Spring 2011

Detection of Folate Binding Protein

Anwer Unnisa
Governors State University

Follow this and additional works at: http://opus.govst.edu/capstones

Part of the Analytical Chemistry Commons, and the Nanomedicine Commons

Recommended Citation
Unnisa, Anwer, "Detection of Folate Binding Protein" (2011). All Capstone Projects. 75.
http://opus.govst.edu/capstones/75

For more information about the academic degree, extended learning, and certificate programs of Governors State University, go to http://www.govst.edu/Academics/Degree_Programs_and_Certifications/

Visit the Governors State Analytical Chemistry Department
This Project Summary is brought to you for free and open access by the Student Capstone Projects at OPUS Open Portal to University Scholarship. It has been accepted for inclusion in All Capstone Projects by an authorized administrator of OPUS Open Portal to University Scholarship. For more information, please contact opus@govst.edu.
Detection of Folate Binding Protein

A Project

Submitted

To

Governors State University

By

ANWER UNNISA

In Partial Fulfillment of the

Requirements for the Degree

Of

MASTERS IN SCIENCE

APRIL 2011

Governors State University

University Park, Illinois.
DEDICATED TO MY FAMILY
ACKNOWLEDGEMENTS

My sincere thanks and gratitude to my Prof. Dr. Walter Henne who was abundantly helpful and offered me invaluable assistance, support and guidance without whom the project would not have been successful.

My deepest gratitude to my committee members Dr. Patty Fu and Prof. Kent for their continuous assistance throughout the project work.

Also special thanks to the Governors State University for providing the financial means and other facilities for conducting this study.
Table of Contents:

Abstract: ........................................................................................................................................... 5

Introduction: ....................................................................................................................................... 6
  Folic Acid: ........................................................................................................................................ 6
  Folate Receptors: .............................................................................................................................. 7

Upregulation of folate receptors: ......................................................................................................... 9

Imaging of Folate Receptor Tumors: .................................................................................................. 10

Folate receptor beta  expressed by tumor-associated macrophages: ........................................... 13

Detection of folate binding protein by optical biosensor analysis: ................................................... 16

Detection of folate binding protein in milk: ...................................................................................... 17
  Analysis with Biacore system ........................................................................................................... 18
  Concentration Analysis ................................................................................................................... 19

Detection of folate binding protein with Megalin: ......................................................................... 20
  Binding of FBP and Megalin: .......................................................................................................... 20

Detection of folate binding protein by Quartz Crystal Microbalance Sensor: ............................ 21

Imaging Studies ................................................................................................................................ 22

Conclusion: ....................................................................................................................................... 23

FIGURES ........................................................................................................................................... 23
  Figure 1: Structure of folic acid ......................................................................................................... 23
  Figure 2: Folate receptor mediated endocytosis ............................................................................. 24
  Figure 4: Surface plasmon resonance (SPR) biosensor principle .................................................. 25
  Figure 5: Overview of the basic interactions at the biosensor surface ......................................... 26
  Figure 6: Sensorgram ...................................................................................................................... 27
  Figure 7: Sensor surface functionalization for the detection of folate binding protein .................. 28
  Figure 8 : Quartz Crystal Microbalance Sensor ........................................................................... 29
  Figure 9: Structure of Megalin featuring regions containing pathogenic epitopes in Heymann Nephritis ................................................................. 29

  Figure 10: Autoradiography showing RAP-inhibitable binding of soluble 125I-labeled FBP to proximal tubules of rat kidney cortex ........................................................................ 30
  Figure 11: Uptake of 125I-labeled bovine milk FBP microinjected into rat nephrons in vivo .......... 31
  Figure 12: Early detection of the inflammatory joint by NIR2-folate ............................................ 31
  Figure 13: Establishment of the KRN serum transfer model ....................................................... 32
  Figure 14 :In vivo near-infrared fluorescent (NIRF) imaging of inflammatory joints in the lipopolysaccharide (LPS) induction model ................................................................. 33
  Figure 15: Immunoperoxidase staining ......................................................................................... 33

References: ....................................................................................................................................... 34
Abstract:

Cancer is the second leading cause of death despite the anti-cancer developments including hormone therapy, radiotherapy, chemotherapy. Chemotherapy includes use of potent drugs such as camptothecin, paclitaxel and mitomycin. These drugs have an effect which is dose responsive, where the drug exposure and cell kill are proportional. This chemotherapy is high dose and has less selectivity for cancer cells and has more toxic effect to normal cells. Such kind of non-specific treatment to tumor is causing hurdles in the treatment of cancer by chemotherapy. To overcome this current limitation to chemotherapy, the anti-cancer drug with high specificity is given in concentration effective to the tumor tissues. [1, 2][1, 2][1, 2] In order to achieve this goal, tumor selective drugs were developed by conjugating antibodies, hormones and vitamin derivatives to anti-cancer drugs. One such compound, folic acid, which is low molecular weight vitamin, has emerged as ligand for delivery of drugs to cancer tissues. Since the folate receptors are over expressed in cancer cells, conjugation of anticancer drugs with folate serves as a highly effective way to treat cancer with fewer side effects. This way, the normal cells can be prevented from the toxic effects of the anticancer drugs. Hence, folate receptors can be used as a useful tool for selective targeting of drugs. [3]
**Introduction:**

**Folic Acid:**

Folate plays an important role as it participates in the biosynthesis of nucleic and amino acids and thus is essential in cell survival. [4] It is a member of vitamin B family. Folate is used to enhance the differential specificity of anticancer drugs as it targets the folate receptor positive cancer cells. By this property it serves as a high affinity ligand. Conjugation of anticancer drugs to folate was found to decrease the side effects and increase the drug selectivity. FR is a glycosylphosphatidylinositol anchored protein, which is associated with tumor. It can internalize the folates which are bound and conjugated compounds bound to folate via receptor mediated endocytosis. The FR density is found to be high in breast carcinomas and low levels are found in normal tissues. Its density was also found to increase as the cancer progresses. A wide range of anti cancer drugs were conjugated and evaluated for their anti cancer activity based on the fact that conjugation of folate helps in targeting the drug molecule and getting endocytosed into cells with positive FR. Such drugs include EC 72, EC 118 and folate mitomycin. These were found to have selectivity for FR positive M109 cells. When these E118 and EC 72 were studied in vivo and in vitro, it was proved that drug specificity and selectivity was enhanced by folate conjugation more over reducing the toxicity. [1]
**Folate Receptors:**

The folate receptor was found to have two isoforms which are glycosyl phosphatidylinositol (GPI) anchored. They are alpha and beta. Epithelial cancers are usually associated with FR-alpha expression and myeloid leukemia and activated macrophages are associated with FR-beta expression. The targeted delivery to FR+ cells is done via receptor mediated endocytosis, the anti FR antibodies and conjugates of folic acid can be uptaken by cancer cells. So the FR alpha is used as a marker in cancer and beta for inflammatory diseases and myeloid leukemia. Thus FR targeting agents find a good potential for treatment of range of diseases.[5]

When the stability and structure of folate binding protein FBP from cow's whey was studied, it was found that it has 22 % helix, 5 % parallel beta strand, 17 % turn 25 % ant parallel beta strand and 31 % random coil structure. Spectroscopy studies showed that when folate binding occurs to folate binding protein, significant changes occurs and there was a 10 % decrease in the anti parallel beta strand. The magnitude of ligand binding was found to be proportional to quenching of FBP tryptophan fluorescence. Further studies revealed that because of the ligand binding to FBP, the folding stability of the molecule was increased which is by the aggregation of FBP caused by ligand. This was also confirmed by fluorescence and spectroscopy.
Current clinical trials show that conjugation with folate increases the potency and reduces the toxicity of many anti-cancer drugs. Drugs that are conjugated to folic acid for selective drug delivery are: Chemotherapeutic agents, Protein toxins, Gene therapy Vectors, Radio imaging agents, Radiotherapeutic agents, Liposomes with entrapped drugs, immunotherapeutic agents, oligonucleotides (SiRNA), MRI contrast agents.[6]

Folate receptors were discovered to be overexpressed on activated macrophages and found to be quiescent on non-activated macrophages. The cause and contribution of activated macrophages to a number of diseases as diabetes, rheumatoid arthritis, ischemia, sarcoidosis, psoriasis, vasculitides, sjogren's disease, led to studies which were done to develop folate conjugated therapeutic agents. To visually examine the delivery of folate conjugates into receptor bearing cells, Susan Wang and Phillip Low used bovine IgG and bovine ribonuclease. This was conjugated to folic acid and 15 nm colloidal gold particles where used to label them. [7] Florescent probes were used to label them and then conjugated to folic acid. By fluorescent microscopy it was revealed that only the fluorescent proteins which are folate conjugated were taken by the folate receptor KB cells.[1]

After the folate protein conjugates are taken up by cancer cells, EM pictures of KB cells revealed the internalization of colloidal gold particles by KB cells through uncoated pits. Mostly the ligands delivered by endocytic pathways into cells are internalized for destruction. That means, the hormones after they transduce their signals or any other foreign particle which are harmful if they remain in the cell are digested by the lysosomes. But the folate conjugates were found to be stable and functional after many
hours of uptake by cancer cells. So the folic acid is internalized for consumption and not for destruction. Thus it was found that after uptake by the FBP, the nucleic acids and proteins remain undigested and enzymes retain their activities. [8]

**Upregulation of folate receptors:**

Until late 1991 and early 1992 the knowledge of FR up regulation on cancer cells was not obtained until it was studied that the FR was actually recognized by the monoclonal antibodies used in tissue biopsies to study cancer cells. Mainly, two different classes of proteins mediate the cellular uptake and absorption of folate [9]. One is the reduced folate carrier (RFC) are the transmembrane proteins, which have micro molar affinity to bind with the reduced folate. The second class is the folate binding proteins family also called as folate receptor. Further learning on the distribution of FR actually gave a new approach to the cancer research that the FR is mainly restricted to malignant tissues rather than normal tissues. From then, FR was used as a tool for selective targeting of drugs to the cancer cells. This study lead to the development of folate linked cytotoxic drugs. When the linkage of folate pseudomonas exotoxin (PE38) and folic acid was studied, it was demonstrated that the effect of the conjugate was depending on the type of the bond between them. With the reducible disulphide bond linkage, maximum potency was observed and with thioether bond, there was a decrease in its potency. Thus with these demonstrations it was shown that the cleavable linker in the folate targeted cytotoxic drug was important in the designs of future conjugated drug designs. [10, 11]
Imaging of Folate Receptor Tumors:

Imaging of folate receptor tumors was found to be feasible using radio labeled folate conjugates. The chelating agents that have high affinity for $\gamma$-emitting metal radionuclide’s are used after folate derivatization. Folate was conjugated with chelator deferoxamine and labeled with 67Ga, this was intravenously injected into mice with xerographic implants from cells cultured with KB. Thus high tumor selectivity was achieved by folate targeted imaging agents. Studies show that it helps in detecting the size, location and clinical stage of neoplastic tissue and thus used in planning the treatment strategy for cancer. Recent studies showed that folate targeted liposomes are being effectively used for gene therapy. [11] The gene therapy agents commonly used are expression plasmids and antisense oligonucleotides. Their uses are limited because of their low physiological stability, and lack of tissue specificity. Because of these disadvantages, these macromolecules are being taken into consideration to enhance their stability and permeability. To protect the antisense oligonucleotides from nuclease digestion, folate targeted liposomes are used which also enhances their intracellular delivery. Treatment of KB cells with free antisense oligos and antisense oligos encapsulated in folate liposomes showed that free oligos had barely growth inhibition and no significant changes. But with the use of folate PEG liposomes, there was measurable growth inhibition and abnormalities in the morphology. Further studies proved that encapsulated oligos showed complete suppression of growth factor receptor thus indicating the efficiency and non destructive delivery of encapsulated oligos into cancer cells.[12] Because of the inefficient escape of plasmids from the cell's endosomal compartments, the use folate targeted carriers has been limited in the tumor specific delivery of
vectors. To overcome this issue, studies were conducted to modify the liposomes such that they deliver their effects only after entering the target cell endosomes. One approach which was used by developing a pH sensitive lipid composition. This becomes lysogenic after entering into acidic endosomes. [13] The other approach uses the fusogenic oligopeptide. This initiates membrane fusion by changing the conformation. By testing cytosine arabinoside, an anticancer drug in folate liposomal preparation, the effectiveness was found to increase by folate targeting. The potency was found to increase further by constructing pH sensitive diplasmalogen. Thus the use of pH sensitive lipid design and peptide will have great impact on efficiency of expression. Furthermore, the use of conjugated nanoparticles with folic acid proved to have targeted effect in treatment of cancer. This effect was shown by particles which have biodegradable polymer core and mixed lipid monolayer shell. Docetaxel when used with the nanoparticles showed a markable increase in the cytotoxic effect. Furthermore another advantage of using the drug with nanoparticles is that the targeting effect can be controlled and adjusted by lipid component ratio of mixed lipid monolayer. This targeting efficiency of lipid monolayer was proved by fluorescent microscopy which shows that controlled and targeted delivery is achieved by folic acid conjugated nanoparticles which can be the drug delivery system where the targeting effect is precisely controlled.[14]

**Folate Conjugates:**

Another type of folate nanoconjugates were synthesized which were found to be magneto fluorescent and cancer specific targeting folate conjugates [15]. These particles were
designed with pendant groups (amino, carboxyl) on their surface with o-carboxymethyl chitosan (OCMC). These groups facilitate in covalent attachment of rhodamine isothiocyanate (RITC) which is a fluorescent dye. The magneto fluorescent nanoparticles were conjugated on to the aminated derivative of folic acid. These magneto fluorescent conjugates were found to be biocompatible, with good dispersibility. In the presence of external magnetic field they were found to have specific magnetic properties. Further studies were done using folate over expressed (HeLa) and normal cells. It was proved that the uptake of these nanoparticles folate conjugates is more in cancer cells than in normal cells.[10]

CdHgTe folate conjugates: These were found to have stability, optical spectra and cancer cell targeting characteristics. They were prepared by covalent conjugation of folic acid and CDHgTe quantum dots. The fluorescent wavelength of CdHgTe folate conjugates was found to be 790 nm. The long and continuous fluorescence imaging was achieved by these conjugates because of their fluorescence stability. The bio distributions of these conjugates were studied in vivo in S180 tumor mouse which proved their tumor targeting efficiency. These CdHgTe folate conjugates can be used for monitoring and imaging because of their high fluorescence intensity. The intracellular delivery against folate receptor is enhanced by heparin-PEG-folate (H-PEG-F). In this, the folate is conjugated with succinylated heparin. Since it has amphiphilic property as it has covalent bonding, it can be used with hydrophobic drug such as taxol. Nanoparticles made with the drug and H-PEG-F have the drug in the core with folate on its surface. [16] By UV technique, the quantitative analysis of folate and taxol is studied and NMR
technique, the particles are characterized for their structure. Size of the nanoparticles is studied by field emission scanning electron microscopy (FESEM). When these nanoparticles were studied for their effect, it was shown that the cellular uptake of the carrier by KB-3-1 cells which are the folate receptor over expressing cell line is more than for the folate receptor deficiency cell line. With the help of flow cytometry, it was depicted that when the KB-3-1 cells were treated with nanoparticles, the G2/M phase of cell cycle was found to be arrested. This effect is similar to the inhibition mechanism of taxol. With the help of these folate conjugated nanoparticles various therapeutic agents can be used for cancer cell targeted delivery. The conjugation of folic acid to chitosan molecules was done via the gamma carboxyl moiety. This helps in retaining a high affinity for folate receptor over expression in colorectal cancer cells. For the detection of colorectal cancer, folic acid conjugated with chitosan nanoparticles is used for carrying 5- amino aevulinic acid (5-ALA). Folic acid-chitosan conjugates were prepared and then 5-ALA was loaded to form fCNA nanoparticles. When these nanoparticles were used to study their uptake, it was found that the fCNA particles were taken up more easily by caco-2 cell lines and HT29 cells. Since the colorectal cancer is often undetected with current colonoscopy practices it is the major leading cause of malignant death. With the use of these folic acid-chitosan conjugates the colorectal specific delivery of 5-ALA appears to be an ideal option in the endoscopic detection of colorectal cancer. [17]

**Folate receptor beta expressed by tumor-associated macrophages:**

According to their response to pro-th1/proinflammatory stimuli or pro-Th2/anti-inflammatory stimuli, activated macrophages are grouped functionally. The folate receptor
beta (FR beta) which is encoded by FOLR2 is expressed with increased folate uptake ability. M-CSF promotes the folate uptake ability of macrophages and thus indicates a link between M2 polarization and FR beta expression. The tumor associated macrophages (TAM) exhibits M2 like functional profile and helps in detecting the FR beta expression. [5] The expression of FR beta mediates folate uptake by CD68(+) CD14(+) CD163(+). The ascetic fluid of the tumor and the medium of tumor cell induces the expression in an M-CSF dependent manner. The high degree of macrophage infiltration is usually associated with tumor associated macrophages (TAMs) found in glioblastomas. In an experiment conducted to study the expression of folate receptors, a rat C6 glioma subcutaneously implanted in nude mice, recombinant immunotoxin was produced to target TAMs with FR beta expression. It consisted of immunoglobulin heavy and light chain portions and pseudomonas exotoxin A. C6 glioma xenografts when injected with the immunotoxin was shown to deplete TAMs and thus inhibiting the growth of the tumor. FR beta expressing macrophages targeted by the immunotoxin provides a good therapeutic tool in the treatment of glioblastomas. These studies indicate that FR beta is a marker in M2 regulatory macrophage polarization.[4]

Folate Quantum Dots:

The folate quantum dots were synthesized with folate poly ethylene glycol conjugates and polyamidoamine (PAMAM) . [18] These are useful in the design and development for bio imaging and optical imaging of cancer cells. The CdSe/ZnS dots were found to encapsulate the ligand by ligand exchange reactions. These ligand exchanged QDs were found to target the tumor cells because of membrane expression of folate receptors. The cellular uptake of FPP coated QDs is more in HeLa cells when compared to QDs.
without folate. The FPP quantum dots show slow endosomal escape after binding to the tumor cell surface. After entering, they release into the tumor cells.[19]

The anticancer drug Irinotecan is used in the treatment of colon cancer but its active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin) which is very hydrophobic was found to be more cytotoxic than the original drug. To improve the stability and solubility SN 38 was formulated into folate nanoparticles. These were produced by emulsification of poly lactide co glycolide polyethylene glycol with SN -38. By coupling of PLGA-PEG-NH diblock copolymer with folic acid, the folate-conjugated diblock copolymer were synthesized. When these conjugates were studied against HT-29 cancer cells, it was shown that the S38 loaded particles have strong cytotoxic effects than with the control. [20] With this study it was demonstrated that for the hydrophilic drugs, folate conjugation is the efficient way for to increase the cytotoxicity of the drug. Folic acid conjugation was done to bovine serum albumin nanoparticles (BSANP). Into the folate conjugated albumin nanoparticles, mitoxantrone was incorporated. These nanoparticles were used to target SKOV3 cells where the folate receptors are over expressed. Incorporation of mitoxantrone to the nanoparticles lead to the cytotoxic activity of MTO-BSANP-folate. This activity was quantitatively determined and was found to be more when compared to just the MTO-BSANP and MTO solution in vivo. Thus it was shown that folate conjugated BSANP have good potential in the cancer chemotherapy. [9]

Glutathione modified Gold Nanoparticles:
Because of the over expression of folate receptors on variety of cancers, the therapeutic agents and folate linked imaging can be targeted to FR expressing tumors. In this way, uptake by the healthy tissues can be avoided which express very less FR. Using gold nano particles, the drug glutathione was modified to form GSH- capped GNPs. These particles have carboxyl group on their surface [20]. Though the reaction of amino group on folic acid, these particles were conjugated. In aqueous solution the folic acid conjugated gold nanoparticles were found to be stable. By transmission electron microscopy it was confirmed that the FA-GSH-GNPs were up taken by the human cervices carcinoma cells which showed high level of folate receptor expression. In cells which lack folate receptor, no uptake was seen. The relative absorbance of the supernatant was measured with HeLa cells incubated with FA-GSH-GNPs. The HeLa cells were detection with the detection limit of 10(2) cells /mL by spectroscopic method. As in the case of methotrexate, which is used in the treatment of rheumatoid arthritis, the targeting mechanism plays an important role in the treatment. MTX because of it plasma protein binding is responsible for its effective distribution in the body. in RA patients, the expression of folate receptor β (FR-β) is found to be high on synovial macrophages. This mediates in high uptake of MTX in the cells. Such studies conclude the FR-β specific therapy and is being employed for drugs other than MTX.[6]

Detection of folate binding protein by optical biosensor analysis:

Biosensor is based on SPR technique. It is the surface plasma resonance technique. In SPR system, there is a laser light source which produces light, detector, gold surface and a glass
prism. The incident polarized light undergoes total internal reflection and this is configured at the interface. The electromagnetic field which is generated gets penetrated into the metal and then it transfers some of its energy to its valence electrons. This produces charge density waves which are known as Surface plasmons[5]. When there is any association or dissociation at the sensor surface, it is recorded as a plot of change in refractive index with time.[21]

Detection of folate binding protein in milk:

In early 1960's Ghitis observed that there is a presence of a macro molecular factor in milk which is heat labile. This factor prevented the adsorption of milk folate onto the activated charcoal. Furthermore observations lead to the conclusion that the high affinity folate binding protein is present in bovine milk which prevents the milk from adsorption on to charcoal. This FBP was found to contain 222 amino acids.[22, 23]

The interactions of folate and FBP are essential for study of metabolism of folate and the bioavailability of folate. In the indirect technique for FBP quantification, the folate content was measured after the unbound folate which is excessive is absorbed onto charcoal. The direct technique for determination of FBP was ELISA. [24] This is done by a antibody which is raised against the FBP in milk. These two techniques do not determine the complexation of FBP with folate. To distinguish the free apo form from the complexed holo form, a technique was discovered which was called the Surface Plasma resonance (SPR). The SPR technique works by measuring the refractive index near the surface. By this method, the apo form of the protein can
be easily measured. The most commonly used SPR system is the Biacore system by GE health care Sweden.[23]

**Analysis with Biacore system:**

The components of Biacore system are: interchangeable sensor chip, micro fluidic sample handling system and SPR detector. The sensor chip consists of a glass slide which is plated with gold. A coupling matrix is used which covers the gold film. This coupling matrix is important because it reduces the mobilization capacity of the molecules and hence for characteristics of the surface. The non-specific absorption of proteins is prevented because of the hydrophilic property of the matrix. The interacting partners are immobilized on to the sensor chip surface and the aqueous samples are injected onto this surface. To control the flow of buffer over the sensor surface, IFC i.e., integrated micro-fluidic cartridge is used which gives a continuous flow [25]. The detector detects the binding of interactant and is quantified in real time. The interaction is detected when analyte which is the binding partner binds to the immobilized molecule on the sensor surface. The coupling of activated dextran to the amine group was not possible because for the binding to occur, a ring system is important. So the sample folic acid which was used were coupled on the sensor surface to the amino groups were in their hydroxysuccinimidyl forms. Because of the amine coupling, the FBP is immobilized on the sensor surface. When this binding takes place, there is an increase in the mass at the surface and this result in change in refractive index. This change is measured real time and a graph is plotted with resonance units or response with time. This plot with Resonance Units and time is called a Sensogram. The sensogram gives information about the interaction rate (association or dissociation) through
which the concentration of analyte can be known, the affinity constants are obtained from the binding level information.[25]

**Concentration Analysis:**

In this assay, only the binding which is specific to the two samples is studied. To determine the amount of analyte bound, the rate at which the analyte binding occurs at the beginning when the sample is injected can be taken into account. The other approach is to measure the total amount of analyte which has binded after the whole sample injection. The rate of binding is directly proportional to the concentration of analyte when the association of the samples is dependent on the rate at which the samples are transported. [26]

This method was shown to be in compliance with ELISA method. It was also found that at FBP concentration of 0.38µg/mL, the repeatability (CV) was found to be 1.2 - 3.6 % within assay for 3 different days. This method is based on interaction of the PGA and FBP, so the folate which is endogenous must be removed to estimate the total FBP content because it can interfere with the assay method. For this assay to be done, the presence of pteridine moiety of folate was important for the interaction to occur. [26]

From the data obtained by the interaction of the analyte and the ligand at the sensor surface, the association, dissociation and the equilibrium constants can be analyzed. By this SPR technique, the free and the apo form of FBP can be differentiated so that the effect of FBP on the stability of folate can be known. For the study of the specific interactions of protein- vitamin, this modern technique is highly useful.[8, 27]
Detection of folate binding protein with Megalin:

Megalin is a member of low density lipoprotein receptor family. It is a multiligand endocytic receptor which is mainly expressed in proximal tubule of kidney, intestine and other absorptive epithelia.[27] This megalin has an affinity to bind to the FBP present in milk. In early 2000, the research conducted on uptake of FBP by megalin showed that megalin has high affinity to bind to the FBP but it cannot bind to the low density lipoprotein receptor protein. When this study was done on immortalized rat yolk sac cells, it showed that the 125I-labeled FBP was up taken by the megalin and this uptake was inhibited by antimegalin antibodies and also by the receptor associated proteins. When 125-I labeled FBP was injected into proximal tubule in vivo, there was endocytotic uptake in the renal tubules. Since the human body cannot synthesize folate, the up regulation of folate is very important in the intestines.[14]

Binding of FBP and Megalin:

The binding of bovine milk apo FBP to immobilized rabbit megalin was shown by SPR techniques. This binding was also observed with 5-methyltetrahydrofolate (5-MTHF), or methotrexate. Autoradiography techniques were used to confirm the binding of FBP to megalin in proximal tubule brush border membranes of kidney. On incubation with 125I labeled bovine milk FBP, autoradiographic grains were shown to be accumulated along the proximal tubule which was inhibited by incubation with receptor associated protein. [8] This receptor associated protein is a known inhibitor of megalin. It inhibits the ligands uptake by megalin, thus it shows that megalin has a very specific binding capacity [8]. The uptake of FBP by megalin was calculated as the sum of cell associated, degraded and non trichloroacetic acid precipitable label.
Detection of folate binding protein by Quartz Crystal Microbalance Sensor:

Because of the fact that FBP are present in both the normal and metastatic states, radio immunoassays are employed for their detection. These involve the use of radioactive labels which are hazardous and also the analysis time is long for the detection. To overcome this, a quartz crystal microbalance method (QCM) was discovered in 2006 which allows the recognition of antigen antibody, assess the surface layer phenomenon and also the capture of viral and bacterial cell. For the detection of folate binding protein, a quartz crystal microbalance biosensor was developed.[5] Detection limit of 30 nm was achieved with Au coated quartz sensor absorbed with simple folate BSA conjugate. At concentrations higher than 1 micro molar in excess of folic acid, the binding of FBP to sensor surface are blocked. This showed the specificity of folate FBP interaction. When there is an association or dissociation of protein to the surface of the quartz sensor, change in the mass of the crystal occurs. This results in the increase or decrease in the frequency. When the analyte gets captured on the crystal surface, the effective surfaces mass increases, and thus the resonance frequency decreases. This decrease in the frequency is because of the binding of the proteins to the quartz surface. Since the molecular weight of the analyte protein could be very small, some secondary ligands such as iron or gold particles are employed. These ligands increase the sensitivity by increase in the mass of the analyte binding to the surface. Thus with the use of quartz crystal microbalance analysis, the sensitivity of the assay was increased significantly.[7, 28]
The basic operation of QSM described by Sauerbrey is given as

\[ (\Delta m_{QCM}) = - \left(\frac{C_{QCM}}{n}\right) \Delta f \]

where \( C_{QCM} \) (17.7 ng cm\(^{-2}\) Hz\(^{-1}\)) is the mass sensitivity constant and \( n \) (1, 3 ...) is the overtone number, \( \Delta m \) is the mass change, and \( \Delta f \) is the change in resonance frequency. [5]

**Imaging Studies:**

In early 2005, further studies were in the field of the in vivo imaging of arthritis which is the inflammatory joint disease was done using a lipopolysaccharide intra articular injection model and a KRN transgenic mice serum induction mouse model for the testing of a recently developed near infra red fluorescence labeled folate probe (NIR2 -folate) [29]. Arthritis is characterized by active macrophages with over expressed FR receptors. In the joints with lipopolysaccharide treated and the control joints, the fluorescence signal intensity of free and NIR2 folate was compared. At the NIR2 folate probe, the fluorescence signal intensity was found to be higher than control normal joints. After the transfer of serum, the NIR2 folate was applied at different time points in the KRN serum transfer model. After the transfer of arthritogenic antibody transfer, the inflamed joints were detected after 30 hours and shown to have an increase of 1.8 fold in the intensity of the signal. Thus it was concluded in this study that using a fluorescent probe, the in vivo detection of arthritis could be done and hence with this receptor targeting method, improved arthritis diagnosis can be facilitated.[30]
Conclusion:

As there is a vast potential for folate targeting anti cancer drugs, the study and detection of folate binding protein provides an essential tool for diagnosis and monitoring the therapy for treatment of cancer. These folate delivery systems are essential to establish the level of folate receptors required for the cancer cells to be treated by the folate targeting drugs. By various SPR techniques, a simple fast and robust method was developed to distinguish the apo and the holo forms of FBP. The future studies in this field are the use of nanomechanical sensors which can further explore the detection capacity of biosensors for eg, in relation to the work done by Dr Henne by using Quartz crystal microbalance sensor, the use of nanomechanical sensors gives a new approach to the folate targeting of the drugs. Applications and development of various other cantilever based sensors is presently being studied by the International Workshop on Nanomechanical Sensors.

FIGURES:

Figure 1: Structure of folic acid [4]
Department of Veterinary Anatomy, Purdue University, West Lafayette, IN

Figure 2: Folate receptor mediated endocytosis [3]

<table>
<thead>
<tr>
<th>Epithelial Cancers</th>
<th>Frequency (Sample Size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian carcinoma</td>
<td>93% (36)</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>91% (11)</td>
</tr>
<tr>
<td>Kidney</td>
<td>50% (20)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>38% (8)</td>
</tr>
<tr>
<td>Lung carcinoma (NSCLC)</td>
<td>33% (18)</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>21% (53)</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>22% (27)</td>
</tr>
</tbody>
</table>

Department of Human Molecular Genetics and Biochemistry, Israel.

Figure 3: Folic acid Expression [14]

Figure 4: Surface plasmon resonance (SPR) biosensor principle. [31]
Figure 5: Overview of the basic interactions at the biosensor surface. [31]
Figure 6: Sensorgram.[31]
Figure 7: Sensor surface functionalization for the detection of folate binding protein. [5]
(a) Folate BSA is adsorbed on the QCM gold sensor surface and used as a receptor to capture FBP. (b) Signal enhancement is obtained by addition of anti-FBP antibody, followed by subsequent protein-A-coated gold nanospheres. (c) Structure of folic acid.[20]
Figure 8: Quartz Crystal Microbalance Sensor

Figure 9: Structure of Megalin featuring regions containing pathogenic epitopes in Heymann Nephritis.[1]

Figure 10: Autoradiography showing RAP-inhibitable binding of soluble 125I-labeled FBP to proximal tubules of rat kidney cortex [1]
Figure 11: Uptake of 125I-labeled bovine milk FBP microinjected into rat nephrons in vivo. Uptake of 125I-labeled FBP is visualized by autoradiography on sections from fixed kidney cortex. Grains are located over proximal tubule profiles.[1]

Figure 12: Early detection of the inflammatory joint by NIR2-folate.
(a) White-light image showed no remarkable swelling at bilateral paws. (b) Merged near-infrared fluorescent signal with a white-light image showed increase fluorescence signal intensity at the dorsal aspect of the right wrist, which has a 1.8-fold increase compared
with the left wrist. (c) H&E-stain histology of the right wrist showed polymorphonuclear cell infiltration at the dorsal aspect of the right wrist (arrow).
(d) Histology of the left wrist showed no remarkable inflammatory cell infiltration. (e) Immunohistochemistry of the right wrist showed Mac-3-positive cell infiltration at sub synovial tissues. [32, 33]

Figure 13: Establishment of the KRN serum transfer model. [7]
(a) Discoloration and swelling (arrow) of the right third proximal interphalangeal joint is noted in a healthy C57BL/6 mouse 4 days after KRN serum transfer. (b) Near-infrared fluorescent imaging of the right paw showed increase fluorescence signal intensity at the inflammatory joint (enhancement ratio = 1.9). (c) Correlated H&E-stain section showed abundant inflammatory cells infiltration with pannus-like formation. (d) Immunoperoxidase staining of Mac-3. Mac-3-positive cell infiltration among polymorphonuclear cells was noted in the pannus.[34]
Figure 14: In vivo near-infrared fluorescent (NIRF) imaging of inflammatory joints in the lipopolysaccharide (LPS) induction model [35]
The NIR2-folate probe was intravenously injected 2 days after LPS intra-articular injection. (a) White-light images obtained 48 hours after intra-articular LPS injection at the right ankle joint; soft tissue swelling was noted at the affected joint. (b) NIRF images obtained 24 hours after NIR2-folate injection. (c) A merged NIRF signal with a white-light image showing specific increased fluorescence signal intensity at the affected joint. (d) H&E-stain section of the right ankle joint showing abundant inflammatory cell infiltration at sub synovial tissues. (e) NIRF images of a longitudinal section of the LPS-treated ankles.[34]

Figure 15: Immunoperoxidase staining
(a) Mac-3 and (b) folate receptor (FR) at an arthritic ankle 72 hours after lipopolysaccharide induction. The Mac-3-positive and FR-positive cells morphologically correlated well in adjacent tissue sections. [7]
References:


