Folate Nanoparticle Conjugates

Safwat A. Masood

Governors State University

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

Part of the Analytical Chemistry Commons, and the Nanoscience and Nanotechnology Commons

Recommended Citation
http://opus.govst.edu/capstones/59

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.
Folate Nanoparticle Conjugates

A project
Submitted
To
Governors State University
By
Safwat A. Masood
In partial fulfillment of the
Requirement for the Degree
Of
Masters of Science
In
Analytical Chemistry
May 2012
Governors State University
University Park, IL-60484
Dedicated to my Parents, and Wife
Acknowledgements

I would really like to give my thanks to Dr. Walter Henne for giving me this opportunity. Dr. Henne was always there and gave his time, support, and encouragement as a professor and as my advisor on this project, which lead to this accomplishment. I would also like to give my thanks to Dr. Fu, Dr. Addison and especially Dr. Kumar for the education, discipline, and excellent work ethics that they have all instilled in me at my years at Governors State University. I would like to thank Dr. D’Arcy, and Professor Kent for their time and insight. These professors are what make GSU a great education. I would also like to thank all of those at Governors State University for making this one of the most memorable and excellent educations. To my wife and parents thank you for your time, patience and being there through my educational years at Governors State University.
Table of Contents

Abstract ........................................................................................................................................................................ 5

Introduction .................................................................................................................................................................. 6

Background ............................................................................................................................................................... 7-9

Figure 1. Molecular structure of Folic acid...................................................................................................................... 7

Pegylation of Folic acid Nanoparticle conjugates .................................................................................................... 9-13

Figure 2. Chemical structure of different PEG backbones .......................................................................................... 11

Figure 3. Transmission electron microscopy images of OPM-1 cells ........................................................................ 12

Figure 4. Representation of PEG-modified AuNP exposed to FR+ and FR- cells ..................................................... 13

Nanoparticle Conjugates for intracellular tracking of payloads and imaging of cancer cells ................................ 13

Mesoporous Silica Nanoparticles .................................................................................................................................. 14-17

Figure 5. FT-IR spectra of NP(FITC)-PEG-folate and NPs(FITC) .................................................................................. 15

Figure 5.1 Diagram for the synthesis of NPs(FITC)-PEG-folate .................................................................................. 16

Figure 5.2 Fluorescence Imaging showing the uptake of NPs(FITC)-PEG-folate by KB cells ..................................... 17

Magnetic Nanoparticles .............................................................................................................................................. 17-20

Figure 6. TEM images of (Fe3O4) magnetite nanoparticles ......................................................................................... 19

Figure 6.1 TEM images of FR- NIH/3T3 cells .............................................................................................................. 19

Figure 6.2 TEM images of FR+ 5RP7 cells .................................................................................................................. 20

Quantum Dots ............................................................................................................................................................... 20-24

Figure 7. Diagram for the synthesis of FA-Si-QD ........................................................................................................... 22

Figure 7.1 Confocal imaging of Panc-1 cells ................................................................................................................ 23

Figure 7.2 Toxicity assays of Si-QDs and CdTe quantum dots ..................................................................................... 24
Gold Nanoparticles .......................................................................................................................... 25-29

Figure 8. Schematic diagram for the synthesis of AuNP-4atp-folate.........................................................26

Figure 8.1 Toxicity assay for HeLa and MCF7 cells.....................................................................................27

Figure 8.2 Percent survival rate of HeLa and MCF7 cells exposed to IPL....................................................28

Figure 8.3 Percent survival rates of HeLa and MCF7 exposed to nano-conjugates and IPL......................29

Liposomes .............................................................................................................................................30-39

Figure 9. Schematic picture of Folate-targeted liposomes........................................................................32

Figure 9.1 ICP-AES analysis in the uptake of 157Gd..................................................................................32

Figure 9.2 Mouse tumor enhancement using folate-targeted and non-targeted liposomes.................33

Figure 9.3 MRI images of tumor bearing mice exposed to folate targeted liposomes............................34

Liposome encapsulated ZnTPP ..................................................................................................................34-39

Table 1. Stability of targeted and non-targeted liposomes......................................................................35

Figure 10. Uptake of ZnTPP encapsulated in folate targeted and non-targeted liposomes.....................36

Figure 10.1 Conc. And irradiation time dependence of phototoxicity for ZnTPP.................................37

Figure 10.2 Phototoxicity of 1 µM ZnTPP on A549 and HeLa cells exposed to LED light......................38

Conclusion ..................................................................................................................................................39

References ..................................................................................................................................................40-42
Abstract

The folate receptor is overexpressed on the surface of numerous cancer cell types including those of the breast, lung, kidney, ovary, and brain [1, 2]. Recent interest has exploded in the use of folate to deliver payloads and imaging agents to folate receptor positive cancer cells based on several positive clinical trials including phase III trials of EC-145, which is poised to become the first folate targeted, FDA approved drug [3]. Given the success of EC-145 and numerous other agents in the pharmaceutical pipeline, there remains a great interest in the exploitation of this technology in the delivery of nanoscale agents to folate receptor positive cancer cells [3]. In this review I will examine the current status and role of folate targeted nanoconjugates for both diagnostic imaging and therapy.
Introduction

Cancer affects the lives of many people in more ways than one and is a leading cause of death in today’s society. Radiation of the patient undergoing treatment for cancer with the use of certain chemotherapies usually results in the destruction of healthy tissue that leaves the patient with unwanted and harmful side effects. In the past two decades scientific research has come up with many innovative procedures in detecting cancer and treating different types of cancer without the unwanted side effects. One major breakthrough in cancer research has been the discovery of the tumor associated antigen known as the folate receptor ($FR$) which has a high binding affinity for the vitamin folic acid. Researchers have discovered ways of conjugating nanoparticles such as BSANPs (bovine serum albumin nanoparticles), mesoporous silica, gold, metallic and quantum dots to folic acid in an effort to selectively target cancerous cells that result in cellular penetration causing apoptosis of the cell tissue that is specifically infected without the harmful side effects that follow conventional treatments. Many nanoparticle conjugates that have been used for selective targeting of cancers show promising results with the large payloads of the drug entering the cell into the cytoplasm via attachment the nanoparticle and FA causing its damage to the cell thus preventing the tumor cells from metastasizing. The greatest benefits of using folate conjugated nanoparticles for cell targeting is the reduction in side effects as well as the increase in the potency of the drug due to the large payload being taken into the cell through endocytosis. The focus of this paper is mainly on the advancements of folate nanoparticle conjugates that have been researched and used for bio-imaging in order to determine where exactly the cancer is located and intracellular delivery of the drug causing the destruction of
cancerous cells by targeting the folate receptor which is normally found in large concentrations on cancerous tissue.

**Figure 1. The molecular structure of folic acid.**

**Background**

Folic acid or pteroyl-L-glutamate belongs to the vitamin B family and is a necessary compound or molecule used in many reactions by the body for red blood cell proliferation and is involved in many reactions for DNA synthesis and repair (See Figure 1.) for chemical structure. In the past decade or so researchers have indicated that there is an increased expression of the folate receptor which has been identified as a tumor marker on many cancerous tissues such as human lung, intestinal tract, kidney, ovary, breast, brain, colon, and endometrium [1, 4]. The folate receptor is a glycopolypeptide with a high binding affinity $K_D < 10^{-9}$ M for folic acid. Human folate receptor falls into a family of genes that is categorized by three types –α, β, and –γ each with its own specific tissue distribution and different binding affinities for folic acid [2, 5]. Folate receptor alpha is the most widely researched and over expressed form of the three and due to the increased expression of FRα in epithelial tumor cells it has been considered as a good targeting marker for the use with new selective anti-cancer drugs [6, 7]. The FRα is also expressed on normal epithelial tissue one major concern is the destruction of healthy tissue throughout the body and surrounding areas where the tumors are located but since the FRα has a
restrictive anatomic distribution on the luminal surfaces of healthy epithelial tissue this spares these tissues from FR targeted agents [6]. When designing folate nano-conjugates environmental pH should be considered. Once folic acid forms a complex with the folate receptor the complex is endocytosed with a change in pH environments, acidic environments that can modify the nano-conjugate properties resulting in destruction, or formation of different complex and early release of the payload as a result of the nano-conjugates coming together. Utilizing nanoparticles conjugated to folate for targeting of the folate receptor should tolerate a wide pH range in order to efficiently target the folate receptor and allow for a more controlled release of the payload once endocytosed.

When using folate as a carrier a major concern is the rapid expulsion or release of the nanoparticles through the reticuloendothelial system which is also over expressed with FRα. Pegylation which is the attachment of nanoparticles to folic acid by polymeric chains allows for longer systematic circulation thus larger concentrations of nanoparticles are able to reach their target allowing for a higher uptake of drugs or other agents into the cells [8]. The compound folic acid is a water soluble compound and has its advantages when used as a targeting agent for tumor cells it has a low molecular weight of 441 Da, it has high affinity for the folate receptor and inexpensive, stable, and non-immunogenic compared with proteins such as monoclonal antibodies [9]. Nanoparticles have been of considerable interest in targeting cancerous cells because of the reduced toxicities as well as the enhanced therapeutic effects. Nanoparticles show sufficient physical and biological stability that facilitates entrapment of a large payload and drug release they have good tolerability of components, as well as a simple conjugation process with folic acid [9]. The development and use of nanoparticles conjugated to folic acid has tremendous advantages in fighting cancer. Metallic nanoparticles are used in bio-imaging due to their
controllable light emission which allows for detection and location of the tumor cells, photodynamic therapy allowing for the destruction of tumor cells in specific regions sparing the surrounding healthy cells [8].

**Pegylated nanoparticles for the enhancement of circulation and targeting of FR+ cancer cells.**

One problem with using conventional anticancer agents is not only do they target the cancerous cells but they also target healthy cells and are easily excreted through the body and systematic circulation. First the toxic effects of the drugs cause unnecessary cell death and second rapid excretion of the drug and the nanoparticle by the body will reduce its targeting efficiency [10, 11, 12]. One major goal is to increase systematic circulation of the drug in order to deliver a large payload as well as increase the binding efficacy of the nanoconjugate without damaging healthy tissue. Prior research has determined that the concentrations of the FR are 100-300 times higher on cancerous cells compared to healthy cells it has also been concluded that as the stage of the cancer increases so does the FR density this benefits the use of nanoparticles conjugated to folic acid allowing for a higher efficiency of binding to the FR and delivery of a large payload or drug to the tumor cells [10, 11]. Targeting of tumors occurs through the EPR method the enhanced permeability and retention effect which is based on the fact that as tumor cells become larger they stimulate the production of new vessels that result in leaky fenestrations that allow passage of macromolecules and NPs into the tumor cells [10, 14] One major drawback of the FR is that high concentrations naturally occur in the proximal tubules of the kidney which allows for excretion of folic acid as well the clearance of the nanoparticles and necessary drugs needed to target the tumor cells. The development of PEG poly(ethylene glycol) backbones has been shown to reduce the clearance of nanoparticles by the
reticuloendothelial system in order to increase the targeting efficiency to the FR by increasing
the half-life of the nano-particles carrying the drug or payload in the blood stream [10, 11, 12].
The use of PEG spacers tethered to liposomes and folic acid for targeting of FR+ cells were
found to enter the target cells at much higher concentrations then the control groups with a cell
death count 85 times greater than the controls [1]. PEG backbones containing varying numbers
of –NH2 groups (Au-PAM4-20K-FA) PEG-tetra-amine molecular weight 20000, (PAM2-2k),
(PAM2-10k), PEG-diamine with molecular weights 2000 and 10000 respectively and (PSH2-2K)
PEG-dithiol with molecular weight 2000 were used to determine the targeting efficacy of
folate decorated nanoparticles to the FR+ cells [12]. (See Figure 2) In this study Battacharya et
el. determined that AuNPs had a much higher binding capacity to the sulphydryl groups on the
PEG-dithiol with PEG-tetra-amine having the second highest binding capacity to AuNPs. It was
also determined that PAM4-20K-AuNPs had a much higher binding efficiency to Folate then
Au-PSH2-2K [12]. Due to its higher binding efficiency to folate Au-PAM4-20K was used for
testing on OV-167 and OPM-1 ovarian cancer cells which show the highest concentration of
folate receptor compared to other cell lines such as OVCAR-5, and RPMI cells. After a 1 hr
treatment of Au-PAM4-20K to OV-167 TEM images show a more intense color compared to
OVCAR-5 which have lower FR expression. The images clearly indicate that nanoparticle
internalization is greater in cells lines that have higher concentrations of FR due to the attraction
of folate to the FR and the pegylation of the nanoconjugate. The uptake of Au-PAM4-20k was
also higher and showed a more intense color in OPM-1 cells compared to cells with lower FR
concentration (See Figure 3) [12]. Altering the attachment of folate to gold nanoconjugates by
using different PEG structures Battacharya et el. showed that amine containing PEG structures
are the choice when tethering folic acid to nano-conjugates and that there is an increase in the uptake of gold nanoparticles by FR+ cells.

Experiments to see which nano-conjugate had a higher cellular internalization were implemented using mPEG–thioacetamide (mPEG-2000T):AuNPs, and FA-mPEG-thioacetamide (mPEG-1500T):AuNPs which were then analyzed by transmission electron microscopy that indicate the folate coated polyethylene glycol nanoparticles had a higher internalization and selective uptake by KB cells then the mPEG-thioacetamide gold nanoparticles alone (See figure 4) [11]. The use of PEG backbones is an excellent way of increasing the circulation of nanoparticles in vitro and in vivo resulting in a higher targeting efficiency as well as the delivery of large payloads to FR+ cells.

**Figure 2.** Chemical structure of different PEG backbones [12]
**Figure 3.** TEM images of OPM-1 cell treated for 1 hr with AuPAM4-20k-FA.

A, Internalization of AuPAM4-20k-FA cells. B, C, Images of A at higher magnification. The micrographs with arrows show the internalization of gold nanoconjugates and their localization in early and late endosomes. D, Representative picture of the intracellular nanoconjugate uptake by OPM-1 cells during a five minute treatment [12].
Folate Nanoparticle Conjugates for Intracellular Tracking of Payloads and in Imaging of Cancer Cells

Nanoparticles conjugated with folic acid are not only beneficial in delivering large payloads of medicine to the FR+ cells for destruction. Nanoparticles are very useful in MRI magnetic resonance imaging for detecting where the cancerous tissue is, as well as a tracking device allowing for real-time visualization in where the medicine is being distributed. Folate nanoparticle conjugates can be tethered to certain fluorescent probes in order to track the bio distribution of the payloads through bio imaging. One of the first bio-imaging agents to be conjugated with folic acid for imaging of cancer cells is $^{111}$In. The light-emitting agent was preferred because of its short half-life and rapid excretion. Imaging agents that remain in FR- cells will tend to contribute to nonspecific background and the therapeutic agents will usually
result in off target toxicity [1]. The use of $^{111}$In-DTPA-FA clearly shows a more intense luminescence throughout the patient that is malignant indicating that $^{111}$In-DTPA-FA has been endocytosed by the cancerous cells resulting in visualization of the cancer for site specific targeting [1, 3].

**Mesoporous Silica Nanoparticles**

There are a number of Multifunctional nanoparticles which can be used to simultaneously deliver large payloads of medicine as well as give real time visualization in the delivery of the nano-conjugates. Mesoporous silica nanoparticles are an excellent source for conjugation to folic acid in which fluorophores such as FITC can be tethered to and used in the imaging of cancer. Silica already exists in the human body as silicone dioxide (28). Silica conjugated by a PEG spacer to folic acid and tethered to FITC fluoresceine isothiocyanate, NPs(FITC)-PEG-Folate are used to deliver as well as track the payload by fluorescence. In order to prove that PEG- Folate was conjugated to The Nps(FITC) FT-IR spectral analysis was done on NPs(FITC)-PEG-Folate and NPs(FITC). As you can see from (figure 5) in the FT-IR spectra there are two distinct peaks at 1500 cm$^{-1}$ and 1657 cm$^{-1}$ and corresponds to the benzene of the folate and the NH2 of the nanoparticle resulting in the successful formation of the nano-conjugate [13].
Figure 5. FT-IR spectra of NPs(FTIC)-PEG-Folate and NPs(FITC) [13].

(Figure 5.1) shows a diagram for the synthesis of NPs(FITC)-PEG-Folate. Observation of KB cells under an inverted confocal microscope clearly indicates that NPs(FITC)-PEG-Folate have higher internalization compared to NPs(FITC) and is indicated by the intense fluorescence for KB cells that are FR+ and little or no fluorescence by those cells that are FR-. (See figure 5.2) The use of the NPs (FITC) folate nano-conjugates with FR+ KB cells in this study researchers were able to show the direct targeting and tracking of the nano-conjugates by fluorescence. [13].
Figure 5.1

Preparation of NPs(FITC)-PEG-Folate. The maleimide end of the maleimide-FITC reacts with thiol groups of 3-mercaptopropyltrimethoxysilane forming thioester linkages. Folate is then activated using ethy(diethylaminopropyl) carbodiimide/N-hydroxysuccinimide and reacted with NH2-PEG-maleimide to form the reactive intermediate maleimide-PEG-Folate which then reacts with NPs(FITC) to produce NPs(FITC)-PEG-Folate [13].
Figure 5.2 FR+ KB cells incubated in Non targeted NPs(FITC) nanoparticles top panel in comparison with targeted NPs(FITC)-PEG-Folate at 37 degrees Celsius for 6 hours [13].

Magnetic Nanoparticles

Supraparamagnetic iron oxide nanoparticles such as magnetite Fe3O4 are also multifunctional nanoparticles very useful in magnetic resonance imaging due their magnetic properties. What makes Magnetic nanoparticles so useful for MRI is that they can be controlled by their shape, size, and composition of their magnetic core [14]. Magnetic nanoparticles that are not modified are very stable at low and high pH. Even though (supraparamagnetic iron oxide nanoparticles) or SPIONs are very stable at different pH values iron oxide agglomeration can occur in vivo resulting in inefficient nanoparticle distribution. To protect the uniformity and stability of the SPIONs as well as provide a multitude of arms for conjugation to medicines and their targeting receptors they can be coated with different polymers such as PEG, dextran, or phospholipids in situ. SPIONs that are coated with PEG are very useful for cell targeting in that
they are able to bypass the reticuloendothelial system resulting in higher concentrations of NPs to their target. Supraparamagnetic iron oxide nanoparticles can be conjugated with linker agents that contain a sulfhydryl group that binds the amino group that is on the SPION allowing for control over the bound ligands. Hogman et al used SPIONs and linked them with transferrin protein by using pyridyl disulfide (PD) and was able to show that there was a 4 fold increase in the binding of Tf molecules for each individual SPION as well as a 10 fold improvement in cellular uptake allowing for a more sensitive SPION for imaging [14]

In another study magnetic nanoparticles are conjugated to folic acid and coupled with methacrylamido to formulate MA-FA-Fe3O4 nano-conjugates which were used to determine the cellular internalization of the nanoparticles as well as to target the cancer cell in order to cause cell death by apoptosis [15]. This study first revealed that a 4.5µg/ml concentration of the folic acid conjugate is all that is required to cause cell apoptosis in 5RP7 cancer cells which have high concentrations of the FR compared to NIH/3T3 cells. TEM images of the magnetic nanoparticles can be seen in (figures 6) the TEM images of MA-FA-Fe3O4 indicate that first cellular internalization of the nano-conjugate has occurred and second the nanoparticles are imaged [15]. The TEM images in (Figure 6.1) of normal cells incubated with MA-FA-Fe3O4 shows that internalization of the nano-conjugate has occurred and indicates high cell viability after 24 and 48 hour treatment of the MA-FA-Fe3O4 with no internal damage due to the cells remaining intact [15]. Comparing the TEM images of the 5RP7 cells incubated with MA-FA-Fe3O4 to those of the NIH/3T3 cells it’s a clear observation (See figures 6.2) that internal cellular damage has occurred leading to apoptosis of the folate receptor positive cells [15]. The increased cell death of the 5RP7 cells was most likely due to the increased uptake of the
magnetic nano-particle conjugates resulting from the formation and endocytosis of the folic acid/FR complex in which NIH3T3 cells are deficient resulting in the higher cell viability.

**Figure 6** TEM images showing of $Fe_3O_4$ nanoparticles [15].

![TEM images of Fe3O4 nanoparticles](image)

**Figure 6.1** TEM images of NIH/3T3 cells treated with 4.5µg/ml of nano-conjugate (a) after 24 hr treatment and (b) after 48 hr treatment [15].

![TEM images of NIH/3T3 cells](image)
**Quantum Dots**

Quantum dots which are semiconductor nanocrystals are a good source for use in bio-imaging of cancer. Quantum dots such as CdSe, CdS, ZnS, PbS, and PSe are very beneficial in that their absorption properties can be tuned from visible to near infrared region which allows them to emit a multitude of colors with very intense signals depending on their size and the wavelength of the excitation source. One of the drawbacks to quantum dots is their long lived circulation in the blood stream which increases their toxicity level [16, 17, 18]. There has been an effort to reduce the toxicity of quantum dots by tethering them to folic acid in conjugation with silicon a non-toxic agent to form FA-Si-QD (**See Figure 7**). The formation of Si-QD
nanoparticles tends to have lower toxicity levels compared to conventional QDs and results in quick expulsion through the renal system as silicic acid [18]. (See Figure 7.1). Folate receptor positive cells that were incubated with FA-Si-QDs and then pre-saturated with folic acid show very low luminescence in Panc-1 cells. Panc-1 cells that are incubated with FA-Si-QDs without any pre-saturation of folic acid result in higher cellular uptake and is clearly visualized by false color confocal images. Si-QDs are good agents for conjugation to folic acid for further research allowing for real time visualization [18]. (See Figure 7.2) Cytotoxicity assays carried out on human pancreatic cells using hydrogen terminated Silicone quantum dots and cysteine capped CdTe QDs indicates that there is a higher cell viability of Panc-1 cells when exposed to Si QDs compared to the conventional QDs such as CdTe [18].
Figure 7  a. hydrosilylation with undecylenic acid; b. EDC coupling to the o-acylisourea ester; c. formation of NHS-ester; d. bioconjugation [18].
Figure 7.1

b. False color confocal images of Panc-1 cells incubated with FA-Si-QDs after saturation with excess folic acid which is preventing the internalization nanoparticles conjugates. c. Panc-1 cancer cells incubated with FA-Si-QD alone. The bright green fluorescence clearly indicates cellular uptake of the nanoparticles [18].
Figure 7.2  Comparison of cytotoxicity assays of Si-QDs and CdTe quantum dots in µg/ml it can be seen that the at high concentration range of Si-QDs cell viability is higher compared to the cell viability cells treated with CdTe quantum dots [18].
Gold Nanoparticles

Gold has had a place in the field of medicine ever since ancient times and has been known to be used by the Chinese for its therapeutic purposes. Red colloidal gold is used by the elderly in India as an ayurvedic medicine for revitalization and rejuvenation. Gold because of its non-toxic properties has worked its way into western medicine for the treatment of certain disorders such as epilepsy and other nervous disorders [12, 16]. Gold is the most stable of all the metals and what makes it so attractive in nano medicine is its biocompatibility, its ease of use when designing new nano-conjugates, and SPR (surface plasmon resonance) bands that allows for easy characterization [11, 16]. Gold nanoparticles exhibit scattering at two wavelengths which are tunable in the visible as well as the near IR region and is due to their SPR. Gold nanoshells made from a silica core with a coating of gold nanoparticles showed resonance at 700-750 nm for a 20 nm gold nanoshell with a 60 nm silica core, the same size silica core with a 5 nm gold nanoshell resonated between 1000 and 1050 nm [16, 17]. Gold is very useful in the delivery of nano-medicines because its stability in vitro and wide pH range (2-12) that it can take on once endocytosed by the cell [11].

The absorbance of light in the visible region by the gold nanoparticles is what makes it so useful in the thermal destruction of cancerous cells in a process called nanophotothermolysis. In the process of photothermolysis AuNPs absorb light and tend to build up heat within a picosecond time scale causing destruction of local cells that are targeted [19]. Zharov et el. determined that a 30 nm AuNP irradiated at a wavelength of 532 nm with a fluence equal to 0.5 J/cm² and after a 20 ns pulse could produce temperatures up to 2500 K which can be used to
destroy cancerous cells [19]. In two separate studies Mansoori conjugated (See Figure 8) (4 atp), 4-aminothiophenol with AuNPs via thiol group to form 4atp-AuNPs which was then conjugated to folic acid by amine binding. Using the AuNP nano-conjugate that were designed Mansoori was able to target HeLa cancer cells that are FR+ as well as cause thermal ablation of the cells by nanophotothermolysis and then compared data of the cellular uptake and cell death in MCF7 cells that are FR- determining that the Folate-4atp-AuNP is a very suitable agent for selective targeting of cancer.

Figure 8. Diagram for the reaction of 4-aminothiophenol to AuNps and then folic acid [20].
To test for the cytotoxicity of the AuNP nano-conjugate on the two different cell lines such as FR- MCF7 cells and FR+ HeLa cells Mansoori et al incubated the cells with Folate-4atp-AuNP for 1, 2 and 4 hour periods and observed that there was no cell lethality for either MCF7 or HeLa cells as can be seen from (figure 8.1) [19, 20].

**Figure 8.1** Cytotoxicity assay of AuNP nano-conjugate on HeLa and MCF7 cell for 1 and 2 hour incubation periods [20].

Folate receptor negative MCF7 cells and folate receptor positive HeLa cells were subjected to an Intense Pulse light (IPL) to determine the cell lethality when exposed to varying pulses of light with treatment parameters that consisted of 15 J/cm² for energy fluency, with a cut off filter of 560 nm and a pulse duration of 3 milliseconds. Both cell lines were exposed to 10, 15, 20, 30, and 40 pulses [19, 20]. From observation of the data in figure 8.2 it can be concluded that there was no cell lethality and that IPL does not cause destruction of either cell lines when exposed to
20 pulses and the correct specifications resulting in a suitable number of pulses to use when irradiating the cells containing the entrapped cargo. Cell viability of both HeLa, and MCF7 cells did drop but only after exposure to pulses of 30 and 40 [19,20].

Figure 8.2 Percent survival of HeLa and MCF7 cells after exposure to varying numbers of IPL with an operating mode: 8 mm x 15 mm; fluency 15 J/cm²; filter wavelength 560 nm; pulse duration of 3 milliseconds [19].

Knowing that Folate-4atp-AuNP indicated no cytotoxic effects on HeLa and MCF7 cells and with the correct number of pulses for IPL Mansoori was able to show that there is a significant difference in cell viability between FR+ HeLa cells and FR- MCF7 cells that are incubated with Folate-4atp-AuNp and exposed to irradiation as observed from (figure 8.3). Each cell line was incubated with a specific concentration of the nano-conjugate then irradiated in 3 millisecond increments to observe the effects of photothermolysis on FR+ cells as well as FR- cells. It’s obvious that exposure to different concentrations of nano-conjugates and (IPL) Intense pulsed light to FR- MCF7 cells shows no cell lethality where the FR+ HeLa cells do indicate cell death. The fastest increase in cell destruction occurred when 5 µg/ml of the nano-
conjugate was added and the incubation time was changed from 1 hour incubation to 2 hour incubation which had only 29% cell survival where the incubation period for 1 hour showed 77% cell survival [19, 20]. Using the AuNP nano-conjugate tethered to 4-aminthiophenol and folate Mansoori was able to show the cellular uptake of the nano-conjugate by FR+ HeLa cells, that no toxicity occurred when the cells where incubated with the nano-conjugate alone as well as the destruction of FR+ HeLa cells.

Figure 8.3 Percent survival of Hela and MCF7 cells after 1, 2, and 4 hour exposure to nano-conjugate and radiated with IPL [20]
Liposomes

Liposomes which are mainly composed of phospholipid bilayers are very useful in the delivery of medicines, enzymes, coenzymes, genetic material and are able to entrap a high concentration of payloads to the site of infection either by passive, or active targeting [21]. As the nature and function of liposomes was further understood researchers were able to attach other functional molecules such as PEG to their surface in order to increase longer circulation in vivo by preventing the non-specific opsonisation by phagocytes. Liposomes have the capability of encasing hydrophilic and hydrophobic molecules and at the same time maintain all cellular activities such as accumulation at the target tissue due to long systematic circulation and controlled drug release [22, 23]. Liposomes are able to infiltrate the tumor cells mainly by passive targeting by way of the enhanced permeability and retention effect through the leaky vasculature associated with the tumor. Liposome infiltration into the tumor cells can be increased by conjugation to folic acid resulting in an increased efficiency and delivery of the payload within the liposome. With the advancements and technology in the treatment of cancer liposomes have been tethered to Gadolonium which allows for MRI imaging of tumor cells. Liposomes have also been used for entrapping and delivery of ZnTPP (zinc-tetraphenylporphryn) a photosensitizers in an effort to minimize the unwanted side effects due to normal tissue damage as well as to destroy the tumor cells with photodynamic therapy.

In a 2009 study which researched the uptake and MRI imaging of cancer cells used (See Figure 9) targeted liposomes consisting of Gd.DOTA.DSA/DOPC/Cholesterol/DSPE-PEG2000/DSPE-PEG(2000)Folate/DOPE-Rhodaamine 30/32/30/4/3/10 mol %, and non-targeted liposome formulation which consisted of Gd.DOTA.DSA/DOPC/Cholesterol/DSPE-PEG2000/DOPE-Rhodamine with a molar ratio of 30/32/30/7/1 mol % (50). IGROV-1 cancer
cells having an overexpression of folate receptor on the cell surface were incubated with targeted and non-targeted liposomes and then compared post incubation. To test for the internalization of 157Gd by IGROV-1 cells ICP-AES measurements were conducted. (See Figure 9.1) ICP-AES analysis of IGROV-1 cells incubated for 5 hours with non-targeted liposomes and 0.03 mol % folate liposomes shows very low concentrations in ppm of Gadolonium-157 being internalized by the cells. Observing IGROV-1 cells that were incubated with targeted liposomes containing 3 mol % of folate shows a very high concentration of 157Gd internalized by the cells indicating that folate conjugated liposomes are more preferably taken up by the FR+ IGROV-1 cells (50).

Targeted and non-targeted liposomes were injected into tumor bearing mice for MRI imaging. MRI scans of the tumor bearing mice were conducted in order to identify the tumor and determine the T1 baseline value using a 4.7 T magnet. Post injection the mice were then imaged at 2, 16, and 24 hrs and the weighted T1 images for each respective time point was determined. From Observation of (figure 9.2) it is clear that there is an increase in The MRI signal after 2 hour post injection and that specific targeting of the tumor cells by folate liposomes has occurred. MRI imaging of tumors that have been exposed to folate targeted and non-targeted liposomes can be seen in (figure 9.3) where it is also observed that there is an increase in signal intensity after 2 hour post injection of folate targeted liposomes [24]. After MRI imagining was completed liver and kidney sections from tumor bearing mice were analyzed by fluorescence microscopy imaging to determine if the folate targeted and non-targeted liposomes have infiltrated healthy liver and kidney tissues. From the microscopy images of the liver and kidneys there is low fluorescence intensity indicating that the liposomes have not infiltrated the healthy cells in these specific organs and that long systematic circulation is occurring due to the
pegylation of the liposomes resulting in accumulation within FR+ IGROV-1 cells of the tumor bearing mice [24].

**Figure 9** Schematic picture of Folate-targeted liposome [24].

![Figure 9 Schematic picture of Folate-targeted liposome](image)

**Figure 9.1** ICP-AES analysis of the cellular uptake of 157Gd after 5 hour incubation period [24].

![Figure 9.1 ICP-AES analysis of the cellular uptake of 157Gd](image)
Figure 9.2 Mouse tumor enhancement using folate targeted and non-targeted liposomes. (a) T1 measurement at 4.7 T. (b) signal enhancement determined from the mean tumor T1 values for pre- and post-liposomal injection [24].
Figure 9.3 MRI images of tumor earing Balb/C nude mice: top images indicate mice injected with folate targeted liposomes at 2, 16, and 24 hours; bottom images are of tumor bearing mice injected with nontargeted liposomes at 2, 16, and 24 hours [24].

Liposome encapsulated ZnTPP

Photodynamic therapy is a light based therapy and has been used in the treatment of many conditions including certain cancers. Photodynamic therapy depends on the photosensitization of a photosensitizer which can be locally applied or injected into a specific area. The photosensitizer once exposed to a certain wavelength of light initiates the production of $^1O_2$ (singlet oxygen) from normal oxygen within the tissue. Singlet oxygen is the main ROS reactive oxygen species thought to be the cause of cell death as a result of photo induction [22]. The main objective with photodynamic therapy is to cause cytotoxic cell death by the activation of $^1O_2$ and at the same time minimize destruction of healthy normal tissue that leads to unwanted side effects.
Garcia Diaz et al. were able to design a nano-conjugates consisting of ZnTPP entrapped in liposomes in a molar ratio of 100:1 lipid/porphyrin and then tethered to folic acid in order to compare the cellular uptake and photocytotoxicity by FR+ HeLa cells and FR- A549 cells. Liposomes were prepared by microemulsification process and consisted of non-targeted liposomes POPC/OOPS with a molar ratio of 90:10 and folate targeted liposomes POPC/OOPS/FA-PEG-DSPE with a molar ratio of 90:10:0.01. With 85% cell survival for both folate targeted and non-targeted liposomes a concentration of 10µM ZnTPP was used as an adequate concentration for the delivery to both cell lines [22]. In this study Garcia et al. was able to determine that encapsulation efficacy of the photosensitizer was close to 90% and that the PS was not affected by the incorporation of the folic acid (See table 10). They also tested for liposome stability to assure that none of the entrapped porphyrin is released from liposomes interacting with other proteins or with one another.

**Table 1.** Stability of folate targeted and non-targeted liposome measured by lipid and photosensitizer content [22].

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time/h</th>
<th>L (%)</th>
<th>P (%)</th>
<th>Zave/nm</th>
<th>ζ pot/mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-targeted</td>
<td>0</td>
<td>90±2</td>
<td>94±8</td>
<td>110±20</td>
<td>-38±5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>97±9</td>
<td>85±10</td>
<td>130±30</td>
<td>-31±3</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>79±3</td>
<td>83±13</td>
<td>140±20</td>
<td>-30±3</td>
</tr>
<tr>
<td>FR-targeted</td>
<td>0</td>
<td>87±4</td>
<td>96±7</td>
<td>140±20</td>
<td>-36±2</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>97±12</td>
<td>93±4</td>
<td>130±30</td>
<td>-34±2</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>78±3</td>
<td>83±5</td>
<td>110±20</td>
<td>-35±4</td>
</tr>
</tbody>
</table>

Data are mean values ± SD of at least three independent experiments.

a L: Lipid content, expressed as the percentage of lipid in the sample with respect to the lipid present at the initial stage of liposome preparation.

b P: Porphyrin content, expressed as the percentage of porphyrin in the sample with respect to the porphyrin present at the initial stage of liposome preparation.

c zave: Average mean.

d ζ: Zeta potential.
Folate receptor positive HeLa cells and Folate negative A549 cells were incubated for 24 hours with folate targeted and non-targeted liposomes containing ZnTPP photosensitizer then lysed and analyzed by fluorescence spectroscopy to determine the uptake of liposomes and PS by the cells. By clear observation of (Figure 10) there is a difference in the uptake of PS by HeLa cells and A549 cells. Comparing the fluorescence intensity of the cell lysates for both HeLa, and A549 cells incubated with both non-targeted, and folate targeted liposomes it is clear that the HeLa cells which are folate receptor positive have a much stronger fluorescence intensity then the folate receptor negative cells indicating that the cellular uptake of the liposomes with entrapped ZnTPP has occurred [22].

To test for photodynamic toxicity, the uptake of folate targeted liposomes, and non-targeted liposomes photosensitization experiments were carried out on HeLa, cells (See figure 10.1) that were incubated for a 24 hour period in both liposomes formulations. HeLa cells were exposed to three different concentrations of ZnTPP encased in liposomes then irradiated with green LED light. As observed from (Figure 10.1A) there is a decrease in the number of viable HeLa cells that have been exposed to different concentrations of the poryphrin as well as light but HeLa cells that were incubated with folate targeted liposomes (Figure 10.1B) indicate that there is a higher increase in toxicity indicating that cell cytotoxicity is dependent on concentration as well as photodynamic response [22]. Garcia et el. also used photodynamic toxicity to compare the folate targeting efficiency of both cell lines with a concentration of 1µM ZnTPP throughout and irradiated with 10 Jcm². Analysis of both HeLa and A549 cells (See figure 10.2) indicates that the HeLa cells which are folate receptor positive cells show a higher percent in cell death then A549 cells which remained at the same percent whether incubated with folate-targeted or non-
targeted liposomes. This data clearly indicates that there is a significant difference in the uptake and increase in toxicity of the nano-conjugates and is dependent on folate receptor binding [22].

**Figure 10.** Cellular uptake of ZnTPP encapsulated in Folate targeted liposomes (■) and nontargeted liposome (△) by (A) HeLa cells and (B) A549 cells in folate depleted DMEM [22].

![Figure 10](image1)

**Figure 10.1** Concentration and irradiation time dependence of phototoxicity of ZnTPP encapsulated in (A) non-targeted liposomes and (B) Folate- targeted liposomes By HeLa cells. Concentrations are (■) 0.1µM, (■) 1µM, and (△) 10 µM [22].

![Figure 10.1](image2)
Figure 10.2 Photodynamic cytotoxicity using 1µM ZnTPP encapsulated in liposomes with light intensity set to 10 Jcm² (A) non-targeted liposomes and (B) Folate-targeted liposomes. p < 0.001 [22].
Conclusion

Discovery of the folate receptor on the membrane surface of many cancers and the use of folic acid conjugated to nanoparticles as a moiety for the delivery of therapeutic and imaging agents has been a great achievement in the fight against cancer, and other diseases. The cellular uptake, toxicity affects, and bio-imaging capabilities for real time tracking of the nano-conjugates as well as site specific imaging of the cancer such as MRI for a variety of nanoparticles conjugated to folic acid has been reviewed. Folic acid conjugated nanoparticles show promising results in the fight against cancer in that there is a better understanding in the functionalities of the nanoparticles. In designing and formulating folate nanoparticles to increase the efficiency in targeting cancer cells pH environments within the cells were considered. The Attachment of ligands on the surface of multifunctional nanoparticles was also implemented and researched to provide better systematic circulation and delivery of a large payload via the nano-conjugate. As time progresses researchers are developing newer and more advanced methods for manipulating folate nanoparticle nano-conjugates leading to a more efficient targeting system for bio-imaging and destruction of the cancer without causing cell death to surrounding healthy tissue.
Works Cited


[12] Resham Bhattacharya, MS, PhD, Chitta Ranjan Patra, MS, PhD, Alexis Earl, Shanfeng Wang, MS, PhD, Aaron Katarya, Lichun Lu, BEng, PhD, Jayachandran N. Kizhakkedathu, MS, PhD, Michael J. Yasemski, MD, PhD, Philip R. Greipp, BS, MD, Debrabrata Mukhopadhyaa, MS, PhD, Priyabrata Mukherjee, MS, PhD. Attaching Folic acid on Gold Nanoparticles using Noncovalent Interaction via Different Glycol Backbones and Targeting of Cancer Cells. *Nanomedicine: Nanotechnology, Biology, and Medicine.* 2007, vol. 3, 224-238.


