MAGNETIC NANOMEDICINE AND HYPERTHERMIA FOR THE TREATMENT OF THYROID CANCER

Thesis Presented

by

Aditi Jhaveri

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**Thesis Title:** Magnetic Nanomedicine and Hyperthermia for the treatment of Thyroid Cancer

**Author:** Aditi Jhaveri

**Program:** Pharmaceutical Sciences

Approval for thesis requirements of the Master of Science Degree in Pharmaceutical Sciences

**Thesis Committee**

Chair ___________________________ Date ____________

Member __________________________ Date ____________

Member __________________________ Date ____________

Director of the Bouvé College Graduate School

_____________________________ Date ____________
ABSTRACT

ATC (Anaplastic thyroid cancer) and MTC (medullary thyroid cancer) account for 1-2% and 5-10% of all thyroid cancer cases, respectively. These also represent the most metastatic forms of thyroid cancer. The 5-year survival rate for metastatic MTC is around 40%, while ATC is known to have one of the most dismal prognoses amongst all cancers with 5-year survival rates from 1-7%. Currently, the main treatment options for thyroid cancer are restricted to surgery, radioiodine therapy and external radiation. However, these two forms of thyroid cancer are somewhat radio-resistant and are unable to concentrate radioiodine. No effective chemotherapy options are thus available for treatment, and amongst those that have been investigated, doxorubicin is the most effective agent with a 30% partial response.

The main objective of our investigation was to use a multi-modality approach to treat thyroid cancer. We aim to develop a delivery system (magnetic cationic liposomes) for treating aggressive thyroid cancer which uses a combination of magnetic drug targeting and hyperthermia effectively in enhancing the effects of the chemotherapeutic agent. We characterized our formulation in terms of size, charge, iron content, magnetization measurements and hyperthermia producing effects. We also observed its effect on the viability of the cell lines used, as well as its ability to accumulate in the thyroid cancer and endothelial cells in vitro. We prepared DOX-loaded (Doxorubicin) magnetic cationic liposomes with different concentrations of magnetite (MAG-C) and observed their ability to release drug over a range of temperatures in addition to various characterization parameters. Finally, we observed the effect of varying MAG-C concentrations on the physical properties of relatively novel liposome preparation types, and selected the
optimal formulation on the basis of particle size, cationic charge potential (which would allow it to target the tumor vasculature) and sufficient iron content (to respond to an external magnet as well as to induce heating effects in an AC field).

The MCLs showed particle sizes and zeta potentials from 125±4.0 nm and 26±5.2 mv without MAG-C to 255±11.1 nm and 11±3.1 mv with 20 mg/ml MAG-C. The iron content varied from about 1.5 mg/ml for 2.5 mg/ml MAG-C to about 10.2±4.8mg/ml for 20 mg/ml MAG-C. The MCLs accumulated well in the thyroid cancer cell lines CAL-62 and TT respectively up to a concentration of 1000 nmol/ml, as observed quantitatively by cell association and qualitatively from fluorescence microscopy studies. At concentrations ≤ 1000 nmol/ml, the MCLs were not toxic to either TT or CAL-62 cells. However, HMEC-1, the endothelial cell line was more sensitive to the effects of MCLs and started showing toxic effects at 500nmol/ml of MCLs. We observed a trend which suggested that upon increasing the MAG-C concentration, it became possible to reach a particular temperature in a much shorter duration of time when compared to formulations containing less MAG-C. We also observed that higher MAG-C concentrations stabilized the liposomes and retained significantly more DOX than liposomes with low concentrations of MAG-C. Higher temperatures also favored the release of DOX from liposomes for all MAG-C concentrations. A pilot study making use of a combination of magnetic drug targeting as well effects of heat was also undertaken with the ATC cell line. The DOX-loaded liposomes thus prepared and characterized are expected to show benefits in vitro as well as in vivo as supported by literature, as well as our current and previous investigations.
ACKNOWLEDGEMENTS

“We need to internalize this idea of excellence. Not many folks spend a lot of time trying to be excellent” -Barack Obama

Being excellent and producing quality work are the two fundamental qualities that I have imbibed during my Master’s research here at Northeastern University. The person who was responsible for instilling this in us is none other than Dr. Robert B. Campbell, my thesis advisor and mentor. It was a pleasure being a part of Dr. Campbell’s research lab, learning the fundamentals as they should be learned and being transformed into a scientist. I express my sincere gratitude to him for his continuous support, guidance and encouragement ever since I joined his lab in 2007. Dr. Campbell’s remarkable ability to explain things clearly, paying attention to details, his in-depth knowledge, and dedication to quality research, have benefitted me greatly during my project and have motivated me a lot. I owe him a great deal for having enough faith in me to entrust the project as well as for providing me with this opportunity.

Next, I would like to thank Dr. Mansoor Amiji and Dr. Shashi Murthy for agreeing to be on the thesis committee and providing valuable comments and feedback on my research. I also express my gratitude to Dr. Srinivas Sridhar, Dr. Dattatri Nagesh and Evin Gultepe from the Physics department at Northeastern for helping out with the hyperthermia as well as SQUID measurements and providing resourceful suggestions. I am very grateful to all members of my lab for their kindness and support. I thank from the bottom of my heart my friends Kshitija, Shraddha and Anu for their unflagging and selfless support which saw me through the best and worst of times in Boston. Last but not least, I thank my parents and family members for being there for me when I needed them the most.
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<tr>
<td>AC</td>
<td>Alternating Current</td>
</tr>
<tr>
<td>AMF</td>
<td>Alternating Magnetic Field</td>
</tr>
<tr>
<td>ATC</td>
<td>Anaplastic Thyroid Cancer</td>
</tr>
<tr>
<td>CA4P</td>
<td>Combretastatin A4 Phosphate</td>
</tr>
<tr>
<td>CAL-62</td>
<td>Human anaplastic thyroid cancer cell line</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcino-embryonic antigen</td>
</tr>
<tr>
<td>CHOL</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DOPE-PEG 5000</td>
<td>1,2-dioleoyl-3-phosphoethanolamine polyethylene glycol 5000</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-dioleoyl-3-trimethyl-ammonium propane</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl-phosphatidylcholine</td>
</tr>
<tr>
<td>emu</td>
<td>Electromagnetic units</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FI</td>
<td>Fluorescence Intensity</td>
</tr>
<tr>
<td>FMTC</td>
<td>Familial Medullary Thyroid Cancer</td>
</tr>
<tr>
<td>FTC</td>
<td>Follicular Thyroid Cancer</td>
</tr>
<tr>
<td>HT</td>
<td>Hyperthermia</td>
</tr>
<tr>
<td>LH</td>
<td>Local Hyperthermia</td>
</tr>
<tr>
<td>MAG-C</td>
<td>Magnetite, citric acid matrix</td>
</tr>
<tr>
<td>MCL, MCLs</td>
<td>Magnetic Cationic Liposomes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MDT</td>
<td>Magnetic Drug Targeting</td>
</tr>
<tr>
<td>MEN</td>
<td>Multiple Endocrine Neoplasia</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTC</td>
<td>Medullary Thyroid Cancer</td>
</tr>
<tr>
<td>Oe</td>
<td>Oersted (unit of magnetizing field)</td>
</tr>
<tr>
<td>PTC</td>
<td>Papillary Thyroid Cancer</td>
</tr>
<tr>
<td>RH</td>
<td>Regional Hyperthermia</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>SQUID</td>
<td>Superconducting Quantum Interference Device</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
</tr>
<tr>
<td>TT</td>
<td>Human medullary thyroid cancer cell line</td>
</tr>
<tr>
<td>WBH</td>
<td>Whole Body Hyperthermia</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by abnormal regulation of cellular growth and reproduction (1). Cancer cells can be characterized by some basic features that include uncontrolled cell proliferation, decreased cellular differentiation, ability to invade the surrounding tissue and ability to establish new growth at ectopic sites (2). Cancer cells are characterized by essential alterations in cell physiology that collectively dictate malignant growth. These are: i) self-sufficiency in growth signals, ii) insensitivity to growth-inhibitory (antigrowth) signals, iii) evasion of programmed cell death and metastasis (3). As per the National Center for Health Statistics, cancer is one of the leading causes of deaths in the U.S, second only to heart diseases. The American Cancer Society estimates 1,437,180 new cancer cases and 565,650 deaths related to cancer in 2008 (4).

<table>
<thead>
<tr>
<th>Rank</th>
<th>Cause of Death</th>
<th>Number of Deaths</th>
<th>Percent (%) of Total Deaths</th>
<th>Death Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All Causes</td>
<td>2,448,017</td>
<td>100.0</td>
<td>798.8</td>
</tr>
<tr>
<td>2</td>
<td>Heart diseases</td>
<td>652,091</td>
<td>26.6</td>
<td>211.1</td>
</tr>
<tr>
<td>3</td>
<td>Cancer</td>
<td>559,312</td>
<td>22.8</td>
<td>183.8</td>
</tr>
<tr>
<td>4</td>
<td>Cerebrovascular diseases</td>
<td>143,579</td>
<td>5.9</td>
<td>46.8</td>
</tr>
<tr>
<td>5</td>
<td>Chronic lower respiratory diseases</td>
<td>130,933</td>
<td>5.3</td>
<td>43.2</td>
</tr>
<tr>
<td>6</td>
<td>Accidents (unintentional injuries)</td>
<td>117,809</td>
<td>4.8</td>
<td>39.1</td>
</tr>
<tr>
<td>7</td>
<td>Diabetes mellitus</td>
<td>75,119</td>
<td>3.1</td>
<td>24.6</td>
</tr>
<tr>
<td>8</td>
<td>Alzheimer disease</td>
<td>71,599</td>
<td>2.9</td>
<td>22.9</td>
</tr>
<tr>
<td>9</td>
<td>Influenza &amp; pneumonia</td>
<td>63,001</td>
<td>2.6</td>
<td>20.3</td>
</tr>
<tr>
<td>10</td>
<td>Nephritis, nephrotic syndrome, &amp; nephrosis</td>
<td>43,901</td>
<td>1.8</td>
<td>14.3</td>
</tr>
<tr>
<td>11</td>
<td>Septicemia</td>
<td>34,136</td>
<td>1.4</td>
<td>11.2</td>
</tr>
<tr>
<td>12</td>
<td>Intentional self-harm (suicide)</td>
<td>32,637</td>
<td>1.3</td>
<td>10.9</td>
</tr>
<tr>
<td>13</td>
<td>Chronic liver disease &amp; cirrhosis</td>
<td>27,530</td>
<td>1.1</td>
<td>9.0</td>
</tr>
<tr>
<td>14</td>
<td>Hypertension &amp; hypertensive renal disease</td>
<td>24,902</td>
<td>1.0</td>
<td>8.0</td>
</tr>
<tr>
<td>15</td>
<td>Parkinson disease</td>
<td>19,544</td>
<td>0.8</td>
<td>6.4</td>
</tr>
<tr>
<td>16</td>
<td>Assault (homicide)</td>
<td>18,124</td>
<td>0.7</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>All other &amp; ill-defined causes</td>
<td>433,800</td>
<td>17.7</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Leading causes of Death in the US, 2005(4)
1.1.1 Thyroid cancer: Incidence and mortality

Thyroid cancer is one of the most common endocrine cancers, and accounts for about 1% of all malignancies in the USA. It is estimated to account for 37,340 new cases and 1,590 (910 women and 680 men) deaths in 2008 (4). Over the past several years, thyroid cancer is one of the few cancers that have increased in incidence rates. In 2008, in the US, there are expected to be 11% more new thyroid cancer cases than 2007 (5). As per the latest statistics, it is the sixth most common malignancy in females (4). Moreover, the incidence of thyroid cancer is higher in females with a male to female ratio about 1:1.5-3 in most countries (6).

<table>
<thead>
<tr>
<th>Estimated New Cases*</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>186,320</td>
<td>25%</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>114,890</td>
<td>15%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>77,250</td>
<td>10%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>51,230</td>
<td>7%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>35,450</td>
<td>5%</td>
</tr>
<tr>
<td>Melanoma of the skin</td>
<td>34,950</td>
<td>5%</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>33,130</td>
<td>4%</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>25,310</td>
<td>3%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>25,180</td>
<td>3%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>18,770</td>
<td>3%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>745,180</strong></td>
<td><strong>100%</strong></td>
</tr>
<tr>
<td><strong>Breast</strong></td>
<td><strong>182,460</strong></td>
<td><strong>26%</strong></td>
</tr>
<tr>
<td><strong>Lung &amp; bronchus</strong></td>
<td><strong>100,330</strong></td>
<td><strong>14%</strong></td>
</tr>
<tr>
<td><strong>Colon &amp; rectum</strong></td>
<td><strong>71,560</strong></td>
<td><strong>10%</strong></td>
</tr>
<tr>
<td><strong>Uterine corpus</strong></td>
<td><strong>40,100</strong></td>
<td><strong>6%</strong></td>
</tr>
<tr>
<td><strong>Non-Hodgkin lymphoma</strong></td>
<td><strong>30,670</strong></td>
<td><strong>4%</strong></td>
</tr>
<tr>
<td><strong>Melanoma of the skin</strong></td>
<td><strong>27,530</strong></td>
<td><strong>4%</strong></td>
</tr>
<tr>
<td><strong>Ovary</strong></td>
<td><strong>21,650</strong></td>
<td><strong>3%</strong></td>
</tr>
<tr>
<td><strong>Kidney &amp; renal pelvis</strong></td>
<td><strong>21,260</strong></td>
<td><strong>3%</strong></td>
</tr>
<tr>
<td><strong>Leukemia</strong></td>
<td><strong>19,090</strong></td>
<td><strong>3%</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>592,000</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

Figure 2. Estimated New Cancer Cases in 2008 by sex (4)

1.2 Anatomy and Normal Functions of the Thyroid Gland

The thyroid gland is a butterfly shaped gland, located in the front of the neck immediately below the larynx on either side of and anterior to the trachea. It usually weighs less than
an ounce (15-20g in adults) (7). The normal thyroid consists of two main types of parenchymal cells. The follicular cells which line the colloid follicles concentrate iodine and are involved in the production of thyroid hormone. The second type of cells is the parafollicular cells or the C cells which produce calcitonin (1).

The thyroid gland secretes two main hormones namely, thyroxine and triiodothyronine, (also known commonly as T4 and T3 respectively), that regulate the metabolism of the body. The secretion of these hormones is controlled primarily by the thyroid stimulating hormone (TSH) secreted by the anterior pituitary gland. It also secretes another hormone called calcitonin which is important for calcium metabolism in the body. Together these thyroid hormones (T3 and T4) play a significant role in regulating the protein, vitamin, fat and carbohydrate metabolism. They also regulate the body weight, body temperature, basal metabolic rate, heart rate and sleep (7). The thyroid gland is composed of a large number of closed follicles, 100-300µ in diameter. These follicles are filled with a secretory substance called colloid and lined with cuboidal epithelial cells that secrete into

**Figure 3A)** Anatomy and location of thyroid gland (Modified from Guyton AC, and Hall, JE. (2006) *Textbook of Medical Physiology, W.B.Saunders Company, USA* (7))

**Figure 3B)** Microscopic structure of thyroid gland (Modified from Guyton AC, and Hall, JE. (2006) *Textbook of Medical Physiology, W.B.Saunders Company, USA* (7))
the interior of the follicles. The main constituent of the colloid is thyroglobulin, a large
glycoprotein which contains the thyroid hormones within its molecule. Once the secretion
has entered the follicles, it must be absorbed back through the follicular epithelium into
the blood before it can function in the body. The hormones T3 and T4 are synthesized
from dietary iodine by selective absorption of iodides from the circulating blood by cells
of the thyroid gland. To form normal quantities of thyroxine, about 50mg of ingested
iodine in the form of iodides are required each year (7).

1.3 Thyroid Cancer

Thyroid cancer is a cancerous growth of the thyroid gland, and is one of the most
common types of endocrine malignancies. Although its overall incidence is only about
1% amongst all cancers, it accounts for almost 90% of the endocrine cancers, as well as
for most deaths due to endocrine cancers (9). Growths on the thyroid gland are referred to
as “nodules”, 90% of which are benign. It is the malignant nodules which lead to cancer
(8). Thyroid cancer is classified into four main types, based on how its cells appear under
a microscope. These include: 1) Papillary thyroid carcinoma 2) Follicular thyroid
carcinoma 3) Medullary thyroid carcinoma and 4) Anaplastic thyroid carcinoma. Thyroid cancer for the most part has an excellent prognosis, leading to high 10 year
survival rates, though the prognosis may vary depending on a particular sub-type. These
cancers are broadly categorized as well-differentiated and poorly differentiated thyroid
cancers. They grow at different rates and can spread to other parts of the body if left
untreated.
The other relatively less known thyroid cancers include those which occur in the stromal and immune cells of the thyroid, known as thyroid sarcoma and thyroid lymphoma respectively (1).

1.3.1 Papillary Thyroid Carcinoma (PTC)

This is the most common amongst all the thyroid cancers falling under the classification of well-differentiated thyroid cancers. It occurs in the follicular cells of the thyroid and accounts for 80-85% of all thyroid cancer cases. It has an excellent prognosis with 5 year survival rates estimated at around 95%, and can be cured completely with surgery (1, 9). These carcinomas grow very slowly and most often spread to the lymph nodes. In 60% of the cases the disease is localized (confined to the gland), one third of the cases show regional disease (regional lymph nodes and nearby organs by direct extension) and less than 5% show distant metastasis (distant lymph nodes and organs) (9). Certain histological variants of papillary carcinoma are aggressive clinically but make up a small proportion of the papillary carcinomas. These are the tall cell variant where the cells are
twice as long as they are wide, the columnar variant which shows a clear cytoplasm and the diffuse sclerosis variant which is more common among young individuals and adolescents. These show overlapping cell nuclei, a characteristic feature of PTC, and have a significantly higher mortality at 5 years, but are very rare (1, 10).

1.3.2 Follicular Thyroid Carcinoma (FTC)

This is the second most common type of thyroid cancer and its prevalence is 10-15% amongst all thyroid cancers. It is common in areas where the iodine intake is insufficient (10). Although a cancer of the follicular cells like PTC, FTC lacks the nuclear changes characteristic of PTC. Moreover, FTC has a propensity to invade veins and not lymphatics and hence, lymph node involvement is quite rare. Lungs and bones are common sites of metastasis for FTC. Hürthle-cell (oxyphilic follicular or oncocytic) carcinoma is classified as a type of FTC and is a cytological variant of the same. It accounts for 4% of thyroid cancers. Its prognosis is not as good as FTC since it is harder to find and treat. The overall prognosis of FTC is still very good, though not as good as PTC, with the five year survival rate being around 90% for all stages (1, 9, 10).

1.3.3 Medullary Thyroid Carcinoma (MTC)

Medullary thyroid cancer is a cancer of the parafollicular or C (calcitonin secreting) cells of the thyroid gland. It accounts for 5-10% of the thyroid cancer cases and has a poorer prognosis than PTC and FTC. The 5 year survival rates lie between 80-90% for regional disease, whereas these rates drop to around 40% with distant metastatic disease (1, 9, 11). MTC occurs in four clinical settings: sporadic, inherited and as part of the inherited multiple endocrine neoplasia syndromes MEN-IIA and MEN-IIB (11). Approximately
70-75% of MTC cases are non-inherited, sporadic lesions. Inherited MTC accounts for 25% of the cases and it could be either MEN-IIA, MEN-IIB or familial MTC (FMTC) (12). MEN are a group of tumors affecting the endocrine glands, and which are inherited in an autosomal dominant fashion. Of the MEN syndromes, MEN-IIA and MEN-IIB are associated with MTC. In MEN-IIA, the patients suffer from MTC, pheochromocytoma and tumors of parathyroid glands. MEN-IIB patients on the other hand, have MTC and pheochromocytoma, but show normal parathyroid gland. These patients show the presence of mucosal neuromas (characteristic bumps at the end of their tongue and also the gut), gastrointestinal disorders, and are characterized by thickened lips and eyelids (13, 14). In FMTC, the thyroid cancer is passed genetically through a family but not in association with the MEN syndrome. The inherited types of MTC are related to mutations in the RET proto-oncogene which is located on chromosome 10, and is the most indolent form of the disease (12). MTC tumors are characterized by high levels of calcitonin and carcinoembryonic antigen (CEA). CEA is produced by neoplastic C cells. Calcitonin and CEA can be used as important markers for MTC (14, 15).

1.3.4 Anaplastic Thyroid Carcinoma (ATC)

Anaplastic thyroid carcinoma is one of the most aggressive, difficult to treat, and lethal solid tumors known to affect humans (1, 16, 17). It is also a cancer of the follicular cells of the thyroid like PTC and FTC. However, it shows morphological features of an undifferentiated, highly malignant neoplasm. It lacks the usual features of thyroid differentiation like follicular structures and colloid formation and the cells do not resemble normal structure and morphology. The cells are generally large and multinucleated which is why it is commonly referred to as undifferentiated or poorly
differentiated thyroid cancer (17). It represents only 2% of the clinically recognized thyroid tumors, but is responsible for more than half of all the deaths attributed to thyroid cancer. It has a uniformly fatal prognosis with a median survival of only 4-12 months from the time of diagnosis (18). The five year survival rates are between 1-7.1% for ATC, a sharp contrast from the well-differentiated cancers. The peak incidence of ATC is found in the elderly who are in their 6th or 7th decades (19). ATC metastasizes rapidly with the most common sites of metastasis being the lungs (75%), followed by bones (6-15%) and the brain (5-13%) (16). Microscopically, there are three patterns of ATC-spindle cell (53%), giant cell (50%) and squamoid (19%), though they have the same prognosis (20). The ATC cells unlike the other thyroid cells cannot take up radioactive iodine (16). Currently no effective treatment exists for ATC.

For the purpose of our studies, we selected the more aggressive forms of thyroid cancer, and used human MTC and ATC cell lines CRL-1803 and CAL-62 respectively.

![Relative incidence for various thyroid cancers](image)

**Figure 5.** Relative incidence of various thyroid cancers
1.4 Treatment of Thyroid Cancer

The choice of treatment for thyroid cancer depends on a lot of parameters. These include the type of thyroid cancer, its stage, size of the nodule and age of the patient. The treatment options for thyroid cancer include:

1) **Surgery:** Surgery is the mainstay of treatment for thyroid cancer. It may include removing all (total or near-total thyroidectomy) or part of the thyroid (lobectomy). Also, in cases of metastasis to the central or cervical lymph nodes, lymph node dissection is indicated (8).

2) **Hormone treatment:** It is generally done after the removal of the thyroid to replace natural thyroid hormone, and is also used as a part of treatment for papillary and follicular thyroid cancer after surgical removal of thyroid. When TSH levels are high they stimulate the normal thyroid (as well as the thyroid cancer and metastasis) to grow and enlarge. Thyroid hormone pills shut down the TSH production and thereby suppress the cancer growth (8).

3) **Radioactive iodine treatment:** Thyroid cells are the only cells in the body which have the property to concentrate iodine and this can be employed in treating thyroid cancer. It is used for PTC and FTC only, since MTC and ATC are relatively radioresistant. $^{131}$I is given either as a liquid or a capsule to swallow, which travels to thyroid cancer cells throughout the body, killing them when the $^{131}$I is taken up in sufficient quantities (8).

4) **External radiation:** This approach is suitable for patients in whom surgical excision is incomplete or impossible and in cases where the tumor tissue does not take up $^{131}$I (10). It is also used for palliative control of distant metastases and for
treating residual or recurrent disease (21). External radiation therapy makes use of high-energy rays to kill cancer cells. The radiation is delivered by a large machine which directs it to the neck and other tissues where the cancer has spread (8).

5) **Chemotherapy:** Chemotherapy is usually indicated for the more aggressive forms of thyroid cancer; predominantly ATC, and is generally used in combination with one or more of the treatment modalities mentioned above. Chemotherapy makes use of drugs to kill cancer cells. It is usually employed when the other forms of treatment have failed or are inadequate. It might also be used to relieve the symptoms of MTC or other cancers (8, 21).

There is no curative systemic therapeutic approach for the treatment of MTC. Surgery however, still remains the main treatment option. MTC cells cannot concentrate radioactive iodine and this cancer does not respond to conventional cytotoxic chemotherapy (22). Total thyroidectomy is usually indicated in MTC due to the presence of bilateral disease. Patients are also given post operative thyroid hormone therapy. External radiotherapy has proved to be of limited value for MTC and has not been adequately studied. It can be given as a palliative treatment for painful or progressing bone metastasis (15, 22). In most cases however, external radiotherapy should be considered in patients with gross residual disease or inoperable tumors (15). For MTC, chemotherapy could be recommended for progressive metastatic disease, which is not very common. Various chemotherapeutic agents have been evaluated in clinical trials including, doxorubicin, 5-fluorouracil, cis-platinum, vincristine, dacarbazine, epirubicin, methotrexate, bleomycin and cyclophosphamide alone or in combinations. Of these, doxorubicin has been the most successful with 30% partial response rate (15).
Treatment for ATC still remains mostly palliative since the disease is rarely cured and has a fatal prognosis. In most cases of ATC (50%), death is attributed to upper airway obstruction and suffocation. In the remaining patients, the cause of death may be the result of a combination of complications of local and distant disease, or therapy or both (22, 23). Various studies have shown that total thyroidectomy in ATC is justified if the cervical and mediastinal disease can be resected with limited morbidity, which may not always be the case due to the locally invasive nature of ATC. Tracheostomies are performed for impending airway obstruction rather than on prophylactic basis (20). ATC is also relatively radioresistant, but radiotherapy has shown benefit in some studies for palliation as well as for pre/post operative therapy and as an adjunct to surgery. Hyperfractionated radiotherapy in the presence of radiosensitizers like DOX is also known to prolong survival in combination with surgery (20, 24).

Half of the ATC patients show distant metastasis, and even those in whom the disease is controlled locally, die of systemic spread of tumor. The role of systemic chemotherapy therefore is crucial to survival of ATC patients. Doxorubicin is the most widely used single agent for therapy so far but with little success. It showed a partial response of only about 20% in some studies but was slightly more effective in combination therapy with other chemotherapeutic agents. Paclitaxel showed a 53% response rate in a phase II trial in patients with ATC, and stimulated further investigation of its effectiveness (16). In recent clinical studies, combretastatin A4 phosphate (CA4P), a vascular targeting agent; has longer lasting effects than paclitaxel and has shown promise in improving the survival for ATC. One patient who took the drug in a phase I study after exhausting all other treatment options was still alive after 36 months (20).
Chemotherapy alone is of limited use in ATC cells due to the expression of P-glycoprotein and MRP (multidrug resistance-associated protein, MRP1 gene product) (19, 20, 24). The best strategy currently for ATC is a combined modality treatment which involves surgery, hyperfractionated external beam radiation and aggressive systemic combination chemotherapy protocols (16, 19, 20, 24). Vascular targeting, as in the case of CA4P, could be a promising treatment option for ATC in combination with other treatment approaches.

1.5 Tumor Vascular Targeting

The growth of tumors depends on their ability to recruit new blood vessels from pre-existing vessels, in a process known as angiogenesis. Tumor vascularization plays a critical role in the progression of a neoplasm from a small localized tumor mass to a significantly larger and more aggressive burden, capable of metastasizing to distant locations within the body (25, 26). The newly formed vasculature supplies the tumor with oxygen and nutrients required for its growth and metastatic potential. The tumor vasculature is thus an attractive target for fighting cancer. Disrupting this established vascular network may lead to destruction of the tumor cells due to hypoxia and lack of nutrients (25). Moreover, some other advantages of targeting the tumor vascular supply over the tumor interstitium are: a) relatively high degree of access to circulating therapeutics compared to the cells in the tumor interstitium, thus circumventing the problem of delivering drugs to the centre of the tumor, a major hurdle for conventional treatments; b) the endothelial cells are not transformed, and are not likely to achieve mutations leading to drug resistance; and c) the death of a single endothelial cell will result in the death of nearly 100 neoplastic cells (25, 27, 28). Antivasculature therapy thus
deals with targeting the endothelial cells rather than the tumor cells (25). The endothelial cells lining the tumor blood vessels possess an overabundance of negatively charged proteoglycans (such as heparan sulfate), and can proliferate much faster in comparison to the endothelial cells in quiescent tissues (28). The presence of these anionic molecules on the tumor vasculature makes it an attractive target for positively charged or cationic drug carrier molecules (29). Various drug carriers have been used to target the vasculature, liposomes being the most popular.

1.5.1 Liposomes as drug carriers

Liposomes are an important class of pharmaceutical carriers for delivery of drugs and have been extensively studied. They are spherical, self-assembled structures formed by one or more concentric bilayers of natural or synthetic lipids. Liposomes have an aqueous core inside and between the lipid bilayers (30, 31). They have the ability to encapsulate drugs in the aqueous core or bilayer depending on the characteristics of drug and the encapsulation process. Polymers such as polyethylene glycol (PEG) when incorporated into liposomes, give rise to stealth liposomes. The stearically stabilized liposomes remain in circulation for extended periods of time as compared to the conventional liposomes, due to a decrease in the rate of clearance by the reticuloendothelial system (RES) (31, 32). Liposomes offer several advantages since they are biocompatible and non-immunogenic. Their physicochemical properties such as size, charge, membrane rigidity and stability can be manipulated with ease; they protect the pharmaceuticals entrapped within from inactivating effects of external conditions, and prevent untimely drug release (30). The phospholipid vesicles have an ability to deliver pharmaceuticals to specific intracellular targets (30, 33).
On the basis of the surface charge potential of liposomes (resulting from the type of lipids used to prepare them), they can be classified as anionic, electroneutral or cationic. Of these, the cationic liposomes preferentially target the tumor vasculature and are not as likely to penetrate the interstitial matrix as the anionic or electroneutral liposomes (33, 34). Cationic liposomes have been shown to target about 28% of the tumor blood vessel area as opposed to 5% of the normal vessels (27, 33). This preferential targeting of tumor blood vessels by cationic liposomes is because of the presence of patchy and irregular anionic sites along the capillary endothelium. The tumor blood flow is sluggish and irregular which favors more interactions between the cationic liposomes and anionic sites on the angiogenic vasculature (33); compared with the normal vascular networks with regular blood flow velocities. The use of PEGylated cationic liposomes increases their circulation time in blood. PEG is known to shield some of the positive charge of the cationic liposomes due to its stabilizing effect. Incorporation of up to 5mol% PEG in the cationic liposomes however does not prevent the accumulation of the cationic vesicles along the tumor vessel wall, while providing adequate protection against premature clearance and rapid elimination (33).

Although cationic liposomes preferentially accumulate in tumors, their distribution along the tumor vessels is not uniform, and there are preferential sites of drug accumulation (33). Though many vessels are targeted using this approach, there are vessels which are not targeted at all (33, 35). In an effort to achieve the maximum therapeutic potential of cationic liposome technology, an approach which targets liposomes to tumor blood vessels uniformly, and more extensively, may be required. One approach being developed to overcome the heterogeneity associated with using cationic liposomes alone
is to combine cationic liposome nanotechnology with magnetic drug targeting (MDT) (35).

### 1.6 Magnetic Drug Targeting

Magnetic drug targeting (MDT) is the site specific delivery of chemotherapeutic agents to their target using magnetic nanoparticles (ferrofluids) bound to these agents, with an external magnetic field generated at the tumor site (36). In this approach, magnetic particles along with the drug are formulated into a pharmaceutically stable formulation which can be held in position at the target site (or tumor) using an external magnetic field (36-38). This approach helps in localizing the drug to the desired site, thereby minimizing the potential for accumulation of the drug in healthy tissues. A combination of improved target selectivity and enhanced duration of drug exposure to the target also reduces the overall amount of drug taken up by the RES. MDT uses much smaller doses of drug leading to several fold increased drug efficacy and reduced drug toxicity (35, 36, 38-41).

**Advantages of MDT**

In addition to some of the key advantages of MDT already mentioned above, the magnetic nanoparticles used in MDT also offer distinct advantages. They are:

a) The magnetic nanoparticles are biocompatible, non-immunogenic and injectable.

b) They are non-toxic, even if injected in larger quantities. They are metabolized by the hepato-renal system and used in the synthesis of hemoglobin.

c) They have controllable sizes (from a few nanometers to tens of nanometers).

d) They have high magnetic susceptibility and show high accumulation in the desired target tissue or organ.
e) They can be made to respond resonantly to an alternating magnetic field, with advantageous results related to the transfer of energy from the exciting field to the nanoparticle, as in case of hyperthermia.

f) They are superparamagnetic, hence they preferentially deliver the ferrofluid bound drug at the desired site under the influence of an external magnet. This reduces the amount of drug required and thus the side-effects owing to the drug (41-43).

1.6.1 Ferrofluids

A magnetic colloid also known as a ferrofluid, is a colloidal suspension of single-domain magnetic particles dispersed in a liquid carrier which is either polar or non-polar (44). The important properties which determine the biocompatibility and toxicity of magnetic nanoparticles are the nature of the magnetically responsive component, such as iron, nickel, cobalt or neodymium-iron-boron and the final size of the particles their core and the coatings (45, 46). For biomedical applications, iron oxide particles such as magnetite (Fe₃O₄) or maghemite (γ-Fe₂O₃) are employed most commonly. Cobalt and nickel are highly magnetic, toxic and are susceptible to oxidation so are of little interest (46). Iron oxide nanoparticles are superparamagnetic; that is, they do not show any magnetism in the absence of an external magnet, but develop a magnetic moment when an external magnet is applied. A common method for synthesis of the magnetite particles is by coprecipitation from a solution of Fe(II) and Fe(III) salts in an appropriate ratio using alkali metal hydroxides (47).
The ferrofluids used in our study were obtained from Chemicell, Berlin, Germany. We used fluidMAG-C (magnetite), a ferrimagnetic substance containing Fe(II) and Fe(III) ions which has a magnetite (Fe₃O₄) core surrounded by citric acid (Figure 6). The magnetic nanoparticles in fluidMAG-C are stabilized with multivalent low molecular weight ions such as phosphates or carboxylates. It is an aqueous dispersion of magnetic nanoparticles in a citric acid matrix, which prevents the particles from aggregation with foreign ions and stabilizes them. Each individual magnetite particle is surrounded by citric acid as shown in the Figure 6.

1.6.2 Magnetic liposomes

Magnetic liposomes or magnetoliposomes are magnetic derivatives of liposomes and can be prepared by entrapment of ferrofluids within the liposomal core. In other words, they consist of magnetic nanoparticles wrapped in a phospholipid bilayer (37, 42). Magnetoliposomes offer a promising tool for passive targeting with regard to the drug delivery of magnetic nanoparticles (42). An advantage in formulating magnetic particles within liposomes is the enhanced protection against aggregation and oxidation when incorporated in liposomes (43). Magnetic liposomes like conventional liposomes have
biokinetic and structural advantages, like their ability to simultaneously encapsulate drugs or genes along with magnetite (42, 48, 49). Furthermore, their surface can be modified chemically to target specific tissues (42).

![Structure of a magnetic cationic liposome (MCL)](image)

**Figure 7.** Structure of a magnetic cationic liposome (MCL)\(^{(42)}\)

Magnetic cationic liposomes (MCLs) are made of positively charged (cationic) and relatively biocompatible lipids. They have a positive charge on their surface, which is useful in the interaction of these liposomes with the negatively charged tumor vascular surface (33). Prior studies in our laboratory have shown that liposomes containing fluid MAG-C (magnetite) can maintain a positive charge potential and hence their potential to target the tumor vasculature in the presence of the magnetic material. The liposomes have been shown to be taken up by the tumor endothelial cells and cancer cells as well. The application of an external magnet can thus enhance the vascular accumulation in tumors. It has also been shown that MCLs show superparamagnetic behavior and with an increase in the MAG-C content the saturation magnetization values of the MCLs significantly increase (35, 48).

For MDT, it is important to know if the ferrofluid-drug complex reaches the tumor micro-circulation and diffuses freely into interstitial space, or whether the complex
remains within the vascular space (35, 50). Other important aspects to be considered for MDT are: 1) The type and concentration of the ferrofluid employed, 2) the magnetic strength of the external magnetic field, 3) the time duration for which the target tissue is exposed to the external magnet, 4) bioavailability of ferrofluid as well as the in vivo desorption time of the drug (35, 50).

One of the advantages of magnetic nanoparticles as stated earlier is that they can be used for generating hyperthermia. This is a very promising approach in cancer therapy (43). In our study, we plan to explore the combined effects of hyperthermia and MDT for the treatment of thyroid cancer.

1.7 Hyperthermia

**Principle:** Hyperthermia (HT) is a minimally invasive method for selective heat treatment in which the target tissues are heated to about 42-46°C. The main principal here is that cancer cells are more susceptible to effects of heat at temperatures above 41°C when compared to normal cells; and hence, hyperthermia treatment brings about the death of the tumor cells (43).

**Types and methods of hyperthermia application:** HT is usually applied as one of three different treatment strategies: local hyperthermia (LH), which restricts the application of heat to the tumor site, regional hyperthermia (RH) which is applied to treat an entire organ or limb and whole body hyperthermia (WBH), which applies heat to the entire body (51). Various methods can be used to apply heat including microwaves, high-frequency radiowaves, special radiant-heat systems, temperature controlled water baths,
high energy magnets, ultrasound, capacitive heating using RF electric field, as well as interstitial and intracavitary probes (51-53).

**Magnetic nanoparticles for hyperthermia:** A major concern with these heating treatments is difficulty in heating the local tumor region to the intended temperature without causing harm to the normal tissues (42, 54). To overcome this, the idea of intracellular hyperthermia was developed. The approach uses submicron magnetic particles for inducing hyperthermia. The concept of intracellular hyperthermia was first proposed in 1979 by Gordon et al., using dextran magnetite nanoparticles (42, 54). However, the first experimental investigations of the application of magnetic materials for hyperthermia were carried out in 1957 by Gilchrist et al. The group heated various tissue samples with 20-100nm size particles of $\gamma$-Fe$_2$O$_3$ exposed to 1.2MHz magnetic field (41). The magnetic particles can be injected into tumors prior to the application of heat. In this case, when temperatures reached between 40 and 41°C the tumors showed an increase in blood supply. However when the heat was applied at higher temperatures (>42°C), it resulted in a compromised microcirculation and eventual tumor-vascular collapse. Thus highly selective uptake of the magnetic particles by the target cells has a potential to restrict hyperthermic effects to diseased areas and hence spare the normal surrounding tissues from injury (51).

**Mechanism of HT generation by magnetic nanoparticles:** The magnetic nanoparticles generate heat in an alternating magnetic field (AMF) or AC magnetic field of sufficient strength, causing the induction of hyperthermia through hysteresis losses (43). The field of HT has been revitalized with the advent of ferrofluids which exhibit superparamagnetic properties. The HT induced by such magnetic or ferrofluids is termed
magnetic fluid hyperthermia. The magnetization of a ferrofluid relaxes back to zero when it is removed from a magnetic field, due to the ambient thermal energy of its environment (41). This relaxation either corresponds to the physical rotation of the particles themselves within the fluid, or rotation of the atomic magnetic moments across an effective anisotropy barrier within each particle (41, 43). Rotation of the particles is referred to as ‘Brownian rotation’ while rotation of the moment within each particle is known as Néel relaxation (43). Such phenomena strongly depend on the size of the particles. For the smaller particles the Néelian process dominates, while large particles relax following Brownian relaxation (43). For magnetite the critical size for the transition from Neel to Brown relaxation is about 13nm (43). The critical properties of magnetic particles which are used for HT generation include non-toxicity, biocompatibility, injectability, high-level accumulation in the target tumor and effective absorption of the energy of the AMF (54).

Hyperthermia using liposomes: Magnetic particles can selectively heat up tumors by coupling AC magnetic field to targeted nanoparticles. As previously noted, liposomes can effectively load magnetite as well as chemotherapeutic agents simultaneously, hence they can be utilized for hyperthermia in combination with chemotherapy or gene therapy (51). The application of hyperthermia is favored if phospholipids with transition temperature slightly above the physiological temperature are incorporated into liposomes. For drug-loaded MCLs, the magnetic nanoparticles embedded within the lipid bilayer get heated by means of an AC field. The thermal energy is then transferred to the liposome bilayer which gets heated to its phase transition temperature. The bilayer then melts and results in efficient release of the incorporated drug substance (43, 55). The interaction of
chemotherapeutic agents and hyperthermia quite often results in synergism (increased cytotoxicity over that predicted for an additive effect obtained by combining the individual effects of the chemotherapeutic agent and hyperthermia) (56). This efficacy can definitely be enhanced if chemotherapeutic agents are selectively delivered to tumor cells and then localized hyperthermia is applied to tumor site (57). Figure 8 shows various mechanisms by which hyperthermia can exert a therapeutic benefit in combination with liposomes as well as by itself. A few examples of randomized trials for assessing the efficacy of hyperthermia have been listed in the Table 1. Some studies investigating the effects of using liposomes and hyperthermia have been listed in Table 2.

Figure 8. Mechanisms of generating therapeutic effects by Hyperthermia (58)
<table>
<thead>
<tr>
<th>Tumor</th>
<th>Trial</th>
<th>Year</th>
<th>Patients</th>
<th>Therapy</th>
<th>End point</th>
<th>Effect without HT</th>
<th>Effect with HT</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>Gibbsten et al.</td>
<td>1988</td>
<td>107</td>
<td>OK</td>
<td>Disease free survival</td>
<td>52%</td>
<td>89%</td>
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<td>Melanoma</td>
<td>EORTC 18832</td>
<td>1998</td>
<td>832</td>
<td>OK</td>
<td>Overall survival</td>
<td>86.2%</td>
<td>98.1%</td>
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<td>Overgaard et al.</td>
<td>1995</td>
<td>70</td>
<td>RT</td>
<td>In-transit metastases</td>
<td>6.6%</td>
<td>3.3%</td>
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<td>2007</td>
<td>341</td>
<td>RT + HT</td>
<td>Overall survival</td>
<td>16.7%</td>
<td>12.6%</td>
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<td>CT/RT/OR</td>
<td>Local progression</td>
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<td></td>
<td></td>
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<tr>
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<td>1996</td>
<td>306</td>
<td>RT</td>
<td>Disease free survival</td>
<td>16.2 m</td>
<td>31.7 m</td>
<td>0.004</td>
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<td>Glioblastoma</td>
<td>Sneed et al.</td>
<td>1998</td>
<td>68</td>
<td>OR + RT</td>
<td>Local response</td>
<td>41%</td>
<td>59%</td>
<td>&lt;0.003</td>
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<td>41</td>
<td>RT</td>
<td>Local failure</td>
<td>31%</td>
<td>17%</td>
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<td>and neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30.4%</td>
<td>28.0%</td>
<td>NS</td>
</tr>
<tr>
<td>Squamous cell cancer - esophageal</td>
<td>Sugimachi et al.</td>
<td>1995</td>
<td>66</td>
<td>RT + HT</td>
<td>Time to progression</td>
<td>33 m</td>
<td>49 m</td>
<td>0.04</td>
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<tr>
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<td>Nauta et al.</td>
<td>1994</td>
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<td>CR</td>
<td>2-year survival</td>
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<td>1998</td>
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<td>OR</td>
<td>5-year survival</td>
<td>24%</td>
<td>69%</td>
<td>0.015</td>
</tr>
<tr>
<td>Pelvic tumors*</td>
<td>Van der Zee et al.</td>
<td>2000</td>
<td>358</td>
<td>OT + HT</td>
<td>Peritoneal recurrence</td>
<td>16</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bladder</td>
<td>Colombo et al.</td>
<td>2003</td>
<td>83</td>
<td>OR + IVC</td>
<td>8-year survival</td>
<td>49%</td>
<td>6%</td>
<td>0.036</td>
</tr>
<tr>
<td>Colorectal Carcinomatosis</td>
<td>Verwaal et al.</td>
<td>2003</td>
<td>105</td>
<td>CT</td>
<td>Local recurrence</td>
<td>39%</td>
<td>24%</td>
<td>NS*</td>
</tr>
</tbody>
</table>

CT + OR/HIPEC

CR, chemoradiotherapy; CT, chemotherapy; HILP, hyperthermic isolated limb perfusion; HIPEC, hyperthermic intraperitoneal chemotherapy; HT, hyperthermia; IVC, intravesicular chemotherapy; IVTC, intravesicular thermochemotherapy; NR, not reported; NS, not significant; OR, surgical excision; RT, radiation therapy.

*Pelvic tumors - consists of bladder, rectal, and cervical cancer.

**Cervical cancer showed a 3 year survival advantage of 51% vs. 27% (p-value 0.009).

Table 1. Randomized trials for assessing the efficacy of hyperthermia \(^{(59)}\)
<table>
<thead>
<tr>
<th>Author</th>
<th>Tumour model</th>
<th>Drug</th>
<th>Liposome</th>
<th>HT Tx</th>
<th>Tx endpoint</th>
<th>(ITT - Lip)/ (ITT - Drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weinstein et al. 1980</td>
<td>L1210</td>
<td>Methotrexate</td>
<td>7:3 DPPC-DSPC</td>
<td>42°C for 60 min, at same time of IV injection</td>
<td>Death of animal</td>
<td>5</td>
</tr>
<tr>
<td>Bosset et al. 1988</td>
<td>MBT-2 transitional cell carcinoma</td>
<td>Methotrexate</td>
<td>4:1 DPPC-DPPG</td>
<td>41°C for 30 min, at same time of IV injection</td>
<td>Unit tumours too large to heat in apparatus</td>
<td>1.76</td>
</tr>
<tr>
<td>Yatvin et al. 1981</td>
<td>Sarcoma 180</td>
<td>Cisplatin derivative</td>
<td>7:1 DPPC-DSPC</td>
<td>42°C for 60 min, at same time of IV injection</td>
<td>13 days</td>
<td>2.5</td>
</tr>
<tr>
<td>Kazimura et al. 1996</td>
<td>Rous sarcoma virus induced glioma</td>
<td>Cisplatin derivative</td>
<td>9:1 DPPC-DSPC</td>
<td>41°C for 30 min, at same time of IV injection</td>
<td>Death of animal</td>
<td>1.35</td>
</tr>
<tr>
<td>Nishimura et al. 1990</td>
<td>SCC VII carcinoma</td>
<td>Cisplatin</td>
<td>9:1 DPPC-DSPC</td>
<td>42°C for 60 min, at same time of IV injection</td>
<td>Time to double tumour volume</td>
<td>2.8</td>
</tr>
<tr>
<td>Iga et al. 1991a</td>
<td>Meth A fibrosarcoma</td>
<td>Cisplatin</td>
<td>9:1 DPPC-DSPC</td>
<td>42°C for 30 min, at same time of IV injection</td>
<td>21 days</td>
<td>1.74</td>
</tr>
<tr>
<td>Li et al. 1995</td>
<td>B16/F0 melanoma</td>
<td>NDOP</td>
<td>10:3:1 PC-Chol-PEG0000/PC</td>
<td>42°C for 60 min, at same time of IV injection</td>
<td>16 days</td>
<td>3.29</td>
</tr>
<tr>
<td>Zhou et al. 1993</td>
<td>Walker Carcinosarcoma 256 in liver</td>
<td>Doxorubicin</td>
<td>DPPC</td>
<td>42°C for 6 min, 2h after injection via hepatic artery or femoral vein</td>
<td>90 days</td>
<td>1.47</td>
</tr>
<tr>
<td>Maruyama et al. 1993</td>
<td>Colon carcinomA 256</td>
<td>Doxorubicin</td>
<td>9:1:0:6 DPPC-DSPC-GM1</td>
<td>42°C for 20 min, at same time of IV injection</td>
<td>5 min after treatment</td>
<td>GM1 - 3.4</td>
</tr>
<tr>
<td>Huang et al. 1994</td>
<td>Colon carcinomA 256</td>
<td>Doxorubicin</td>
<td>HISPC-Chol-alpha tocopherol 5:5:5:6:1:38:2:0:2 PEG200-DSPC-Chol-alpha tocopherol 5:5:5:6:1:38:2:0:2</td>
<td>42°C for 30 min, 1h. 24h. or both after IV injection</td>
<td>30 days</td>
<td>No GM1 - 3.1</td>
</tr>
<tr>
<td>Uzczuki et al. 1994</td>
<td>Colon carcinomA 256</td>
<td>Doxorubicin</td>
<td>9:1:0:3 DPPC-DSPC-Chol-alpha tocopherol 5:5:5:6:1:38:2:0:2</td>
<td>42°C for 20 min, at same time of IV injection</td>
<td>Death of animal or 70 days</td>
<td>1.14</td>
</tr>
<tr>
<td>van Bree et al. 1996</td>
<td>R-1 rhabdo-myosarcoma</td>
<td>Daunorubicin</td>
<td>Thermostable liposomal daunorubicin (DX)</td>
<td>42°C for 60 min, 1h after IV injection</td>
<td>Time to double tumour volume</td>
<td>1.78</td>
</tr>
<tr>
<td>Chevri &amp; Rathau 1993</td>
<td>Fibrosarcoma</td>
<td>Epirubicin</td>
<td>7:1:0:6 eggPC-Chol-ethanol</td>
<td>43°C for 60 min, at same time of injection. Treatment repeated on alternate days for 4 weeks</td>
<td>Tumour volume measured for 1 month, survival observed for 100 days</td>
<td>PEG - 4.2</td>
</tr>
<tr>
<td>Chevri et al. 1995a</td>
<td>B16F10 melanoma</td>
<td>Melphalan</td>
<td>9:1 DPPC-DSPC</td>
<td>42°C for 60 min, at same time of injection. Treatment repeated on alternate days for 4 weeks</td>
<td>Tumour volume measured for 1 month, survival observed for 4 months</td>
<td>1.32</td>
</tr>
<tr>
<td>Chevri et al. 1995b</td>
<td>B16F10 melanoma</td>
<td>Melphalan</td>
<td>7:0.38:2:1 eggPC-Chol-ethanol methanol</td>
<td>43°C for 60 min, at same time of injection. Treatment repeated on alternate days for 4 weeks</td>
<td>Tumour volume measured for 1 month, survival observed for 3 months</td>
<td>1.36</td>
</tr>
<tr>
<td>Maekawa et al. 1987</td>
<td>A166 ascites in lymph nodes</td>
<td>Bleomycin</td>
<td>7:3 DPPC-DSPC</td>
<td>42°C for 20 min, 4h after IV injection</td>
<td>50 days after treatment</td>
<td>8.46</td>
</tr>
</tbody>
</table>

Table 2. Studies investigating tumor growth delay using hyperthermia and liposomes to treat tumors. (58)
1.8 Doxorubicin

As mentioned already in the treatment section, Doxorubicin (DOX) is the most extensively used chemotherapeutic agent to treat metastatic thyroid cancer. It can be used either as a single agent or in combination with other drugs. It belongs to the anthracycline class of antibiotics and is isolated from different species of Streptomyces. It has a characteristic four-ring structure that is linked to daunosamine, an amino sugar via a glycosidic bond (60).

**Mechanism of action:** DOX is cytotoxic through a number of varied mechanisms. It can be directly toxic to the cell membrane without entry into tumor cells. Once inside the DNA it can alkylate DNA and intercalate with it through covalent binding which leads to DNA crosslinking. It also inhibits isomerase I, II, can interfere with the DNA strand separation and helicase activity, and can lead to enzyme catalyzed iron-mediated free radical formation and lipid peroxidation. All these effects ultimately lead to induction of apoptosis (60, 61).

![Figure 9. Structure of Doxorubicin](image)

DOX is biotransformed into active and inactive metabolites in the liver. It extensively binds to plasma proteins and tissues (70%), is excreted primarily in the bile (40-50%) and persists in plasma for prolonged periods. The extensive binding of DOX in tissues
accounts for the large apparent volume of distribution of approximately 25L/kg (62). DOX shows short term dose-limiting toxic side effects like myelosuppression and mucositis, while cardiotoxicity is its major long-term dose limiting side effect. DOX also sensitizes the tissues to the effects of radiation therapy, hence in combining it with radiotherapy caution should be exercised (61).

**Dose:** DOX is usually given as a single IV dose of 60-75mg/m² infused slowly over about 4-5 min observing precautions and avoiding extravasation. Alternatively, it can be administered at the same dose as a continuous infusion over 2-4 days through a central venous access or it can be divided into two or three daily bolus injections. Doses are repeated every 3 weeks with all of these schedules (61).
2 STATEMENT OF HYPOTHESIS

1. We hypothesize that magnetic drug targeting will improve the accumulation of drugs in thyroid cancer cells in the presence of an external magnetic field in vitro.

2. We hypothesize that magnetic drug targeting followed by the application of heat will significantly enhance the cytotoxic effect of drug-loaded MCLs when compared to drug-loaded liposomes and heat alone.

3. We hypothesize that an increase in magnetite content in magnetic cationic liposomes will increase the heat-generating properties of the liposomes.

4. We hypothesize that an increase in magnetite content will favorably alter the physicochemical properties of doxorubicin-loaded cationic liposomes.
3 SPECIFIC AIMS

**Aim #1** To characterize TT (medullary thyroid cancer) and CAL-62 (Anaplastic thyroid cancer) and HMEC-1 (Human dermal microvascular endothelial cells) cell lines in terms of their susceptibility to the effects of treatment with MCLs and/or heat, as well as their association with magnetic cationic liposomes.

**Aim #2** To demonstrate that drug-loaded magnetic cationic liposomes accumulate to a greater extent in thyroid cancer cells in the presence of an external magnet when compared to MCLs in the absence of a magnet.

**Aim #3** To optimize the concentration of MAG-C for future hyperthermia studies in terms of particle size, zeta potential, iron content and heat-inducing effects of the formulation.

**Aim #4** To demonstrate the effectiveness of chemotherapy *in vitro* in the presence of an external magnet and heat using drug-loaded magnetic cationic liposomes.
4 MATERIALS AND METHODS

4.1 Materials

DPPC (Dipalmitoyl-phosphatidylcholine), DOTAP (1,2-dioleoyl-3-trimethyl ammonium propane), Cholesterol, DOPE-PEG5000 (1,2-dioleoyl-3-phosphoethanolamine polyethylene glycol 5000) and rhodamine-DPPE (1,2-dipalmitoyl-3-dipalmitoylphosphatidylethanolamine-N-Lissamine rhodamine B sulfonyl)-dipalmitoylphosphatidylethanolamine) (Ammonium salt) were purchased from Avanti Polar Lipids (Alabaster, AL); Doxorubicin hydrochloride, trichloroacetic acid, 1% acetic acid, ethanol and SRB (sulforhodamine B) were purchased from Sigma Aldrich (St. Louis, MO); fluid MAG-C (aqueous dispersion of magnetite (Fe₃O₄) iron content-72%) was purchased from Chemicell (Berlin, Germany); 0.9% sodium chloride (saline) irrigation USP was purchased from B.Braun Medical Inc. (Irvine, CA); permanent magnets were purchased from Master Magnetics, Inc. (Castle Rock, CO). For in vitro and in vivo experiments ceramic (0.4 T (Tesla)) and neodymium (1.2 T) magnets were used, respectively.

4.2 Cell Culture and Media

TT (CRL-1803) a human medullary thyroid carcinoma cell line originally derived from a 77-year old female with medullary thyroid carcinoma was purchased from ATCC (American Type Culture Collection, Manassas, VA); CAL-62 (ACC-448), a human anaplastic thyroid carcinoma cell line originally established from the thyroid gland of a 70-year old woman with anaplastic thyroid carcinoma was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany (German Collection of Microorganisms and Cell cultures). HMEC-1 (human dermal microvascular
endothelial cells) was obtained from Centers for Disease Control and Prevention (Atlanta, GA). TT cells were maintained in F-12K medium (Kaighn’s modification of Ham’s F-12K) with L-glutamine supplemented with 10% FBS (fetal bovine serum) while CAL-62 cells were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium) with L-glutamine supplemented with 10% FBS. F-12K, DMEM and trypsin-EDTA were purchased from ATCC. HMEC-1 cells were maintained in EBM-2 consisting of 10% FBS and required growth supplements, purchased as a single bullet kit (CC-3202) from Cambrex Bioscience (Walkersville, MD). FBS (fetal bovine serum)-Fetaclone I (optimized for hybridomas) was purchased from Hyclone (Logan, UT) and PBS (phosphate-buffered saline) was purchased from Biowhittaker (Walkersville, MD). All cell lines were grown in a Revco ELITE III cell culture incubator (Kendro Laboratory, Asheville, NC) with 5% CO₂ at 37°C.

**Cell Lines Used:**

<table>
<thead>
<tr>
<th>TT</th>
<th>CAL-62</th>
<th>HMEC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Doubling time: 83h)</td>
<td>(Doubling time: 24h)</td>
<td></td>
</tr>
<tr>
<td>Light Microscope images</td>
<td>Light Microscope images</td>
<td>DIC images</td>
</tr>
</tbody>
</table>

**Figure 10.** Images of cell lines used (TT, CAL-62 and HMEC)
4.3 Preparation of Liposomes

Liposomes were prepared using the thin film evaporation method (63). The lipid stocks were stored at -80°C under an inert atmosphere. DPPC, DOTAP, cholesterol and DOPE-PEG5000 in the ratio 60:20:15:5 were the standard lipids used for preparing the liposomes. The fluorescent label rhodamine-DPPE was around 5mol% of the total liposome preparation and was included for studies involving fluorescence detection. The required amount of lipid (typically around 10µmol/ml) was dissolved in chloroform and transferred to a pyrex tube or a round bottom flask. Lipids were mixed thoroughly in specific ratios and the chloroform used to prepare lipid stocks was evaporated using a Buchi Rotovapor R-200 (Buchi Labortechnik AG, Flawil, Switzerland). The temperature of water-bath was held slightly above the phase transition temperature of the dominant lipid (DPPC) in the mixture until a thin film was formed inside the wall of the glassware. The lipid film was then allowed to freeze-dry for 4 h in a vacuum environment using a Labonco freeze dryer (Labonco, Kansas City, MO) to remove any trace amounts of the organic solvent.

The dried film was hydrated with saline or different concentrations of MAG-C (2.5, 5, 10, 20 mg/ml) in saline. MAG-C solution was prepared in saline as per the concentration required and pH of this solution was adjusted to 7.0. Before adding MAG-C solution to the film, it was sonicated for 10 min. The specific volume added to the dry film depended on the experimental purpose. The newly formed preparation was warmed to a temperature slightly above the phase transition temperature of DPPC, and vortexed intermittently until the film was completely resuspended (63). The preparation was kept overnight in a water-bath maintained at 37°C. To prepare a homogenous mixture of small
unilamellar vesicles, liposome preparations were sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) for 10 min. Liposomes were centrifuged for at 1000xg for 15 min at 25°C to remove unincorporated MAG-C in the preparation (48, 49). The liposomes were finally sterilized by passage through a 0.22µ filter.

To prepare drug loaded liposomes doxorubicin stock solution was first prepared in ethanol and then added to lipid components (DPPC/DOTAP/cholesterol/PEG) at a specific ratio prior to the evaporation of organic solvent for a 3mol% drug to lipid ratio. In order to separate free doxorubicin from drug-incorporated preparations, the liposomes were subjected to dialysis after centrifugation. Dialysis was carried out using Spectra/Por CE dialysis membranes (Spectrum Laboratories, Inc., Racho Domingues, CA) with a MWCO (molecular weight cutoff) of 1000g/mol, using saline as the dialysis buffer. The preparation was sterilized by passage through a 0.22µ filter.

4.4 Characterization of Liposomes

4.4.1 Size and charge

The particle size and zeta potential of the liposomes were measured at 25°C in distilled water using a 90PLUS particle size and zeta potential analyzer (Brookhaven Instruments, Holtsville, NY)

4.4.2 Total iron content

To determine the total iron content of MAG-C liposomes the following mixture was prepared: 100µl of MAG-C liposomes, 100µl of concentrated HCl, 10µl of H₂O₂ concentrate and 1ml of 1% potassium thiocyanate solution. A red colored complex was
formed upon mixing and this was quantified using a UV absorbance spectrophotometer at 480nm (Bio-Tek® Instruments Inc., VT) (64).

4.4.3 Magnetization measurements using SQUID magnetometer

We wanted to determine the nature of magnetization that our MCLs possessed, and hence we decided to acquire magnetization measurements for MCLs with different MAG-C concentrations (0-20mg/ml). These measurements were carried out using a superconducting quantum interference device (SQUID) MPMS XL-5 from Quantum Design, USA. The liposome samples (volume 100µl) were sealed in glass NMR tubes using a blow torch, so as to prevent leakage of the contents. The magnetic susceptibility was measured using a magnetic field between -1T to 1T at a temperature of 300K.

4.5 Hyperthermia Measurements

Liposomes were prepared as mentioned (see Preparation of Liposomes) using all the different concentrations of MAG-C (0, 2.5, 5, 10 and 20 mg/ml). Heating experiments were carried out using 1kW radio frequency induction power supply (Hot Shot 1, Ameritherm Inc., NY). Samples were placed in the center of a 4 cm, 3 turn, water cooled copper tubing coil. Heating was achieved at operating at 270 kHz, 1.4 kW and 260.0 A. Liposome formulations with varying concentrations of magnetic NPs were heated for 25 min. Temperature was measured in situ using a fiberoptic thermometer (Luxtron Inc, CA). A graph of temperature (°C) vs. time (min) was plotted for the respective formulations.
4.6 Cytotoxicity Studies

The percent of cell viability was assessed to determine the effect MAG-C liposomes on the viability of human thyroid cancer cells. An amount of 5 mg/ml MAG-C was utilized for cell viability studies. Cell viability was assessed using the SRB assay (35, 65, 66). 

Cells (CAL-62, TT and HMEC-1) were seeded at $1 \times 10^4$ cells/well in a 48 well plate (Fisher Scientific, Pittsburgh, PA). Cells were allowed a sufficient amount of time to adhere to well plate (typically 24-48 h), after which they were treated with different amounts of MCLs containing 5 mg/ml MAG-C (typically ranging from 10 to 1000nmol). After 24 h, the well plates were washed with PBS to remove unbound liposomes and cellular debris. Cells were fixed to the plate by adding 100µl of 50% TCA into each well followed by storage at 4°C for a 1 h. After 1h, each well was washed 4 times with distilled water and the cells were then stained with 200µl of 0.4% sulforhodamine B for 30 min. SRB is a bright pink aminoxanthene dye with two sulfonic groups, and under mildly acidic conditions, SRB binds to basic amino acid residues in TCA-fixed cells (65). After 30 min, the wells were washed with 1% acetic acid to remove the unbound stain, and the plate was air-dried in a laminar flow hood. Fluorescence intensity (FI) was
measured by dissolving bound sulforhodamine with 1ml of 1x PBS at an excitation and emission wavelengths of 545/40 and 590/35nm respectively using FLX 800 Fluorescence Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT). Percent cell viability was calculated by the following formula:

\[
\text{Percent of cell viability} = \frac{\text{F.I of treated cell population}}{\text{F.I of untreated cell population (control)}} \times 100
\]

4.7 Cell Association Studies

4.7.1 Cell association in the absence of external magnet

These studies were carried out to measure the extent to which MCLs associated with the cells. The cells (CAL-62, TT and HMEC-1) were seeded at 2x10^4 cells/well in a 24-well plate. They were allowed to adhere to the plate overnight and then rhodamine-labeled MCLs (50-500nmol) containing 5 mg/ml MAG-C were added to the plate. After 24 h the wells were washed with PBS to remove unbound liposomes and cellular debris. The total fluorescence was measured using FLX 800 Fluorescence Microplate Reader. Total fluorescence was measured at excitation and emission wavelengths of 545/40 and 590/35nm respectively using a fluorescence microplate reader.

4.7.2 Cell association in the presence of external magnet

These studies were carried out to measure the extent to which MCLs associated with the cells under the influence of an external magnet. We used one of the two thyroid cancer cell lines, CAL-62, and the endothelial cell line HMEC-1 for this study. Cells were seeded at 4x10^4 cells/well in two 12-well plates for each concentration of MAG-C used.
They were allowed to adhere to the plate for 24 h. After 24 h, the cells in both plates were exposed to rhodamine labeled MCLs (50-500nmol) containing 5 mg/ml and 10mg/ml MAG-C respectively and incubated for 1 h. One of the cell-culture plates was placed on an external magnet of 0.4 T during this incubation. During this 1 h period, the well plate was placed on a Stovall Belly Dancer Shaker (Fisher Scientific, NJ) on slow continuous rotation. After 1 h the plate was removed from the magnet and shaker. Following this, the wells were washed with PBS to remove unbound liposomes and cellular debris. The total fluorescence was measured using FLX 800 Fluorescence Microplate Reader. Total fluorescence was measured at excitation and emission wavelengths of 545/40 and 590/35nm respectively using a fluorescence microplate reader.

**Figure 12.** The Belly Dancer (Source: Fisher Scientific, NY)
4.8 Fluorescence Microscopy

Fluorescence microscopy was used to evaluate the nature of liposome-cell interactions and intracellular accumulation of liposomes. Rhodamine-DPPE labeled MCLs (5 mg/ml MAG-C) were used to investigate the areas of localization within cells using fluorescence microscopy. The cells (CAL-62, TT and HMEC-1) were seeded at $5 \times 10^5$ cells/well on sterile cover slips in 6-well plates. They were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO$_2$. Rhodamine labeled MCLs at concentrations from 50-500 nmol/ml were added to the cells, and they were incubated for additional 24 h. After this incubation period, the required growth media was removed, the cover slips were washed with PBS to remove the unbound liposomes and other cellular debris. Cover slips were next mounted on glass microslides (Corning, NY) using Trevigen flour mounting medium (Trevigen, Gaithersburg, MD) or SlowFade Gold antifade reagent with DAPI (Invitrogen, Eugene, OR), and analyzed using differential interference contrast (DIC) microscopy and fluorescence microscopy with a BX61 WI Olympus fluorescence microscope from Optical Analysis Incorporation (Melville, NY). Images were acquired using 20x and/or 40x magnifications and recorded with an intensified CCD camera.

4.9 Drug Loading

We prepared DOX-loaded liposomes containing different concentrations of MAG-C and studied the percentage of drug loaded in the liposomes. We used fluorescent properties of DOX to study the drug loaded in MCLs. Liposomes were prepared as mentioned previously. The liposomes were then centrifuged and sonicated. The fluorescence intensity of the preparation was measured at excitation and emission wavelengths of 485 and 590 nm respectively at room temperature. Liposomes were then subjected to dialysis.
overnight using Spectra/Por CE dialysis membranes (Spectrum Laboratories, Inc., Racho Domingues, CA) with a MWCO of 1000g/mol and saline as the dialysis buffer. The fluorescence intensity of DOX-loaded liposomes was again measured after dialysis. The % drug loaded was calculated using the formula:

\[
\% \text{ DOX loaded} = \frac{\text{F.I of after dialysis}}{\text{F.I before dialysis}} \times 100
\]

4.10 Drug Release Studies

We wanted to study the release of DOX from DOX-loaded MCLs as a function of temperature as well as concentration of MAG-C (and hence the iron content) for its implication in hyperthermia. DOX release was studied at temperatures from 22 °C to 60°C making use of the intrinsic fluorescence properties of DOX. An understanding of preparation type susceptibility to the effects of heat should reveal the influence of MAG-C on properties regulating stability. The transition temperature for our formulation containing DPPC/DOTAP/cholesterol/PEG5000 was estimated to be covered adequately using this temperature range. DOX-loaded MCLs were prepared using different concentrations of MAG-C (0-20 mg/ml) as described (see Preparation of Liposomes). After centrifugation, the prepared liposomes were divided into three different pyrex tubes, each of which was incubated at different temperatures (22, 42 and 60°C) for 1 h using a water bath. The F.I was measured for the formulation kept as a control (22°C) at 485nm and 590nm, the excitation and emission wavelengths respectively, for DOX (67). This served as the control for all three samples. The samples from all the three tubes were then transferred into Spectra/Por CE dialysis membranes (Spectrum Laboratories, Inc., Racho Domingues, CA) with a MWCO of 1000g/mol and dialyzed overnight using saline
as buffer. After dialysis, the fluorescence intensities were measured again for all the samples. The total fluorescence was measured using FLX 800 Fluorescence Microplate Reader. The drug release was calculated using the following equation:

\[
\% \text{ Drug release} = 100 - \frac{\text{F.I of after dialysis}}{\text{F.I before dialysis (control)}} \times 100
\]

### 4.11 Effect of Heat on Cells as a Function of Time

We carried out a study to observe the effect of exposing anaplastic thyroid cancer cells to a temperature of 42°C for various time-points. This is the temperature which is also utilized for producing hyperthermia. We used the SRB assay to check the viability of cells 24h after exposing them to heat from 0 to 30 minutes. Seven 48-well plates were seeded with $1 \times 10^4$ cells/well. The plates were allowed to incubate for 24h and on the following day they were exposed to heating in a water bath. The water bath was temperature controlled and was set at 42°C. The covered plate was placed in the water bath, taking care that the water level was enough to cover the bottom of the plate uniformly, but was not too much, so as to avoid risk of water getting into the wells and contaminating the cells. The plates were exposed for 0, 5, 10, 15, 25 and 30 minutes respectively. They were incubated for 24h after the exposure, and the cell viability was carried out using SRB assay as mentioned previously.
4.12 Effects of External Magnet and Heat- Pilot Study

This study was undertaken to observe the combined effects of heat and magnetic drug targeting on thyroid cancer cells *in vitro*. Heating was carried out using a temperature-controlled water bath maintained at 42°C as mentioned in the previous section (4.12). CAL-62 cells were seeded at 1x10⁴ cells/well in six 48 well plates. After an incubation of 24h, DOX-loaded liposomes were added to all plates from 0-500nmol/ml of media. The plates were designated as: A) DOX-loaded liposomes only B) DOX-loaded liposomes+ magnet (1h) C) DOX-loaded liposomes + heat (5min) D) DOX-loaded liposomes+ magnet (1h) + heat (5min) E) DOX-loaded liposomes + heat (10min) and F) DOX-loaded liposomes+ magnet (1h) + heat (10min). The study was performed for three different time points of incubation of the cells with the drug-loaded liposomes. For the first set of experiments, DOX-loaded liposomes were added to the plates after 24h incubation and the plates were incubated with the liposomes at the different conditions stated above for 1h. Following 1h of incubation with the drug loaded liposomes, media was removed from all six 48-well plates and the plates were washed twice with PBS. Fresh media was replaced in the plates and they were allowed to incubate for an additional 23 h before carrying out the cell viability assay.

The effects of these treatments on cells were observed using the SRB assay for cell viability. For plates exposed to the external magnet and heat, the plate was heated immediately after removal from the magnet. Similarly, two other sets of experiments were carried out with all conditions the same but with 4h and 24h incubation times with the drug loaded liposomes. For 4h time point, fresh media was replaced after 4h of incubation with the drug-loaded liposomes, while for the 24h time point, the media was left in the plate and was washed off only during the SRB assay.
4.13 Statistical Analyses

To evaluate significant differences between the experimental groups, analysis of variance (ANOVA) and student’s t-tests were performed. The level of statistical significance reported for tests was set at $p \leq 0.05$ unless otherwise mentioned. Post-hoc tests were performed. Analysis was performed using the statistical package SPSS 16.0 (SPSS Inc, Chicago, IL).
5 RESULTS

5.1 Physicochemical Characterization of Liposomes

5.1.1 Particle size and zeta potential

The size and charge of liposomes are important determinants for maximum vascular targeting efficiency (28, 29, 33). For example, cationic liposomes between 100 and 150 nm and with zeta potential values ranging from 20-25 mv have been shown to target the tumor vasculature up to an extent of 28% (33). We prepared MCLs without any drug with different MAG-C concentrations (0-20 mg/ml) and determined the size, charge as well as the iron-content of all the preparations. We also studied the effects of MAG-C incorporation on these parameters. The particle size of the liposomes ranged from 125±4.0 nm for liposomes without any MAG-C to 255±11.1 nm for MCLs containing 20 mg/ml MAG-C. The zeta potentials values ranged from 26±5.2 mv for liposomes without MAG-C to 11±3.1 mv for 20 mg MAG-C liposome preparations. We found that as the MAG-C concentration was increased there was an increase in the particle size but a decrease in zeta potential of the same liposomes. A similar trend was also seen for DOX-loaded liposomes. The particle sizes of the drug-loaded liposomes ranged from 137±7.7 for liposomes without MAG-C to 245±45.6 for liposomes containing 20 mg/ml MAG-C while the corresponding zeta potential values ranged from 35±7.7 to 13±3.7 respectively. Figures 13 and 14 show graphs comparing the effects of increasing concentrations of MAG-C on particle size and zeta potential.
5.1.2 Total iron content

We next determined the iron content values for MCLs prepared with different MAG-C concentrations. We wanted to make sure that the fraction of magnetite loaded within the liposomes was sufficient to respond to an external magnet. The amount of iron incorporated within liposomes was determined spectrophotometrically for all MAG-C concentrations used in the study (48, 64). The iron content in MAG-C is 72% (see materials). So based on this 72% iron content, 2.5, 5, 10 and 20 mg/ml MAG-C correspond to 1.8, 3.6, 7.2, and 14.4 mg/ml of iron, respectively. The values obtained for iron content increased from 1.49 mg/ml for 2.5 mg/ml MAG-C to 10.2±4.8 mg/ml for 20 mg/ml MCLs. For MCLs loaded with DOX, the iron content values followed the same trend in going from 2.5 mg/ml to 20 mg/ml MAG-C. The iron content was found to be 1.3±0.6 for 2.5 mg/ml MAG-C while it was 7.4±2.9 for 20 mg/ml DOX-loaded MCL preparation. Table 3 includes the particle size, zeta potential as well as the iron content values for all the concentrations of MAG-C used.
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Concentration of MAG-C (mg/ml)</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mv)</th>
<th>Iron Content (mg/ml)</th>
<th>Std. Iron content (72%) values #</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC/DOTAP/CHOL/PEG 5000 (60:20:15:5)</td>
<td>0</td>
<td>125±4.0</td>
<td>26±5.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>121±6.4</td>
<td>24±6.7</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>143±8.7</td>
<td>17±4.4</td>
<td>3.1±0.4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>199±9.7</td>
<td>13±3.0</td>
<td>5.2±0.7</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>255±11.1</td>
<td>11±3.1</td>
<td>10.2±4.8</td>
<td>14.4</td>
</tr>
</tbody>
</table>

#Std. concentration of iron in MAG-C

**Table 3A)** Physicochemical characterization of liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Concentration of MAG-C (mg/ml)</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mv)</th>
<th>Iron Content (mg/ml)</th>
<th>Std. Iron content (72%) values #</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC/DOTAP/CHOL/PEG 5000/3mol% DOX (60:20:15:5)</td>
<td>0</td>
<td>137±7.7</td>
<td>35±7.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>141±4.2</td>
<td>25±4.3</td>
<td>1.3±0.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>138.±3.2</td>
<td>19±5.2</td>
<td>2.4±0.2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>189 ±27.9</td>
<td>16±5.6</td>
<td>5.7±1.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>245±45.7</td>
<td>13±3.7</td>
<td>7.4±2.9</td>
<td>14.4</td>
</tr>
</tbody>
</table>

#Std. concentration of iron in MAG-C

**Table 3B)** Physicochemical characterization of DOX-loaded liposomes
**Figure 13A**) Graph of particle diameter vs. concentration of MAG-C loaded in magnetic cationic liposomes.

**Figure 13B**) Graph of zeta potential vs. concentration of MAG-C loaded in magnetic cationic liposomes.

*P*≤0.05 One way ANOVA
**Figure 14A**  Graph of particle diameter vs. concentration of MAG-C loaded in DOX-loaded magnetic cationic liposomes

**Figure 14B**  Graph of zeta potential vs. concentration of MAG-C loaded in DOX-loaded magnetic cationic liposomes

*p*≤0.05 One way ANOVA
5.1.3 Magnetization measurements using SQUID magnetometer

We investigated the magnetic susceptibility of MCLs containing different concentrations of MAG-C (0-10mg/ml). Figure 15A shows the magnetization curve as a function of the magnetic field for each preparation type. Cationic liposomes without any MAG-C showed the typical curve for diamagnetic materials, confirming a lack of magnetic susceptibility. The magnetization curves for MCLs (2.5-10mg/ml) did not show any hysteresis loop, confirming the superparamagnetic behavior of magnetite. The saturation magnetization value of MCLs containing 5mg/ml of MAG-C (1.92 x 10^{-2} emu/100µl) was significantly higher than MCLs containing 2.5mg/ml (1.06 x 10^{-2} emu/100µl) and that of 10mg/ml MAG-C (4.22 x 10^{-2} emu/100µl) was significantly higher than the 5mg/ml MAG-C preparation as shown in Fig. 15B.

**Figure 15A** SQUID magnetization curves for MCLs with different magnetite (MAG-C) concentrations
5.2 Hyperthermia Generation

Ferrofluid-loaded liposomes can be heated using an AC field of sufficient strength, and the magnetic nanoparticles can cause the induction of hyperthermia through hysteresis losses (43). The heating experiments were carried out to determine whether the iron content determined for various concentrations of MAG-C-loaded liposomes commensurate with their ability to generate heat, as well as to compare different formulations for their heat producing capacity, and to optimize them based on four properties including their size, charge, iron content and capacity to be heated. It is evident from Figure 16 that formulations containing a higher concentration of MAG-C took lesser time to heat up and reach a particular temperature. If we choose a particular time-point, and compare the temperatures achieved by different formulations of MCLs at that
time-point, we can make a clear comparison between all the formulations. When we consider the 5 min time point in Figure 16A, we observe the following: The sample without any MAG-C reached only 31.8°C, the 2.5 mg/ml sample reached 42.9°C, the 5 mg/ml sample achieved a temperature of 47.2°C while the 10 mg/ml and 20 mg/ml reached temperatures of 67.5°C and 74°C respectively. We thus observe a trend which suggests that as we increase the concentration of MAG-C, it becomes possible to reach a particular temperature in a much shorter duration of time when compared to a formulation which contains less MAG-C. This trend was observed for liposomes without drug as well as the ones loaded with DOX. The heating of magnetite nanoparticles within the liposomes did not seem to be affected by the presence of drug at the concentrations used.

Figure 16A) Temperature vs. Time graph for MCLs with different MAG-C concentrations.

The figure represents the results of two separate experimental determinations, and these results were generally very reproducible.
The figure represents the results of two separate experimental determinations, and these results were generally very reproducible.

The different liposome formulations were heated for approximately 25 min. Heating was performed using 1kW radiofrequency induction power supply using Hot Shot 1 (Ameritherm Inc., NY) which was operated at 270 kHz, 1.4 kW and 260.0 A.

5.3 Cytotoxicity Studies

We evaluated the effect of MCLs on the cell viability of CAL-62, TT and HMEC-1 cells at concentrations ranging from 0-1000 nmol/ml of media, using SRB assay as mentioned previously. The % of viable cells remained nearly at 100% up to 1000 nmol/ml MCLs using 5 mg/ml MAG-C for CAL-62 as well as TT cells (Figure 17). The conditions of the SRB assay needed to be optimized for TT cells, since they had a higher doubling time, and were prone to being washed away during the assay. The incubation time was raised to 48 h for these cells to allow more time for the cells to attach to the well plate, as compared to 24 h incubation for CAL-62 cells. For HMEC-1, no toxicity was observed.
up to 250nmol/ml of MCLs while concentrations higher than that (500-1000nmol) showed significant toxicity. The endothelial cells were more sensitive to the effects of MCLs when compared to both the thyroid cancer cell lines.

Figure 17. Cell viability studies for CAL-62, TT and HMEC-1 cells using 5 mg/ml MAG-C loaded liposomes up to 1000 nmol/ml of liposomes. Cells were seeded at 1 X 10^4 cells/ml in a 48 well plate incubated at 37 °C. Percent cell viability was determined following 24 h exposure to the MCLs. Percent cell viability was determined using the formula provided in the Materials & Methods section. For statistical purposes ANOVA was used to compare differences among different concentrations in a cell line (* p< 0.05 One-way ANOVA).

5.4 Cell Association Studies

5.4.1 Cell association in the absence of external magnet

We carried out cell association studies to evaluate the ability of MCLs to associate with the both cell lines and determine this association quantitatively. We used liposomes containing 5 mg/ml MAG-C for the studies. We found that the tendency of MAG-C
liposomes to associate with cells increased with increasing liposome concentrations. The highest concentration (500nmol) of liposomes showed the maximum association with both cell thyroid cancer cell-lines (Figure 18). For HMEC-1 however, the maximum association with MCLs was observed at 250nmol/ml of MCLs after which the association dropped as we increased the amount of liposomes added. This was consistent with the cell viability studies for HMEC-1 which also showed significant toxicity beyond 250nmol liposomes.

**Figure 18.** The association of magnetic liposomes with CAL-62, TT and HMEC-1 cells

Cells were seeded at 2 X 10^4 cells/ml in a 24 well plate and incubated at 37 °C. The relative extent to which each liposome type interacted with cells was determined 24h following cell exposure to rhodamine labeled MCLs (5 mg/ml MAG-C) (0-500 nmoles). The control group was untreated. ( *p≤0.05 One-way ANOVA)

### 5.4.2 Cell association in the presence of external magnet

We evaluated the ability of MCLs to interact with CAL-62 as well as with HMEC-1 cells in the presence and absence of an external magnet of 0.4T. With CAL-62 we found that the application of an external magnet did not affect the association of MCLs (with
5mg/ml MAG-C) at any of the concentrations used. With the 10mg/ml MCL preparation, we could see a significantly higher association at 50nmol/ml of MCLs. At higher concentrations however, there was no benefit seen with the external magnet. Probably, the amount of MCLs associated with the cells was high enough to cause saturation, which is why we could not observe a benefit of using a magnet. Figure 19 shows the association of MCLs with CAL-62 cells at 5 and 10mg/ml MAG-C in the presence and absence of an external magnet. With HMEC-1 also, no effect with the magnet was seen at 5mg/ml magnetite concentration, however at 10mg/ml concentration of MAG-C a significantly greater association was seen in the presence of 500nmol/ml of MCLs (Figure 20).
Figure 19. Cell association of MCLs with CAL-62 at 5mg and 10mg/ml MAG-C concentration in the presence and absence of an external magnet

*p≤0.05 student’s t-test
Figure 20. The association of MCLs with HMEC-1 at 5mg and 10mg/ml MAG-C concentration in the presence and absence of an external magnet

*p<0.05 student’s t-test

5.5 Fluorescence Microscopy

This study was carried out to evaluate ability of MCLs (containing 5 mg/ml MAG-C) to associate with both thyroid cancer cell lines as well as HMEC-1. We used DIC and fluorescence microscopy to observe the interaction and localization of liposomes in the cells. The images were taken at 20X and 40X magnifications. DIC was used to observe the cell morphology, DAPI (SlowFade® Gold antifade reagent with DAPI) was used as a
mounting medium to stain the nucleus and rhodamine-DPPE was used to visualize the location of liposomes with respect to the nucleus. CAL-62 cells showed a peri-nuclear uptake of liposomes as shown in Figure 21. For TT cells the liposomes seemed to have accumulated more or less uniformly within the cells and not just the peri-nuclear areas as shown in Figure 22. HMEC-1 cells also showed peri-nuclear accumulation of MCLs (Figure 23).

**Figure 21.** Fluorescent images of CAL-62 cells in the absence of external magnet

MCLs (50 nmol/ml) were added to a 6-well plate containing 5x10^5 cells and incubated for 24 h after which images were taken using a fluorescent microscope **A)** DIC image at 40x **B)** Rhodamine channel image (red) **C)** DAPI channel image (staining the nucleus) **D)** Merged image from rhodamine and DAPI channels showing the association of liposomes with cells with respect to the nucleus.
Figure 22. Fluorescent images of TT cells in the absence of external magnet

MCLs (500 nmol/ml) were added to a 6-well plate containing $5 \times 10^5$ cells and incubated for 24 h after which images were taken using a fluorescent microscope A) DIC image at 40X B) Rhodamine channel image (red) C) DAPI channel image (staining the nucleus) D) Merged image from rhodamine and DAPI channels showing the association of liposomes with cells with respect to the nucleus.
Figure 23. Fluorescent images of HMEC-1 cells in the absence of external magnet

MCLs (100 nmol/ml) were added to a 6-well plate containing $5 \times 10^5$ cells and incubated for 24 h after which images were taken using a fluorescent microscope A) DIC image at 20X B) Rhodamine channel image (red) C) DAPI channel image (staining the nucleus) D) Merged image from rhodamine and DAPI channels showing the association of liposomes with cells with respect to the nucleus.
5.6 Doxorubicin Loading in Liposomes

We carried out this study to see the effect of varying MAG-C concentrations on the efficiency of drug loading. We found that the loading of DOX significantly increased as we increased the concentration of MAG-C in the liposomes. Previous studies in our lab have also shown that addition of 5mol% PEG to liposomes significantly increased the incorporation of drugs loaded in magnetic cationic liposomes (48). Since the preparations that we used also had PEG it is reasonable to believe that it conferred a stabilizing effect on liposomes which is one reason why the incorporation of DOX increased. Increasing concentrations of MAG-C also seem to show a beneficial effect in terms of drug loading within MCLs as per the results obtained.

![% DOX Loading vs. Concentration of MAG-C](image)

* p<0.01 One-way ANOVA

**Figure 24.** % DOX loaded in magnetic cationic liposomes with different MAG-C concentrations

The percent DOX loaded in the liposomes was calculated based on the fluorescence intensity of the liposomes before and after dialysis overnight to remove the unincorporated drug from the liposomes.
5.7 Doxorubicin Release from Liposomes as a Function of Temperature and Varying MAG-C Concentrations

The release of DOX from liposomes was studied with respect to temperature across different concentrations of MAG-C. We observed a common trend among the liposomes at all four different MAG-C concentrations employed. The release of DOX from liposomes increased at increasing temperatures. From 22°C to 60°C the amount of DOX released increased drastically for all the concentrations. The heating of liposomes results in melting of the bilayer of liposomes, and thus facilitates the release of the encapsulated drug.

Hyperthermia can be viewed as a way of triggering the release of the drug from the liposomes in the body in response to a specific temperature. Hyperthermia can be used to target and trigger drug release and is advantageous because it can be controlled by external means and thus, can be easily modified to the desired conditions unlike methods that depend on more inherent properties of the tissue (like pH or specific surface antigens) (58).
Figure 25. % DOX release at different temperatures as a function of MAG-C concentration

DOX release from liposomes with different concentrations of MAG-C was studied using fluorescence properties of DOX. The total fluorescence intensity of the liposomes was measured before and after dialysis at all the temperatures employed.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>22°C</th>
<th>42°C</th>
<th>60°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MAG-C</td>
<td>0</td>
<td>38±1.1</td>
<td>40±2.8</td>
<td>74±1.5</td>
</tr>
<tr>
<td>2.5 mg/ml</td>
<td>0</td>
<td>49±0.6</td>
<td>51±1.0</td>
<td>67±0.6</td>
</tr>
<tr>
<td>5 mg/ml</td>
<td>0</td>
<td>17±1.6</td>
<td>29±1.2</td>
<td>40±1.1</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>0</td>
<td>8±3.4</td>
<td>7±3.1</td>
<td>24±1.3</td>
</tr>
</tbody>
</table>

Table 4. DOX release as a function of temperature at different MAG-concentrations
5.8 Effects of Heat on CAL-62 as a Function of Time

This study was carried out to ascertain the effect of exposing CAL-62 to heating at 42°C for various lengths of time. The rationale and conditions for this experiment are relevant for our future hyperthermia studies. Seven 48-well plates were subjected to heat effects in a temperature controlled water-bath from 0-30 min. SRB assay was carried out 24 h after the plates were incubated at their designated intervals of time.

Figure 26. Effects of application of heat on the viability of CAL-62 cells.
We found that the cell viability started decreasing significantly beyond 10 min of heating. At 25 and 30 min of heating nearly 10% of the cells were found to be dead.

5.9 Effect of External Magnet and Heat- Pilot Study

This study was carried out as a pilot study with one of the thyroid cancer cell lines. We used CAL-62 for this study, and used a relatively low concentration of DOX for this first study to assess the heat contribution effects. As described in the methods section, we used 3 sets of experiments, each with a different period of incubation of the cells with DOX-loaded MCLs (0-500 nmol/ml). We used the SRB assay to determine the effect of various treatments that the cells were subjected to during the study. The cells were incubated with DOX-loaded liposomes for 1 h, 4 h or 24 h as explained in the methods section. Figure 27A, 27B and 27C show the results of the SRB assay for all the three time-points respectively.

**Figure 27A** 1h incubation with DOX-loaded MCLs
**Figure 27B)** 4 h incubation with DOX-loaded MCLs

**Figure 27C)** 24 h incubation with DOX-loaded MCLs
We could not see very drastic changes in the % viability as a result of various treatments at the concentration of DOX employed. We were however, able to observe (at some of the concentrations) that certain effects seemed dominant at all the incubation time points. We found that the drug+magnet+hyperthermia (10min) treatment group (orange bars) seemed to affect the cell viability more than the other treatment groups across all the incubation intervals. However, we need additional studies to confirm the exact mechanism of the observed effects as well as to optimize the drug concentration and other parameters in the study.
6 DISCUSSION

We prepared and characterized magnetic cationic liposomes using 2.5, 5, 10 and 20mg MAG-C, in the presence and absence of DOX. We found that these liposomes generate a hyperthermia-inducing effect in the presence of an AC magnetic field, varying as a function of the iron content of the liposomes. On increasing the MAG-C concentration in the preparations, we found that the iron content of the samples increased as well as the ability to generate hyperthermia in a shorter duration of time. We carried out SQUID measurements for MCLs with different magnetite concentrations in order to determine their magnetic susceptibility as well as to know the type of magnetic behavior that they exhibited. SQUID measurements clearly depicted that the MCLs were superparamagnetic. Their saturation magnetization values increased with an increase in the MAG-C content which correlated with their iron content. Our findings are consistent with studies conducted previously involving the use of similar formulations (48). Magnetic behavior is characteristic of the type of magnetic material incorporated in the liposomes and is not affected by the presence or absence of any chemotherapeutic agent. It would however be affected if the magnetic material within the liposomes is changed. When the MAG-C concentration in the liposomes was increased from 2.5 mg/ml to 20 mg/ml, we found that the time required for the liposomes to get heated and reach a particular temperature reduced markedly. This property of the liposomes did not change when DOX was included, and the temperature vs. time curves for all the formulations followed the same trend even in its presence. We also found that a higher MAG-C content influenced the particle size and zeta potential values. The particle sizes of the MCLs increased with increase in MAG-C content. Furthermore, we observed that the
zeta potentials significantly decreased with increase in MAG-C content. MAG-C is known to partly shield the cationic charge potential of the liposomes, being negatively charged itself (48). However, the 5 mg/ml formulation that we used for our studies did retain sufficient cationic charge which is essential for targeting the vasculature, following intravenous administration.

We carried out *in vitro* studies using 5 mg/ml MAG-C liposomes, since they had a good balance of particle size, zeta potential, iron content, and hyperthermia producing effects. Liposomes falling between the size range of 100-200nm are typically used for tumor vascular targeting purposes (28). This range covered majority of the formulations that we prepared. The zeta potential was positive for all the formulations from preparations without MAG-C to 20 mg/ml MAG-C, which is favorable for vascular targeting. The MCLs were susceptible to the effects of an alternating magnetic field and could be heated as a function of their MAG-C concentrations. We observed a trend which showed that as the MAG-C concentration increased within the liposomes, they were able to reach a particular temperature in a much shorter duration of time. The MCLs also associated well with TT, CAL-62 and HMEC-1 cell lines as seen qualitatively from fluorescence microscopy studies, and quantitatively from cell association studies. We found that the cell association of MCLs with CAL-62 and HMEC-1 was favored using an external magnet for 10mg/ml MAG-C but not with the 5mg/ml preparation. The MCLs were not toxic to the cells and 100% of cells were viable up to 1000 nmol/ml of the MCLs with both the thyroid cancer cell lines used. However, the endothelial cell line HMEC-1 seemed to be more susceptible to the effects of the MCLs and showed significant cytotoxicity as the concentration of the MCLs was increased beyond 250nmol/ml.
Endothelial cells are an attractive target for chemotherapeutic agents owing to their numerous advantages as supported by several studies, and can be exploited to benefit chemotherapy (29, 68).

The MCLs were also capable of loading DOX efficiently, and higher MAG-C concentrations seemed to have a stabilizing effect on the liposomes thus increasing the amount of drug loaded. We also observed that increasing the temperature favored the release of DOX from the liposomes. The maximum release of DOX was found at 60°C for all MAG-C concentrations evaluated. This suggests that the application of heat triggered the release of drug from the liposomes by melting the bilayer and favoring the release of the encapsulated agent.

We also determined the effects of heat as a function of time at 42°C on thyroid cancer cells. It was observed that beyond 15 min significant cytotoxic effects were observed on the viability of cells. Previous investigations using Doxil in a lung tumor model have shown that hyperthermia for a longer duration (30 min) was more effective than hyperthermia for a shorter duration (5 min) (69). Although our study did not utilize any drug, the trend observed was similar. We saw significant cytotoxicity at 30 min compared to 5 min at 42 °C. We finally carried out a pilot study combining the aspects of MDT and effects of heat at 42 °C over three different time-points of incubation with DOX-loaded MCLs. CAL-62 cells were incubated at 1 h, 4 h and 24 h with drug-loaded liposomes and cell viability was assessed for all the different experimental groups employed. However, there were no major differences observed amongst the different experimental groups. We need further investigations to ascertain the interaction of DOX with the thyroid cancer cells at the concentrations employed, as well as the effects of MAG-C if any, on this
interaction. The concentration of DOX needs to be optimized for the study, for the cell line used in order to show any benefits of the treatment. Moreover, endothelial cells were more susceptible to the effects of the MCLs as seen from the cell viability studies, and they might be more susceptible to the effects of DOX-loaded liposomes than CAL-62 cells. We do expect to observe an enhanced effect in the presence of a magnet since a greater amount of liposomes can be retained due to the effects of the magnetic field, and the application of hyperthermia should enable the release of drug from these liposomes as well.
7 CONCLUDING REMARKS

- The particle size of MCLs as well as their iron content increased with an increase in the MAG-C concentration, whereas the zeta potential decreased at higher MAG-C concentrations.
- The hyperthermia-inducing effects increased with an increase in MAG-C concentration, and higher temperatures were achieved in a shorter duration of time.
- SQUID magnetization measurements confirmed that the MCLs were superparamagnetic and magnetization values for preparations containing a higher concentration of magnetite were significantly greater than that for MCLs containing lower magnetite content.
- No cytotoxicity was observed up to 1000 nmol/ml of 5 mg/ml MAG-C containing MCLs with TT as well as CAL-62 cells. For HMEC-1, the maximum concentration of MCLs that could be added without any toxic effects was 250nmol/ml.
- MCLs containing 5 mg/ml MAG-C associated well with both the thyroid cancer cell lines, and showed a concentration dependent increase in cellular association with MCLs. With HMEC-1 the association of MCLs increased and reached a maximum at 250nmol/ml, but then dropped as the amount added was increased up to 500nmol/ml.
- Beneficial effects of using an external magnet were seen using 10mg/ml MAG-C loaded liposomes for CAL-62 as well as HMEC-1 cells.
- Fluorescence microscopy studies using rhodamine and DAPI showed peri-nuclear uptake of MCLs (containing 5 mg/ml of MAG-C) by the CAL-62 and HMEC-1 cells whereas, liposomes were taken up almost uniformly by TT cells.
- Increasing concentrations of MAG-C had a stabilizing effect on DOX-loaded MCLs improving drug retention.
- The release of DOX from liposomes was favored on increasing the temperature for all concentrations of MAG-C employed.
- Heating of CAL-62 cells at 42°C as a function of time showed significant cytotoxic effects above 15min.

Our study thus showed that MCLs thus prepared and modified suitably by optimizing various parameters like particle size, charge, iron content and susceptibility to an alternating magnetic field can be used for targeting tumor as well as endothelial cells. These MCLs have the potential to simultaneously load drug as well as the magnetic materials and show a superparamagnetic behavior. They can be used for the purpose of magnetic drug targeting as well as for generating hyperthermia under the influence of an AMF. This study serves as the first phase of an investigation where we have been successful in optimizing the formulation parameters, as well as in characterizing the cell lines CAL-62, TT and HMEC-1 for their susceptibility to the effects of the MCLs, and their ability to take up MCLs.
REFERENCES


8. What you need to know about thyroid cancer 2007. US Dept. of Health and Human Services, NIH.


