 wells and incubated for 30 minutes on ice whereas in the remaining 3 wells ice cold 1X PBS were added. Hot folate solution was prepared by dissolving 6.9 ng of \[^{3}H\]-folic acid in 6 mL of PBS. The solution from previous step was discarded and 1 mL of freshly prepared hot folate (\[^{3}H\] folic acid) solution was added in all the wells and the well plate was incubated for 1 hour on ice. After one hour incubation, cells were washed with 1 mL of ice cold 1X PBS for 1 minute and this
procedure was repeated one more time. Cells were further washed with acidic buffer for 1 minute. 1 mL of acidic wash solution was added to 10 mL of scintillation fluid. Radio label counting was done using Beckman scintillation counter.

2.4.4. Conjugation of folate to FITC:

1) 2 mg of folic acid was dissolved in 1 mL of freshly prepared 0.1 M NaHCO₃ (pH 9.0).

2) 1 mg of FITC was dissolved in 1 mL of anhydrous DMSO.

3) 50 µL of FITC solution prepared in step 2 was added very slowly in aliquots of 5 µL to folic acid solution prepared in step 1 with continuous stirring.

4) The reaction mixture was incubated at 4 ºC in the dark for 8 h.

5) To this reaction mixture 55.3 µL of 1 M NH₄Cl was added and incubated further at 4 ºC in the dark for 2 hours.

6) Conjugated Folate-FITC was desalted by solid phase extraction.

2.4.4.1. Solid phase extraction of conjugated Folate-FITC:¹⁶

1) C18 Sep-Pak cartridge was washed with 1 mL of ethanol and this was repeated 2 more times.

2) Cartridge was further washed 3 times with 1 mL of water.

3) Sample was then applied 5 times on to a cartridge.

4) Cartridge was washed with 1 mL of water.

5) Conjugated Folate-FITC was eluted with 1 mL of acetonitrile.

6) Conjugated Folate-FITC was dried using vacuum evaporator. The dried sample was stored at -20 ºC until further use.

7) The dried sample was dissolved in 50:50 ACN: H₂O, 3.7% FA (Mobile Phase) to run liquid chromatography- mass spectrometry (LCMS).
2.4.5. HPLC-ESI-Q-TOF mass spectrometric analysis of conjugated Folate-FITC:

The HPLC system used for separation of Folate-FITC consists of LC-20AD binary pump and DGU-20A3 Vacuum degasser (Shimadzu technologies, Addison, IL, USA). Chromatographic separation was performed on a Vydac C18 5 µm (250 × 2.1 mm i.d.) reverse phase column (Vydac, Deerfield, IL, USA). The mobile phase A was HPLC grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.7% formic acid. The flow rate was 0.1 mL/min and injection volume was 5 µL. Elution gradient was applied by keeping the amount of B at 5% for 5 minutes and then increasing the amount of B from 5 to 20% in 10 minutes, from 20 to 35% in 30 minutes, from 35 to 45% in 10 minutes, from 45 to 95% in 10 minutes, and from 95 to 5% in 10 minutes. Run was stopped after 80 minutes. Excitation and emission wavelength used for fluorescence detection were 492 nm and 518 nm, respectively. The described conditions above were optimized conditions used for separation of labeled and unlabeled folate.

The ESI-LC-MS analysis was performed on a Q-TOF mass spectrometer (Waters Inc., Milford, MA, USA) in positive ion mode in the range of m/z 200-900. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. Desolvation temperature was set at 350 ºC and desolvation gas flow rate was 500 L/h. The source temperature was 90 ºC and collision energy was set at 4.0 V. MCP detector was set at 2.35 kV. The scan time was 1.0 s and the interscan time was set to 0.1 s. Data processing was performed using MassLynx software version 4.1.

2.4.5.1. Collection of conjugated Folate-FITC:

After successful separation, separation conditions were further optimized to collect conjugated Folate-FITC as described here. Elution gradient was applied by
increasing the proportion of B from 5 to 20% in 15 minutes, from 20 to 35% in 30 minutes, from 35 to 95% in 1 minute, and from 95 to 5% in 8 minutes. Run was stopped after 55 minutes. The flow rate was 0.3 mL/min and injection volume was 20 µL. Excitation and emission wavelengths used for fluorescence detection were 492 nm and 518 nm, respectively. 20 fractions were collected for conjugated Folate-FITC at the elution time specific for conjugated analyte. Collected fractions were dried completely using vacuum evaporator.

One of the collected conjugated Folate-FITC fraction from HPLC was dissolved in 50:50 ACN: H2O, 3.7% FA. Direct infusion of the dissolved sample was made to ESI-MS to confirm the purity of the collected conjugate. A positive ESI-LC-MS was performed in full spectrum acquisition mode in the range of m/z 250-900. The spray voltage was set at 3.0 kV and sample cone voltage was 40 V with a desolvation temperature was of 130 ºC and desolvation gas flow rate was 350 L/h. The source temperature was 90 ºC and collision energy was set at 4.0 V. MCP detector was set at 2.35 kV. The scan time was 1.0 s and the interscan time was set to 0.1 s.

2.4.6. Excitation and emission spectrum of FITC and Folate-FITC:

Emission spectra of FITC and Folate-FITC were measured using the luminescence spectrometer LS 50B from Perkin Elmer (Beaconsfield, Buckinghamshire, England). To measure the excitation spectrum for conjugated Folate-FITC, excitation wavelength was set in the range from 350 nm to 500 nm and emission wavelength was set at 520 nm. Also, to measure the emission spectrum for Folate-FITC, emission wavelength used was in the range from 460 nm to 700 nm while keeping excitation wavelength constant at 443 nm. Similarly, for FITC excitation spectrum, excitation was
set in the range between 350 nm to 500 nm and emission wavelength was set at 520 nm. For FITC emission spectrum, the emission wavelength used was in the range from 470 nm to 670 nm and excitation wavelength set at 420 nm.

2.4.7. Imaging of live cells and fixed cells:

2.4.7.1. Preparation of Poly-lysine treated cover slip:

Four coverslips were placed in a Petri dish and left in the clean hood with UV light on for 15 minutes. After 15 minutes the UV light was turned off and approximately 4-5 drops of poly-lysine stock solution was spread on each cover slip. The cover slips were left for 3 hours. After 3 hours, each coverslip was washed 3 times with autoclaved distilled water. After washing the coverslips, cover slips were left overnight for drying. After the coverslips were dried, they were placed in the small Petri dish until further use.

2.4.7.2. Preparation of 4% Para-formaldehyde solution:

4 g of para-formaldehyde was dissolved in 100 mL of 1X PBS buffer. The prepared solution was heated in the hood at 70 ºC with continuous stirring.

2.4.7.3. Preparation of mounting media:

10X PBS stock solution was prepared. Stock solution of 20% (w/v) n-propyl gallate in dimethyl sulfoxide was prepared. 1 mL of prepared 10X PBS stock solution was added to 9 mL of glycerol. To this solution, 100 µL of prepared n-propyl gallate stock solution was added with constant stirring.

2.4.7.4. Fixed and live cell labeling:

KB cells were washed 3 times with 1X PBS solution. After washing, cells were suspended in 1X PBS, and labeled with FITC (5 µL of 1 mg/mL FITC solution in DMSO) and folate-FITC (conjugated folate- FITC fraction collected using HPLC). Cells
were incubated at 4 °C for 15 minutes in the dark. After the incubation, cells were washed 3 times with 1X PBS and resuspended in 1 mL of 1X PBS solution. 200 µL of KB cells suspension in 1X PBS solution was added to poly-lysine treated coverslip. The coverslip was left for 45 minutes in the dark. After 45 minutes, 1 mL of para-formaldehyde solution was added to the poly-lysine treated coverslips. The coverslips were left for 30 minutes in the dark. After 30 minutes, coverslips were washed 3 times using 1 mL of 1X PBS solution for 5 minutes each.

For live cell imaging, cells grown in a 6-well plate at 75% confluency were left on ice for 10-15 minutes. Cells were washed with 1 mL of ice cold 1X PBS for 1 minute in each well. 1 mL of freshly prepared folate (10 µM folic acid in ice cold PBS) was added in 2 wells and incubated for 30 minutes on ice whereas in the remaining 4 wells ice cold 1X PBS was added. The solution was discarded and 1 mL of conjugated folate-FITC solution was added in 2 wells and in the other 2 wells FITC solution was added. In the remaining wells PBS was added and the well plate was incubated on ice for 1 hour. After one hour incubation, cells were washed with 1 mL of ice cold 1X PBS for 1 minute and this was repeated one more time. Cells were further washed with 1X PBS and imaged using light microscope.

2.4.7.5. Preparation of microscope slides for fixed cells:

One drop of mounting media was added onto the microscope slide. Coverslip was mounted carefully on the drop of mounting media. Prepared microscope slides were placed at 4 °C until further use after sealing the coverslip around the edges with nail polish.
2.4.7.6. **Cell imaging:**

Cells were imaged by Nikon Eclipse 80i upright microscope after incubation with conjugated Folate-FITC and FITC. 10X Plan Fluor (numerical aperture, NA = 0.3), 40X Plan Fluor (NA = 0.75), and 100X Oil Plan Apo (NA = 1.4) objectives were used for imaging by fluorescence and DIC microscopy. For fluorescence imaging, a FITC filter cube was used. For cell imaging by DIC, one high-resolution oil condenser (NA = 1.4) was used. An X-Cite 120 illuminator (EXFO Life Sciences, Mississauga, Canada) coupled to a liquid light guide was used for fluorescence imaging for illumination of cells, while a halogen lamp was used in DIC imaging for illumination of cells. A Photometrics CoolSNAP ES2 CCD camera (Tucson, AZ) was used for acquisition of cell images. Acquired images were analyzed using NIS Elements imaging software (Nikon).

2.4.8. **Flow cytometric analysis:**

In order to investigate selective cellular uptake of folate-FITC and FITC, KB cells were incubated with conjugated folate-FITC and FITC for 30 minutes and cells were washed three times with 1X PBS. Finally, cells were analyzed by flow cytometry (Accuri Cytometers Inc, Michigan) using FITC-specific excitation and emission settings.

2.5. **Extraction and Purification of Folate Binding Protein**

Cells were grown as described in section 2.4.2.

2.5.1. **Preparation of folate–sepharose gel:**

Folate-sepharose gel was prepared as described by Sawad et al.\textsuperscript{35} Briefly, 8 grams of epoxy activated sepharose 6B was washed with distilled water for 2 minutes. To the hydrated epoxy activated sepharose, 100 mL of 0.1 M NaHCO\textsubscript{3} (pH 10.0, adjusted with 6 M NaOH) was added and resulted reaction mixture was shaken well and equilibrated for
5 minutes. The supernatant was decanted and 100 mL of 0.01 M folic acid (dissolved in 0.1 M sodium bicarbonate, pH 10.0) was added to the gel. The reaction mixture was shaken at 30 °C for 16 hours. The gel was washed on sintered glass filter with 400 mL of 0.1 M NaHCO₃ followed by washing with 100 mL of distilled water, 100 mL of 0.5 M NaCl in 0.1 M NaHCO₃ (pH 8.0), 100 mL of 0.5 M NaCl in 0.1 M sodium acetate (pH 4.0), and 100 mL of distilled water. Above procedure was repeated 4 times with 100 mL of 0.01 M folic acid dissolved in 0.1 M NaHCO₃ (pH 10.0). After 4 repetitions, 80 mL of 1 M monoethanol amine was added to the brownish yellow gel and the gel was shaken for 8 hours at 30°C in the dark. The gel was washed with 400 mL of NaHCO₃ followed by was hing with 100 mL of distilled water, 100 mL of 0.5 M NaCl in 0.1 M NaHCO₃ (pH 8.0), 100 mL of 0.5 M NaCl in 0.1 M sodium acetate (pH 4.0), and 100 mL of distilled water. After preparation of folate-sepharose 6B gel, the column was loaded with the folate-sepharose gel in the cold room to the bed volume of 25 mL. The column was loaded at a flow rate of 1 mL/min. After loading, the column was stored in the cold room at 4 °C.

2.5.2. Sample Preparation for purification of FBP from KB cell lysate and media:

The purification of folate binding protein from the cell culture medium and cell lysate was carried out as described by Ratnam et al. 22

2.5.2.1. Preparation of activated charcoal slurry:

The volume of the slurry was equal 10% of the volume of media and 10% of the volume of cell lysate. To prepare slurry, activated charcoal was added to 10 mM sodium acetate, pH 3.5/1% Triton X-100 at the concentration of 0.5 g of charcoal per mL. The solution was mixed well with the help of a glass rod to form slurry. The pH of the slurry
was maintained at 3.5. The slurry was placed on ice until the temperature of the slurry reached 4 °C and was left on ice until further use.

2.5.2.2. Treatment of cell lysate for purification:

Cell pellets stored at -80 °C were resuspended in 10 mM sodium phosphate and left on ice for half an hour. Cell suspension was freezed and thawed several times. Cells were spun down at 500 rpm for 5 minutes and the supernatant was decanted. Cell lysate was washed with 10 mM sodium acetate (pH 3.5) followed by washing with 1X PBS. Cell lysate was solubilized in 1X PBS/1% Triton X-100. Solubilized cells were centrifuged at 500 rpm for 30 seconds and supernatant was collected. 50 µL of supernatant was used to measure the amount of FBP present in KB cells using [^3]H folic acid binding assay.

50% Triton X-100 was added to the rest of the supernatant to obtain a final concentration of 1% Triton X-100. The solution was placed on ice tray at 4 °C with constant stirring and the pH was adjusted to 3.5 with 1 N HCl.

When the slurry had reached 4 °C, it was transferred to the above solution with constant stirring. The transfer was done on ice to maintain the temperature at 4 °C. The solution was stirred for 5 minutes on ice. It was then transferred to a centrifugation tubes and centrifuged in a refrigerated Beckmann centrifuge at 4 °C at 10,000 rpm for 10 minutes. The clear supernatant was transferred to another beaker which was placed on ice tray to maintain the temperature at 4 °C with constant stirring and the pH was adjusted to 7.5 with drop wise addition of 1 N NaOH. The supernatant was centrifuged again at 4 °C at 10,000 rpm for 10 minutes to remove any cloudy material and the supernatant was collected. 50 µL of supernatant was used to measure the amount of FBP present using

2.5.2.3. Treatment of cell media for purification:

50% Triton X-100 was added to the cell culture media to obtain a final concentration of 1% Triton X-100. The solution was placed on ice tray at 4 °C with constant stirring and the pH was adjusted to 3.5 with 1 N HCl.

When the slurry had reached 4 °C, it was transferred to the solution with constant stirring. The mixed solution was maintained at 4 °C using ice bath. The solution was stirred for 5 minutes. It was then transferred to centrifugation bottles and centrifuged in a refrigerated Beckmann centrifuge at 4 °C at 10,000 rpm for 10 minutes. The clear supernatant was transferred to another beaker which was placed on ice tray to maintain the temperature at 4 °C with constant stirring and the pH was adjusted to 7.5 with drop wise addition of 1 N NaOH. The supernatant was centrifuged again at 4 °C at 10,000 rpm for 10 minutes to remove any cloudy material, and supernatant was collected. 50 µL of supernatant was used to measure the amount of FBP present using [^3]H folic acid binding assay.

2.5.2.4 [^3]H folic acid binding assay:

The entire assay was performed on ice using the following procedure.

1) The solution of 1X PBS/1% Triton X-100 (pH 7.4) was prepared. The solution was placed in ice bath.

2) The suspension of 80 mg/mL of activated charcoal in 1X PBS/1% Triton X-100 was prepared and placed in ice bath.

3) 0.4 µL of hot folate was added to 2 mL of 1X PBS/1% Triton X-100. The solution was kept in ice bath.
4) 500 µL of solution prepared in step 3 was added to 2 mL eppendorf tube. 50 µL of supernatant from cell culture media was added to the tube as well.

5) To the 500 µL of solution prepared in step 3, 50 µL of supernatant from cell lysate obtained before treatment to activated charcoal slurry was added.

6) 500 µL of solution prepared in step 3 above was added to 2 mL eppendorf tube. 50 µL of supernatant obtained from cell lysate after the treatment with activated charcoal slurry was added to the tube.

7) The solutions in step 4, 5, and 6 were incubated at 37 ºC for 1 hour.

8) After 1 hour the solutions in step 7 were placed in an ice bath for 10 minutes. After 10 minutes, 500 µL of solution prepared in step 2 was added and the solutions were vortexed gently.

9) The vortexed solutions were then centrifuged at 10,000 rpm for 10 minutes in the cold room. The supernatants of all the samples were carefully added to separate tubes containing 5 mL of scintillation fluid.

10) The solutions were mixed well and counted for the radioactivity using scintillation counter.

2.5.2.5. Column chromatography for purification of FBP from cell media:

Using a peristaltic pump the folate-sepharose 6B column was equilibrated with 1X PBS for ~ 3-4 hours. The column was washed with 1 L of 1X PBS /1% Triton X-100 followed by 1 L of the same buffer containing 0.5 M NaCl, then with 500 mL PBS with no detergent. The supernatant from cell culture media was applied to the equilibrated folate-sepharose 6B column. The folate binding protein was eluted with 10 mM sodium acetate (pH 3.5) in 5 mL fractions and 20 fractions were collected. Each fraction was
neutralized by adding a pre-titrated amount (~100 µL) of 1 M sodium phosphate buffer (pH 7.5) to bring the pH up to ~7.0. 50 µL of each fraction was used to measure the amount of FBP using [³H] folic acid binding assay.

2.5.2.6. [³H] folic acid binding assay:

The assay was performed on ice using the following procedure.

1) The solution of 1X PBS/1% Triton X-100 (pH 7.4) was prepared. The solution was placed in an ice bath.

2) The suspension of 80 mg/mL activated charcoal in 1X PBS/1% Triton X-100 was prepared and placed in an ice bath.

3) 3.6 µL hot folate was added to 18 mL of 1X PBS/1% Triton X-100. The solution was placed in an ice bath.

4) 500 µL of solution prepared in step 3 was added to 21 eppendorf tubes. To these tubes, 50 µL of each fraction collected from the column were added.

5) The solutions were mixed well. The mixed solutions were incubated at 37 ºC for 1 hour.

6) After 1 hour, the solutions were placed on ice bath for 10 minutes. After 10 minutes, 500 µL of solution prepared in step 2 was added to each tube and the solutions were vortexed gently.

7) The solutions were centrifuged at 10,000 rpm for 10 minutes in the cold room. The supernatant of all the tubes were carefully added to separate tubes containing 5 mL of scintillation fluid. The solutions were mixed well and were counted for the radioactivity with the help of scintillation counter.
2.5.3. *Column chromatography for purification of FBP:*

2.5.3.1. *Column chromatography for purification of FBP from cell lysate:*

The folate-sepharose 6B column was equilibrated with 1X PBS for ~ 3-4 hours. The column was washed with 1 L of 1X PBS /1% Triton X-100 followed by 1 L of the same buffer containing 0.5 M NaCl, then with 500 mL PBS with no detergent. The supernatant from cell lysate was applied to the equilibrated folate-sepharose 6B column. The folate binding protein was eluted with 10 mM sodium acetate (pH 3.5) in 5 mL fractions and 10 fractions were collected. Each fraction was neutralized by adding a pre-titrated amount (~100 µL) of 1 M sodium phosphate buffer (pH 7.5) to bring the pH up to ~7.0. 50 µL of each fraction was used to measure the amount of FBP using [³H] folic acid binding assay.

2.5.3.2. [³H] folic acid binding assay:

The assay was performed using the procedure as described in the section 2.5.2.6.

2.5.4. *Measurement of absorption spectra of the fractions collected from column chromatography:*

Absorption spectra of the fractions of cell culture media collected during purification were measured in quartz cuvettes across the wavelength range from 200 nm to 820 nm using the Nicolet Evolution 300 Spectrophotometer from Thermo Electron Corporation (Madison, Wisconsin). The blank used for fractions of media was the elution buffer. The absorption spectra for all of the above samples were measured using the spectrometer with PDA detector.
2.5.5. Measurement of excitation and emission spectra of the fractions collected from column chromatography:

Emission spectra of fractions of cell culture media collected during purification were measured in quartz cuvettes using the luminescence spectrometer LS 50B from Perkin Elmer. To measure the emission spectrum, excitation wavelength was set at 270 nm and emission wavelength used was in the range from 290 nm to 500 nm. Also, to measure excitation spectrum, the emission wavelength was set at 370 nm and excitation wavelength range used was 200 nm to 340 nm. To measure the emission spectrum of folic acid, 1 mg of folic acid was dissolved in elution buffer (5 mL of sodium acetate (pH 3.5) and 150 µL of 1M sodium phosphate (pH 7.5) was added to adjust pH to 7.0). The excitation wavelength used was 270 nm and emission wavelength range was between 290 nm and 500 nm.

2.5.6. Coomassie Blue stain analysis of collected fractions:

2.5.6.1. 12% SDS-PAGE gel was prepared as described in section 2.2.1.

2.5.6.2. Preparation of samples for SDS-PAGE and Coomassie staining:

Samples were prepared to run on the SDS-PAGE as described:

1) 1 mL of fraction numbers 5 to 14 of cell culture media collected from the folate sepharose column were taken and the samples were dried using vacuum evaporator. The dried samples were stored at -20 °C until further use. Dried samples were redissolved in 50 µL of H2O before loading on the SDS-PAGE.

2) 50 µL of cell lysate supernatant before treatment with activated charcoal slurry was used to load on the SDS-PAGE.

3) 50 µL of cell lysate supernatant after treatment with activated charcoal slurry was used
to load on the SDS-PAGE.

47.5 µL of sample buffer and 2.5 µL of 2-mercaptoethanol were added to all of the above samples. All samples were heated at 90 °C for 10 minutes. Samples were allowed to cool down.

2.5.6.3. SDS-PAGE analysis for Coomassie staining:

Prepared 12% SDS gel was plunged into the gel electrophoresis apparatus. Then, prepared 1X SDS buffer was added to the gel tank. 5 µL of standard markers and 20 µL of each sample were loaded on the gel. To run the gel, the voltage applied was 200 V. After 45 minutes, the instrument was stopped and the gel was removed from the gel tank. Gel was then placed in the gel box and washed with deionized water for 3 times, 5 minutes each. Gel was imaged using a Typhoon scanner. After Typhoon imaging, the gel was left in coomassie blue for an hour with continuous shaking. After 1 hour, the gel was again washed with deionized water and image was taken using gel scanner.

2.5.7. Silver stain analysis of collected fractions:

2.5.7.1. Preparation of samples for silver staining:

1 mL of fraction numbers 5 to 14 of cell culture media collected from folate sepharose column was taken and the samples were dried using vacuum evaporator. The dried samples were stored at -20 °C until further use. Dried samples were redissolved in 50 µL of H2O before loading on the SDS-PAGE. 47.5 µL of sample buffer and 2.5 µL of 2-mercaptoethanol were added to all of the above samples. All the samples were heated at 90 °C for 10 minutes. Samples were allowed to cool down.
2.5.7.2. *Preparation of silver staining solution*:

Silver staining solution was prepared 10 minutes before use as described. To 35 mL of deionized water, 5 mL of silver complex solution, 5 mL of reduction moderator solution, and 5 mL of image development reagent were added. The prepared solution was stirred with the help of magnetic bar. To this solution, 50 mL of the room temperature development accelerator solution was added (12.5 g of development accelerator was added to 237.5 mL of distilled water. The solution was stirred with the help of stirrer bar. Water was added till 250 mL, and solution was stored at 4°C until further use).

2.5.7.3. *SDS-PAGE analysis for silver staining*:

Prepared 12% SDS gel was plunged into the instrument. Now, prepared 1X SDS buffer was added to the gel tank. 5 µL of standard markers and 20 µL of each sample were loaded on the gel. To run the gel, voltage applied was 200 V. After 45 minutes, the instrument was stopped and the gel was removed from the gel tank. Gel was then placed in the gel box and washed with deionized water for 3 times, 5 minutes each. Gel was imaged using Typhoon scanner. Gel was then placed in 200 mL of fixative enhancer solution with constant shaking for 20 minutes. After 20 minutes, the solution was decanted and washing of the gel was done 3 times with 200 mL of deionized water, 10 minutes each. 50 mL of silver staining solution was quickly added to the gel box. The solution was left in the box for 20 min. The solution was decanted and 200 mL of 5% acetic acid solution was added to stop the staining reaction for 15 minutes. After 15 minutes, gel was washed with HPLC grade water for 10 minutes. The gel image was taken using gel scanner.
Chapter 3: Results and Discussion

Folate binding protein is currently the focus of extensive research for cancer diagnosis and treatment due to the role of this glycoprotein as an important tumor-associated antigen. Hence, it is essential to fully understand the structural characteristics of the molecule. The proteolytic processing and N-glycosylation sites of FBP have been investigated, but no studies have defined the N-glycan structures present on this molecule. Here, we describe structural studies on the N-glycans present in commercial FBP originating from two species: *Bos taurus* and *Homo sapiens*. We have also worked on imaging of FBP in cancer cell lines and its isolation from cancer cells.

3.1. Electrophoretic and Western blot analysis of FBP originating from bovine and human milk

SDS-PAGE and Western blot analyses were initially employed to confirm the purity and identity of the commercial FBP samples. Coomassie blue staining of FBP samples indicated heterogeneity of the purified proteins and their distributions on the gel due to glycosylation and aggregation, with latter being especially noticed in the sample of human FBP (Figure 4). Several bands corresponding to glycosylated FBP from bovine milk showed apparent molecular weight around 30 kDa. These bands were easily detected by Western blot analysis using the affinity-purified rabbit anti-FBP antibody in combination with alkaline phosphatase conjugated goat anti-rabbit antibody (Figure 4). Several high molecular mass bands were detected by Coomassie staining and Western
blotting of FBP from human milk. According to the manufacturer, these high molecular mass bands are due to aggregates of FBP.

**Figure 4.** Electrophoretic and Western blot analysis of FBP from human and bovine milk. Western blotting was performed using affinity-purified rabbit anti-FBP antibody as the primary antibody (A). The gels were also stained with Coomassie (B). Molecular masses of markers are indicated for each panel.
3.2. Mass spectrometric analysis of biomolecules

3.2.1. Analysis of N-linked glycans from bovine folate binding protein using mass spectrometry:

Glycans were enzymatically cleaved from glycoprotein using enzyme PNGase F. The released glycans were separated from protein and were analyzed by matrix-assisted laser desorption/ionization (MALDI)-MS and electrospray ionization (ESI)-MS after purification using a C18 Sep-Pak cartridge and an activated charcoal column. Purified native glycans were permethylated and extracted.

3.2.1.1: MALDI-TOF and tandem mass spectrometric analysis of native and permethylated N-linked glycans:

Released N-glycans were examined by reflectron MALDI mass spectrometry using 2,5-DHB as the matrix. N-glycans present in glycoprotein have a composition consistent with high mannose (H₅N₂, H₆N₂, H₇N₂, H₉N₂) and hybrid/complex (H₄N₄, H₅N₄, H₆N₄) N-linked glycans. Glycan profile of native N-glycans was maintained even after permethylation using iodomethane. For example, H₅N₂, H₆N₂, H₅N₄, H₆N₄ glycan ions which were present in MALDI-MS of native N-glycans (Figure 5), were still obtained in MALDI-MS of permethylated N-glycans (Figure 7). These glycan structures were detected as singly charged ions using MALDI-MS. Putative structures of N-linked glycans were assigned based on measured MS data, and compared to structures of bovine glycans found in the Functional Glycomics Glycan Database as shown in Tables 1 and 2. The glycan profile obtained from MALDI-MS was similar to the glycan profile recorded by ESI-MS as described in the following section.
Figure 5. MALDI-TOF mass spectrum of native N-linked glycans from bovine FBP.

Several N-glycan ions observed in MALDI-TOF-MS were further selected and subjected to fragmentation using LID to assist sequence assignment. The singly charged $[\text{M+Na}]^{+1}$ ion (m/z 1663.6) (Figure 6 a), yields various daughter ions characteristic of these oligosaccharides: the most intense B-ions (e.g., 1442.578, 1239.332, 550.002,
Figure 6 (a). LID-MALDI-TOF/TOF mass spectrum of native N-linked glycan (H₂N₄) from bovine FBP [(M+Na)⁺, m/z = 1663.6].

387.834), less intense Y-ions (e.g., 1501.463, 1298.359, 446.920, 243.851), and several other daughter ions resulting from cross-ring fragmentation. An abundance of LID-formed cross-ring fragmentation ions was obtained which is unusual for these daughter ions due to sufficient energy needed to produce such fragmentation.³⁰ The presence of α1-6 linkage for β antennae was suggested by ⁰₄A₄ ion (m/z = 609.033). The presence of α1-3 linkage for α antennae was suggested by ²⁴X₂ ion (m/z = 1077.276). ⁰₂X₃α/B₃β.
fragment ion (m/z = 428.960) shows the presence of β1-2 linkage. Other cross-ring fragment ions were also observed in the MALDI spectrum as shown in the inset of Figure 6 (a). One of the most intense ion observed in MS/MS spectra which results from the cleavage of 3-linked antenna and two reducing-end GlcNAc (i.e. B₄/Y₃α, m/z = 712.057) corresponds to D ion. The presence of this daughter ion suggests and confirms the identity of native H₂N₄ glycan ion as the structure shown in the inset of Figure 6 (a).
Figure 6 (b). LID-MALDI-TOF/TOF mass spectrum of native N-linked glycan (H$_5$N$_2$)
from bovine FBP [(M+Na)$^+$, m/z = 1257.4].
The abundance of glycosidic bond fragmentation was observed in the case of high-mannose N-glycan at m/z = 1257.4 (H₅N₂). LID MS/MS spectrum of this parent ion is shown in Figure 6 (b). This spectrum exhibits extensive glycosidic bond cleavage yielding fragment ions (B ions, Y ions) with high intensity in addition to the ⁰²A₅ and ¹³X₀ cross-ring fragment ions. The B series of fragment ions was observed (B₂α, m/z 508.468; B₃, m/z 832.764; B₄, m/z 1036.140). ⁰⁴X₂ fragment (m/z = 711.649) confirms the presence of α₁-6 linkage for α antennae and ¹⁵X₃β ion (m/z = 1124.611) confirms the presence of α₁-3 linkage for the β antennae. One of the most intense ions observed in MS/MS spectra results from the cleavage of 3-linked antenna and two reducing-end GlcNAc (i.e., B₃/Y₃α, m/z 670.634), and corresponds to D ion.³⁰ These daughter ions confirm the sequence and structure of a parent ion with m/z of 1257.4 (H₅N₂) as shown in the inset of Figure 6 (b).
Figure 7. MALDI-TOF mass spectrum of derivatized N-linked glycan released from bovine FBP.
Similarly, permethylated N-glycan ion at m/z 2070.3 ($H_5N_4$) was selected and fragmented. The singly charged $[M+Na]^{+1}$ ion (m/z = 2070.3) (Figure 8), upon LID, yields various daughter ions characteristic of this oligosaccharide: the most intense C-ions (i.e., 1811.269, 1565.725, 708.971, 504.864, 259.846), less intense Y-ions (i.e., 1852.317, 1606.709, 1402.657, 544.971, 299.847) and B-ions (i.e., 1793.219, 485.866) and several other daughter ions resulting from cross-ring fragmentation. The presence of $^{0.4}X_2/C_5$ (m/z = 1084.023) cross-ring fragmentation ion indicates α1-6 linkage for the β antennae and $^{2.4}X_2$ ion (m/z = 1339.482) indicates α1-3 linkage for α antennae.

**Figure 8.** LID-MALDI-TOF/TOF mass spectrum of permethylated N-linked glycan ($H_5N_4$) from bovine FBP ($[M+Na]^{+1}$, m/z = 2070.3).
3.2.1.2: MALDI-TOF mass spectrometric analysis of permethylated N-linked glycans:

Released N-glycans were also examined by reflectron MALDI mass spectrometry with 2,5-DHB as the matrix in the Department of Chemistry at Bowling Green State University. Similar N-glycans were observed as discussed above (data not shown).

3.2.1.3: ESI-Q-TOF and tandem mass spectrometric analysis of permethylated N-linked glycans:

Profiling of glycans was also performed on an ESI-Q-TOF mass spectrometer. The MS spectrum obtained is shown in Figure 9. The peaks represent different glycan structures present at the glycosylation sites. MS analyses indicated that FR contains putative structures that correspond to high-mannose, hybrid, and complex N-linked glycans. Triply and doubly charged ions were assigned to the N-glycan structures. Putative structures of N-linked glycans were assigned based on measured MS as represented in Table 2 and compared to structures of bovine glycans found in Functional Glycomics Glycan Database.36
Several N-glycan ions observed in ESI-Q-TOF-MS were further selected and subjected to fragmentation using CID-ESI-MS/MS to assist sequence assignment. For example, parent ion \((\text{H}_4\text{N}_4\text{+2Na})^{+2}\) (m/z = 944.4) was subjected to ESI-MS/MS which produced a doubly charged \((\text{C}_5^{+2})\) fragment ion at m/z 814.56 (\(\text{H}_4\text{N}_3\text{+2Na})^{+2}\) and singly charged \((\text{Y}_1^{+}, \text{C}_4^{+}, \text{Z}_2^{+}, \text{B}_{1a}^{+}\)) fragment ions at m/z 282.1 (\(\text{N+Na})^{+1}\), m/z 1361.6 (\(\text{H}_4\text{N}_2\text{+Na})^{+1}\), m/z 527.3 (\(\text{N}_2\text{+Na})^{+1}\), and m/z 268.1 (\(\text{H}+\text{Na})^{+1}\) as shown in Figure 10 (a).

The presence of these fragment ions confirms the identity and structure of parent ion.
(H₄N₄+2Na)⁺² as shown in Table 2. Another parent N-glycan ion (H₅N₄+2Na)⁺² (m/z = 1046.5) produced doubly-charged (C₄⁺² and C₅⁺²) fragment ions at m/z 783.4 (H₃N₂+2Na)⁺³ and m/z 916.9 (H₅N₃+2Na)⁺² and singly-charged (C₄⁺ and Z₂⁺) fragment ions at m/z 1565.8 (H₅N₂+Na)⁺¹ and m/z 527.3 (N₂⁺+Na)⁺¹ as shown in Figure 10 (b). The presence of fragment ions confirms the structure of the parent ion as H₅N₄ as shown in Table 2. The nomenclature by Domon and Costello was used for fragmentation processes.³¹
Figure 10 (a). CID-ESI-MS/MS spectrum of permethylated N-linked glycan (H₄N₄) from bovine FBP [(M+2Na)⁺², m/z = 944.42].
Figure 10 (b). CID-ESI-MS/MS spectrum of permethylated N-linked glycan (H$_5$N$_4$) from bovine FBP [(M+2Na)$^{+2}$, m/z = 1046.5].

3.2.1.4: ESI-ion trap mass spectrometric analysis of permethylated N-linked glycans:

N-linked glycans were released from FBP by enzymatic digestion with PNGase F and purified with Sep-Pak cartridges. Released and purified N-glycans were analyzed by ESI-ion trap-MS and results were compared with ESI-Q-TOF instrument. Mass spectrum (Figure 11) shows that most of the N-glycans obtained were similar to those obtained by ESI-Q-TOF instrument. Most of doubly, triply and singly charged ions were detected. The spectrum indicates that FBP is rich in N-linked glycans, having a composition
consistent with high-mannose structures (Hex$_{6-8}$HexNAc$_2$) and complex/hybrid type glycan structures. But hybrid and complex N-linked glycans (Hex$_{3-9}$HexNAc$_{3-9}$) mostly do not contain fucose and sialic acid building blocks in their composition. The data consists of characteristic features: 1) N-linked glycans correspond to high mannose, complex and hybrid type N-linked glycans. 2) The amount of fucose in N-linked glycan structures obtained was relatively low. Putative structures of N-linked glycans were assigned based on measured MS data as represented in Table 2 and compared to structures of bovine glycans found in Functional Glycomics Glycan Database.\textsuperscript{36}

\textbf{Figure 11.} ESI-Ion Trap mass spectrum of permethylated N-linked glycans from bovine FBP.
Table 1. MALDI-MS assignments of native FBP glycans originating from bovine milk. a

<table>
<thead>
<tr>
<th>m/z measured</th>
<th>charge</th>
<th>m/z calculated</th>
<th>Proposed glycan</th>
<th>putative structure</th>
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<td>1257.433</td>
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<td></td>
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<td>1+</td>
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<td>1+</td>
<td>1905.645</td>
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</tr>
</tbody>
</table>

a S represents sialic acid (diamonds); F represents fucose (triangles); H represents hexose (mannose open circles, galactose solid circles); N represents N-acetyl glycosamine (solid squares) and N-acetyl galactosamine (open square).
Table 2. Assignments of permethylated FBP glycans originating from bovine milk.\(^a\)

<table>
<thead>
<tr>
<th>m/z measured</th>
<th>charge</th>
<th>m/z calculated</th>
<th>Proposed glycan</th>
<th>putative structure</th>
</tr>
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<td></td>
</tr>
</tbody>
</table>

\(^a\) S represents sialic acid (diamonds); F represents fucose (triangles); H represents hexose (mannose open circles, galactose solid circles); N-represents N-acetyl glycosamine (solid squares) and N-acetyl galactosamine (open square).
3.2.2: MALDI-TOF mass spectrometric analysis of deglycosylated folate binding protein from bovine milk:

FBP protein after deglycosylation using enzyme PNGase F was subjected to MALDI-TOF-MS and molecular weight of deglycosylated folate binding protein was found to be approximately 26.4 kDa as shown in Figure 12. Besides a singly charged peak for deglycosylated protein, peaks that correspond to singly charged multimers of deglycosylated FBP, dimer and trimer of the FBP were also found by MALDI-MS. The mass of the deglycosylated FBP protein is less than the mass of FBP glycoprotein shown by Western blotting which confirms that we successfully released the glycans from the protein.

Figure 12. MALDI-TOF mass spectrum of deglycosylated FBP from bovine milk.
3.2.3. MALDI-TOF and tandem mass spectrometric analysis of N-linked glycans from human folate binding protein using mass spectrometry:

Released N-glycans from commercial sample of FBP from human milk were examined by MALDI mass spectrometry in positive reflectron mode with 2,5-DHB as the matrix. Native N-glycans have composition consistent with predominating neutral complex-type chains of bi- and tri-antennary structures and one to three fucose residues (Fuc0-3Hex3-6HexNAc2-5) as shown in Figure 13 and Table 3. Permethylated N-glycans showed masses that are compatible with high mannose structures (Hex5-6HexNAc2) whereas the remaining masses correspond to complex-type N-glycans of bi-antennary

![MALDI-TOF mass spectrum](image)

**Figure 13.** MALDI-TOF mass spectrum in positive ion mode of native N-linked glycans from human FBP.
structures \((\text{NeuAc}_{0.1}\text{Fuc}_{0.1}\text{Hex}_{3.6}\text{HexNAC}_{3.5})\), as shown in Figure 15 and Table 4. Permethylated N-glycans profile shows the presence of sialylated N-glycans which were not present in the native N-glycan profile due to sialic acid loss. These glycan structures were detected as singly charged ions using MALDI-MS. Putative structures of N-linked glycans were assigned based on measured MS data.\(^{36}\)

Several of the major components (native and permethylated N-glycan ions) observed in MALDI-TOF-MS were further selected and subjected to fragmentation using MALDI-TOF/TOF to assist sequence assignment. The most abundant singly charged

**Figure 14.** LID-MALDI-TOF/TOF mass spectrum of native N-linked glycan \((\text{F}_{1}\text{H}_{5}\text{N}_{4})\) from human FBP \([\text{M+Na}]^{+1}, \text{m/z} = 1809.7\).
Fuc$_1$Hex$_5$HexNAc$_4$ ion (m/z 1809.7) derived from human milk, revealed various daughter ions characteristic of this oligosaccharide: the most intense Y-ions (i.e., 1444.127, 1282.247, 592.894) and B-ions (i.e., 1442.309, 1238.761, 549.237, 387.210) and several other daughter ions resulting from cross-ring fragmentation as shown in Figure 14. $^{0,4}$A$_4$ fragment ion (m/z = 609.236) confirms the α1-6 linkage and $^{1,3}$X$_2$/C$_5$ fragment ion (m/z = 873.430) confirms the presence of α1-3 linkage for the 3-linked antennae. The intense Y$_{1y}$/Y$_{5α}$ ion at m/z 1501.421 indicates loss of terminal fucose species. One of the most intense ions observed in the MS/MS spectra which results from the cleavage of 3-

**Figure 15.** MALDI mass spectrum of permethylated N-linked glycans released from FBP.
linked antenna and two reducing-end GlcNAc (i.e., B_4/Y_3α, m/z 711.272) corresponds to D ion. The glycosidic bond and cross-ring fragmentation confirms the sequence and structure of parent ion with m/z 1809.7 (F_1H_5N_4) shown in the inset in Figure 14.

Similarly, permethylated N-glycans ion at m/z = 2244.231 (F_1H_5N_4) was selected and fragmented to determine the structure of the glycans. The singly charged sodiated ion upon LID yields various daughter ions as described: The complete Y-series of ions (2056.822, 1781.764, 718.874, and 474.375) was observed along with a C-ion (260.219) and a B-ion (486.343) as shown in Figure 16. The presence of Y_1γ ion (2056.822)

![Figure 16. LID-MALDI-TOF/TOF mass spectrum of derivatized N-linked glycan (F_1H_5N_4) from human folate receptor [(M+Na)^+], m/z = 2244.186.](image-url)
indicates that the fucose residue is present at the terminal GlcNac residue. A number of cross-ring fragmentation ions were also observed which were very important to determine the linkages for terminal fucose and 3-linked antennae. For example, $^{3,5}\text{A}_{6}/\text{Y}_{4\alpha}$, (m/z = 1593.074) $^{0,4}\text{X}_{1\gamma}/\text{Y}_{2}$, (m/z = 676.421) and $^{1,3}\text{X}_{1\gamma}/\text{Y}_{2}$ (m/z = 631.473) ions indicate $\alpha1$-6 linkage for the terminal fucose. Similarly, $^{0,4}\text{X}_{2}/\text{B}_{4}$ (m/z = 821.778) ion suggests the presence of $\alpha1$-6 linkage for the 6-linked antennae.

**Table 3.** Assignments of native FBP glycans originating from human milk. a

<table>
<thead>
<tr>
<th>$m/z$ measured</th>
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<th>$m/z$ calculated</th>
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a S represents sialic acid (diamonds); F represents fucose (triangles); H represents hexose (mannose open circles, galactose solid circles); N-represents N-acetyl glycosamine (solid squares) and N-acetyl galactosamine (open square).
Table 4. Assignments of permethylated FBP glycans originating from human milk.\(^a\)

<table>
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</table>

\(^a\) S represents sialic acid (diamonds); F represents fucose (triangles); H represents hexose (mannose open circles, galactose solid circles); N-represents N-acetyl glycosamine (solid squares) and N-acetyl galactosamine (open square).
3.3. Live and fixed cell imaging using light microscopy

Live and fixed cells were imaged to measure the level of folate binding protein in cells using fluorescence microscopy after labeling with folic acid conjugated to fluorescein isothiocyanate (FITC).

3.3.1. Levels of FBP in HeLa and KB cell lines:

HeLa and KB cells were grown in 6-well plates. Live cells were subjected to $[^3\text{H}]$ folic acid binding assay in order to measure the level of FBP. Cells were incubated with $[^3\text{H}]$ folic acid in the absence and presence of an excess of cold FA and the amount of $[^3\text{H}]$ folic acid bound to FBP was measured. When an excess of cold FA was added, the amount of $[^3\text{H}]$ bound was reduced to background levels. This assay confirms the presence of FBP in live cells in both cell lines. The binding assay also indicates the presence of more FBP in KB cells than in HeLa cells (data not shown). Therefore, further studies were done using KB cell lines. The KB cells were lysed by repetitively freezing and thawing them and the lysate was assayed for the binding of $[^3\text{H}]$ folic acid. A significant amount of intracellular $[^3\text{H}]$ folic acid-binding protein was detected in the cells (Table 5).

3.3.2. HPLC-ESI-Q-TOF mass spectrometric analysis of conjugated Folate-FITC:

Folic acid was conjugated with FITC using the manufacturer’s protocol (Sigma Inc). Conjugated folic acid was separated from free FITC and free folic acid using reverse phase high-performance liquid chromatography (RP-HPLC). The separated analytes were detected and characterized by fluorescence and MS detectors (Figure 17). Successful separation of conjugated folate-FITC is achieved after labeling of folic acid with FITC. Conjugated folate-FITC was separated from free folate and free FITC.
Retention times of 17.2 minutes, and 25.3 minutes were measured for free folate and conjugated Folate-FITC, respectively (Figure 17). Separated Folate-FITC was collected from the HPLC outlet and further used for labeling of the FR in live and fixed cells. The mass spectrum of collected folate-FITC is shown in Figure 18.

![Figure 17. HPLC-MS separation of conjugated Folate-FITC reaction mixture. Chromatograms were recorded by a spectrofluorometric detector (blue trace) and a mass spectrometer (pink trace).](image)
Figure 18. ESI-Q-TOF mass spectrum of conjugated folate-FITC.

3.3.3. Excitation and emission spectrum of FITC and Folate-FITC:

Emission spectra of FITC and purified folate-FITC were obtained using emission wavelength range from 510 to 580 nm while keeping excitation wavelength at 494 nm. After comparison of two emission spectra, we concluded that folic acid was labeled successfully with FITC (data not shown). A broad emission band was observed for Folate-FITC conjugate with an emission maximum at 518 nm.

3.3.4. Imaging of cells after labeling with derivatized folic acid:

Studies suggest that many carcinomas overexpress folate binding protein. In contrast, normal cells lack measurable folate binding protein. FBP expressing cells can be selectively labeled in vivo or in vitro by fluorescent folate conjugates that bind FBP with nanomolar affinity. To identify the potential of the FITC-Folate conjugate for
fluorescent tagging of FBP in cells, KB cells were labeled with folate-FITC and FITC. Fluorescence signals of folate-FITC and FITC in cells were collected through optical filters, and imaged using a charge-coupled device (CCD) camera. Cells were incubated with dyes for 15 minutes at 4 °C in the dark. After 15 minutes, cells were washed with PBS for clearance of unbound dye. Figures 19 (a) and (b) show the overlap between the fluorescence and DIC images of KB cells labeled with Folate-FITC and FITC. FITC

![Figure 19](image)

**Figure 19.** (a) Overlap of DIC image and fluorescent image of KB cells labeled with conjugated Folate-FITC, (b). Overlap of DIC image and fluorescent image of KB cells labeled with FITC.

binds to the cellular proteins while folate-FITC binds to the folate binding protein present in the cell. The distribution of folate-FITC throughout the cell indicated that the conjugate was also transferred into the cell by endocytosis. After cells were imaged it was clear that cells labeled with Folate-FITC provide brighter fluorescence signals than cells labeled with FITC. These results were supported by flow cytometry experiments.
3.3.5. Flow cytometric analysis:

To investigate binding of folate-FITC to the folate binding protein in the cancer cells, KB cells were employed as FBP positive cancer cells. As shown in Figure 20, the results of flow cytometry of the KB cells show a greater fluorescence of cells labeled with folate-FITC than cells incubated with FITC. This confirmed that FITC conjugated folic acid specifically bound to KB cells.

**Figure 20.** Flow cytometric analysis of the KB cells treated with Folate-FITC (a) and FITC (b).

3.4. Isolation and purification of FBP from KB cell line

KB cells were cultured in F-FRPMI media supplemented in 10% FBS, pencillin-streptomycin and L-glutamine. Figure 21 shows the DIC image of live KB cells. Separation and purification of folate binding protein in KB cells and cell culture medium was performed on a folate-sepharose column using procedure from Ratnam et al. After treating the cell lysate and cell culture media with activated charcoal slurry, the lysate
and media supernatant were assayed by the $[^3]H$ folic acid binding assay. No significant amount of FBP was detected in the lysate and the media (Table 5). Supernatant was loaded on the epoxy activated folate-sepharose column to isolate and purify FBP. The folate binding protein was eluted from the column with 10 mM sodium acetate, pH 3.5 in 5 mL fractions. Each fraction was neutralized by adding pre-titrated amount of 1 M sodium phosphate buffer, pH 7.5 to bring pH to 7.0.

Figure 21. Microscope image of live KB cells.

Table 5. $[^3]H$ folic acid binding assay counts per minutes (CPM) obtained for FBP from KB cells, KB cells lysed and solubilized using Triton X-100, KB cell lysate treated with activated charcoal, and KB cell culture media.

<table>
<thead>
<tr>
<th>FBP</th>
<th>CPM1</th>
<th>CPM2</th>
<th>Avg</th>
</tr>
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<tbody>
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<td>13371</td>
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<tr>
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<td>37989</td>
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<td>KB cell culture media (Treated with activated charcoal)</td>
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</table>
3.4.1. Detection of FBP in collected fraction:

Fractions obtained from folate-sepharose column were monitored for the presence of protein by different techniques: [³H] folic acid binding assay, absorption spectrometry, fluorescence spectrometry, and SDS-PAGE.

3.4.1.1. [³H] Folic acid binding assay:

Fractions collected from folate-sepharose column were subjected to binding assay using [³H] folic acid. No significant amount of folate-binding protein was detected (data not shown).

3.4.1.2. Measurement of absorption spectrum of the fraction collected from column chromatography:

![Figure 22](image.png)

**Figure 22.** Fluorescence intensity profile of fractions collected from epoxy-activated sepharose column
Fractions collected from folate-sepharose column were tested for the presence of characteristic absorption peaks for the protein in absorption spectroscopy. No peaks were obtained which could be due to absence of protein in the collected fractions.

3.4.1.3. *Measurement of excitation and emission fluorescence spectra of the fractions collected from column chromatography:*

Collected fractions were also tested for the presence of protein using the native fluorescence of natively fluorescent amino acid tryptophan. The emission spectrum was recorded in the wavelength range from 300 nm to 500 nm while the excitation wavelength was kept constant at 270 nm. Fluorescence signals were detected in fractions 6, 7 and 8, showing that some protein was present in these fractions (Figure 22). To confirm that detected protein is FBP, electrophoretic analysis was done using silver staining.

3.4.1.4. *Electrophoretic analysis of collected fractions:*

Collected fractions were analyzed by SDS-PAGE and the gel was stained with silver (Figure 23). The apparent molecular weight of the protein purified from the cell culture medium and subjected to gel filtration through epoxy activated folate-sepharose with buffer containing Triton X-100 was ~20 kDa.

*Figure 23.* SDS-PAGE of the purified FBP. The gel was stained using the Bio-Rad silver-staining kit.
The electrophoretic analysis showed the presence of protein, but bands that correspond to FBP were not present (Figure 23). Therefore, FBP from cell lysate and cell media was not purified successfully by folate-sepharose chromatography.
Chapter 4: Conclusion

This work showed that FBP originating from bovine and human milk contains abundance of high mannose and hybrid/complex type glycans. After deglycosylation of FBP samples, we found by MS that bovine FBP N-glycans mostly do not contain fucose and sialic acid. However, many glycans containing fucose and sialic acid are present in human FBP. We found N-linked glycan structures originating from human milk, which were not reported so far in the Functional Glycomics Glycan Database. The composition and structure of these novel glycans is represented in Table 6. These novel glycans found in human FBP have the potential to serve as biomarkers.

We also reported a convenient method for successful conjugation of folic acid to commercially available FITC fluorophore. The preliminary DIC and fluorescence microscopy imaging of KB cells showed that folate-FITC can be used to label the folate receptor and monitor endocytosis. Conjugates between FITC and folate analogues can be used further for localization of anticancer drugs in cancer cells.
Table 6. List of novel FBP glycans originating from human milk.\textsuperscript{a}

<table>
<thead>
<tr>
<th>(m/z) measured</th>
<th>charge</th>
<th>(m/z) calculated</th>
<th>proposed glycan</th>
<th>putative structure</th>
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<td>1+</td>
<td>1999.009</td>
<td>(F_1H_3N_3)</td>
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<tr>
<td>2111.108</td>
<td>1+</td>
<td>2111.072</td>
<td>(H_4N_5)</td>
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<tr>
<td>2186.218</td>
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<td>2186.093</td>
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<td>2314.196</td>
<td>1+</td>
<td>2315.172</td>
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<td>2360.182</td>
<td>(S_1F_1H_5N_3)</td>
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<tr>
<td>2472.305</td>
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<td>2472.246</td>
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<tr>
<td>2676.409</td>
<td>1+</td>
<td>2676.246</td>
<td>(S_1H_3N_5)</td>
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\textsuperscript{a} S represents sialic acid (diamonds); F represents fucose (triangles); H represents hexose (mannose open circles, galactose solid circles); N-represents N-acetyl glycosamine (solid squares) and N-acetyl galactosamine (open square).

At last, we successfully monitored and detected the presence of FBP in HeLa and KB cells. We also worked on methodologies used for isolation of FBP from these cells and cell growth media. Future experiments will involve isolation and extraction of folate receptor from these cell lines in order to isolate sufficient amounts of FBP for further studies of this bio-medically relevant protein.
References

(1) http://www.gemzar.com/pages/pat400_ovarian_cancer.aspx?WT.seg_1=&DCSext.ag=OvarianCancer&WT.srch=33


(10) Luhrs, C.; Pitiranggon, P.; Da Costa, M.; Rothenberg, S. P.; Slomiany, B. L.; Brink, L.; Tous, G. I.; Stein, S. “Purified membrane and soluble folate binding proteins from cultured KB cells have similar amino acid compositions and molecular weights but differ in fatty acid acylation,” *Proc. Natl. Acad. Sci* 1987, 84, 6546.


(36)[http://www.functionalglycomics.org/glycomics/molecule/jsp/carbohydrate/searchCarbMolecule.jsp](http://www.functionalglycomics.org/glycomics/molecule/jsp/carbohydrate/searchCarbMolecule.jsp)