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Polymeric Superparamagnetic Nanoparticles for Drug Delivery Applications

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Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Engineering Science

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ABSTRACT

Drug delivery systems based on magnetic nanoparticles present a promising avenue for controlled targeted therapeutics, especially in cancer therapy. Conventional systematic therapeutics encompasses numerous side effects due to its limited selectivity between healthy and cancerous cells. In this thesis, novel polymeric-metallic hybrid nanoaggregates were developed to address this challenge. Magnetite nanoparticles were synthesized via precipitation of iron oxide and was surface modified using a unique chitosan derivative, glycol chitosan (GC), loaded with progesterone for potential hormonal therapy application for breast cancer. Surface characterizations techniques, in vitro drug release kinetics, investigation of progesterone release mechanism by mathematical modeling, and cell cytotoxicity were performed. In the size range of 10-20 nm, the synthetized nanoparticles with various GC compositions showed sustained progesterone release influenced by different polymer concentrations and found to be pH-responsive. The prepared nanoaggregates can be considered as a good potential for biocompatible controlled drug delivery applications.

Keywords: Controlled drug release, superparamagnetic nanoparticles, iron oxide, glycol chitosan, drug delivery, mathematical modeling, polymer coating.
CO-AUTHORSHIP STATEMENT

This thesis is an integrated article of two papers. The Review article is written in Chapter two and is accepted for publication. Chapter three is a research article in preparation for submission.

Chapter 2

Title: Bioactivity of Hybrid Polymeric-Magnetic Nanoparticles and Their Application in Drug Delivery.

Authors: Leena Mohammed, Doaa Ragab, and Hassan Gomaa.

Article Status: Accepted for publication in Journal of pharmaceutical Design.

Leena extracted the most updated information and wrote the literature review. Doaa Ragab, a post-doctoral fellow, also contributed in writing a couple of sections in the paper. Dr. Gomaa, Leena and Doaa worked on editing and reviewing the manuscript prior to publication.

Chapter 3

Title: Synthesis and Characterization of Dual Stimuli Responsive Glycol Chitosan-Fe3O4 Core-Shell Magnetic Nanoparticles for Controlled Drug Delivery of Progesterone

Authors: Leena Mohammed, Doaa Ragab, and Hassan Gomaa, Shigang Lin, and Kibret Mequanint

Article Status: In preparation for submission.

This paper was supervised by Dr. Hassan Gomaa. Leena Mohammed conducted the experiments, analyzed data and wrote the manuscript of this paper. Doaa Ragab helped in conducting and writing the drug release experiment. A post-doctoral fellow under the supervision of Dr. Mequanint, Dr. Shigang Lin, preformed the in vitro cell study. Leena Mohammed, Doaa Ragab, and Dr. Hassan Gomaa contributed to the editing the manuscript.
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<th>Description</th>
<th>Unit</th>
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<tbody>
<tr>
<td>$\varepsilon_0$</td>
<td>Initial porosity</td>
<td>-</td>
</tr>
<tr>
<td>$a$</td>
<td>Scale parameter</td>
<td>-</td>
</tr>
<tr>
<td>$A$</td>
<td>Cross sectional area</td>
<td>cm$^2$</td>
</tr>
<tr>
<td>$b$</td>
<td>Shape parameter</td>
<td>-</td>
</tr>
<tr>
<td>$c$</td>
<td>Drug concentration</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$C_0$</td>
<td>Drug initial concentration</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$c_i$</td>
<td>Concentration of species i</td>
<td>mol/m$^3$</td>
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<tr>
<td>$C_{ms}$</td>
<td>Drug solubility</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$C_s$</td>
<td>Concentration of drug in matrix bulk</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$C_t$</td>
<td>Concentration of drug in liquid layer surrounding membrane</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$D$</td>
<td>Drug diffusion coefficient</td>
<td>m$^2$/day</td>
</tr>
<tr>
<td>$dc/dt$</td>
<td>Rate of change in drug concentration</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$D_{ip}$</td>
<td>Represent the diffusion coefficient of species i</td>
<td>m$^2$/s.</td>
</tr>
<tr>
<td>$D_m$</td>
<td>Diffusion coefficient</td>
<td>m$^2$/s</td>
</tr>
<tr>
<td>$j_i$</td>
<td>Mass flux of species i</td>
<td>mol/m$^2$. s</td>
</tr>
<tr>
<td>$k$</td>
<td>Boltzmann constant</td>
<td>J/K</td>
</tr>
<tr>
<td>$K$</td>
<td>Constant</td>
<td>-</td>
</tr>
<tr>
<td>$K$</td>
<td>Drug specific volume</td>
<td>m$^3$/kg</td>
</tr>
<tr>
<td>$K_u$</td>
<td>Universal axial anisotropy</td>
<td>erg/Cm</td>
</tr>
<tr>
<td>$L_p$</td>
<td>Permeability coefficient</td>
<td>cm/day</td>
</tr>
<tr>
<td>$m$</td>
<td>Accumulated drug released</td>
<td>mg/ml</td>
</tr>
<tr>
<td>$Mt/M_\infty$</td>
<td>fraction of drug released at time t</td>
<td>mg/day</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Unit</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>Q</td>
<td>Cumulative amount of drug released</td>
<td>mg</td>
</tr>
<tr>
<td>r</td>
<td>Particles radius</td>
<td>nm</td>
</tr>
<tr>
<td>r₀</td>
<td>Radius of the matrix</td>
<td>m</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
<td>s</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
<td>K</td>
</tr>
<tr>
<td>Tᵢ</td>
<td>Location parameter</td>
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</tr>
<tr>
<td>W₀</td>
<td>Initial amount of drug in a single dosage</td>
<td>mg</td>
</tr>
<tr>
<td>Wᵣ</td>
<td>Remaining amount of drug in a single dosage</td>
<td>mg</td>
</tr>
<tr>
<td>x</td>
<td>Position</td>
<td>m</td>
</tr>
<tr>
<td>β</td>
<td>Peak width</td>
<td>radians</td>
</tr>
<tr>
<td>δ</td>
<td>Thickness of the device</td>
<td>μm</td>
</tr>
<tr>
<td>δ or ε</td>
<td>Porosity</td>
<td>-</td>
</tr>
<tr>
<td>Δπₛ</td>
<td>Osmotic pressure of water</td>
<td>atm</td>
</tr>
<tr>
<td>θ</td>
<td>Bragg diffraction angle</td>
<td>degrees</td>
</tr>
<tr>
<td>κ</td>
<td>Release rate constant</td>
<td>K/day</td>
</tr>
<tr>
<td>λ</td>
<td>X-ray wavelength</td>
<td>Å</td>
</tr>
<tr>
<td>σ</td>
<td>Reflection coefficient</td>
<td>-</td>
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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CLIOs</td>
<td>Cross-linked iron oxide</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CMS</td>
<td>Carboxymethyl starch</td>
</tr>
<tr>
<td>CTX</td>
<td>Chlorotoxin</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>DMBA</td>
<td>Dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen-mediated</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>GC</td>
<td>Glycol chitosan</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GNPs</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>GP</td>
<td>Gamma probe</td>
</tr>
<tr>
<td>GTA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IDA</td>
<td>Iron deficiency anemia</td>
</tr>
<tr>
<td>IONPs</td>
<td>Iron oxide nanoparticles</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>MDT</td>
<td>Magnetic drug targeting</td>
</tr>
</tbody>
</table>
MEC  Minimum effective concentration
MF  Gene Therapy and Magnetofection
MFH  Magnetic fluid hyperthermia
MMP  Matrix-metalloproteinase
MNPs  Magnetic nanoparticles
MRI  Magnetic resonance imaging
MS  Manual magnetometer
MSC  Marrow derived stromal cells
MTC  Minimum toxic concentration
MTX  Methotrexate
NEDD  Nano-enabled drug delivery
NGPC  N-glycyrrhetinic acid-polyethylene glycol (PEG)-chitosan
NPs  Nanoparticles
ODNs  Oligodeoxynucleotides
PAA  Polyaacrylic acid
PCL  Polycaprolactone
PCL  Polycaprolactone
PEG  Polyethylene glycol
PEG  Poly (ethylene glycol)
PGD  Poly(caprolactone) grafted dextran
PLGA  Poly(lactide-co-glycolide)
PVA  Poly (vinyl alcohol)
PVP  Poly (vinyl pyrrolidone)
PVP  Poly(vinyl pyrrolidone)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Term</th>
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<tbody>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SLN</td>
<td>Sentinel lymph nodes</td>
</tr>
<tr>
<td>SLNs</td>
<td>Solid lipid nanoparticles</td>
</tr>
<tr>
<td>SPIONs</td>
<td>Superparamagnetic iron oxide nanoparticles</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermo-Gravimetric Analysis</td>
</tr>
<tr>
<td>Tmx</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>VB1</td>
<td>Violamycine B1</td>
</tr>
<tr>
<td>VSM</td>
<td>Vibrating Sample Magnetometer</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
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CHAPTER 1

1 Introduction

1.1 The puzzling disease of our time- research motivation

Cancer is the second leading cause of death in the world and is expected to exceed heart disease as the top cause of death in the coming few years (1). In 2015, the number of cases diagnosed with cancer were 1,658,37 in the United States (1) and 196,900 in Canada (2). Approximately half of Canadians are expected to develop cancer in their life time and a quarter of these cases are expected to result in death (2). Among women, breast cancer is the most common type of cancer after lung cancer, with 68 Canadian women diagnosed with breast cancer every day, with a death rate of 14 women per day (3). In addition to the unclear causes of cancer, its treatment is exceptionally challenging and daunting. The available therapeutics are one of or a combination of chemotherapy, radiation, and surgery, all of which are not guaranteed to be truly effective. Chemotherapy and radiation intend to destroy cancer cells yet have significant detrimental effects on active healthy cells. This is mainly due insufficient governing of cellular targeting, and if the cells of interest are targeted, the anticancer drug release rate is usually uncontrolled (4,5). However, the underlining difference between them is that radiation therapy results in localized damage to the radiated areas, while with chemotherapy side effects are systemic. In 20 studies including one quarter million women who had undergone radiation treatment worldwide, it was reported that the beneficial effects of radiotherapy in breast cancer women were offset by a 30% increase in heart disease-death rate (6,7). On the other hand, surgery is an invasive approach that is not preferred by most breast cancer patients as it is accompanied by risks, complications and serious limitations. Frequently, patients express their willingness to be subjected to these treatments even if the expected survival rates are very low (8). Clearly, there is a desperate need for a much more effective breast cancer treatment in which a precise targeting of the affected tissue is achieved.
1.2 Nano-enabled drug delivery (NEDD) nanoparticles for breast cancer treatment

Recently, the growth of nano-medical technology has burst mainly in the fields of nanopharmaceuticals and drug delivery. With both diverse and extensive research, it is quoted that targeted therapy represent one of the most promising options in breast cancer treatment. Nano-enabled drug delivery (NEDD) focuses on specific cell targeting and drug release strategies for direct administration to the needed site (9). This approach allows using a wide variety of therapeutics/drugs offering many advantages over conventional treatment methods. The main advantages are improved patient compliance, increased treatment efficacy, decreased toxic side effects, reduced dose, controlled biodistribution, and better drug localization (10). The tailored NEDD systems nowadays in literature are extremely diverse. This includes but is not limited to: nanofibers, viral vectors, nanoparticles, hydrogels, quantum dots, nanocapsules, and carbon nanotubes (11,12). Not to mention, each of these systems has many subsystems and categories, which is beyond the scope of this thesis. Here, we will focus on the different kinds of nanoparticle systems, which are biomaterial aggregates in the size range of 1-100 nm (13).

1.2.1 Polymeric nanoparticles

In the field of oncology, there has been extensive research on chemically-modified polymeric nanocarrier systems due to their capability of carrying a wide range of drugs in a controlled manner for sustained period of time to tumor sites. They are advantageous over other nanoparticle systems, due to the ease of their preparation from well-understood polymers and have high stability in biological fluids as well as during storage (14). The fabrication of these systems is greatly dependent on their morphology and composition of the periphery and the core, hence, they are characterized by their physicochemical structures. They include polymeric nanoparticles (NPs), dendrimers, polymeric micelles, polymersomes, polymer conjugate, polymer-lipid hybrid, and polyplex (15). Polymeric NPs are solid colloidal systems in which the drug is entrapped, encapsulated, dissolved or absorbed into the natural or synthetic polymer matrix. Contingent to their design, they can form either nanosphere where drug molecules are bound to the surface or nanocapsules where drug is enclosed inside a polymeric membrane (16,17). Various polymers such are poly(lactide-co-glycolide) (PLGA), chitosan, dextran, polyglycolide, polycaprolactone (PCL), and polyethylene glycol (PEG)
have been employed in fabrication of NPs and controlled targeting and release of cancer therapeutic agents. Using both passive and active targeting strategies, suppression of breast cancer cells were efficiently achieved (15). Among many examples, PLGA, an FDA approved biodegradable polymer, was applied in this regards. Tamoxifen (Tmx)-loaded PLGA nanoparticles (Tmx-NPs) were prepared via an emulsion diffusion evaporation method by Jain et al. (18), reporting an entrapment efficiency of over 85%. Oral antitumor efficacy in 12-dimethylbenz(a)anthracene (DMBA) induced breast cancer model of Tmx-NPs was performed; the tumor size was reduced to 41.56% compared with the control, untreated cells, where tumor size increased to more than 158.66%. Also, the targeting efficiency was proven, as hepatotoxicity was significantly less in comparison with free Tmx citrate as shown by histopathological examination of liver tissue. In another research study, poly(caprolactone) grafted dextran nanoparticles (PGD-NPs) were synthesised by modified oil/water emulsion method and were loaded with vinblastine as the anticancer drug (19). The fluorescent loaded PGD-NPs were tested in-vitro for cellular uptake and cancer cell viability using MCF-7 breast cancer cell line following their characterization and release study. Internalization efficiency of PGD copolymers was shown to be almost double in the MCF-7 group versus the control group (untreated cells). Lower viability rate of 50% was demonstrated after 48 h incubation mainly due to reduction in cell adhesive interactions in cells treated with drug-loaded NPs.

1.2.2 Lipid-based nanoparticles

Lipid-based nanoparticles are currently the most broadly investigated class of nanoparticles and are already in clinical use (20,21). Their biocompatibility and ability to enhance drug bioavailability have made them suitable for drug delivery and cell targeting (22). Also, they bear the advantage of being the least toxic nanoparticle type in-vivo (23). They include Liposomes, micelles, solid lipid nanoparticles (SLNs), exosomes, and bolalipid vesicles (23). Xing et al. (24) developed liposomes functionalized with a 26-merguanosine-rich DNA aptamer AS1411 (a single stranded oligonucleotides with excellent targeting affinity specially to the nucleolin, an overexpressed protein in breast cancer). The functionalized liposomes were loaded with doxorubicin (DOX)- an anticancer drug. After testing both in vitro and in vivo, selective internalization was observed, with improved cytotoxicity to MCF-7 breast cancer cells and earlier tumor inhibition in mice bearing xenograft MCF-7 tumors. According to studies performed on SLNs, they have shown remarkable effects on MCF-7 cell line (25–
The design of surface modified DOX-loaded SLNs using mannose was developed by Jain et al. (28), and its breast tumor targeting potential was investigated. Mannosylated SLNs demonstrated the highest cytotoxicity on MCF-7 cells compared to non-mannosylated SLNs and free DOX, where the ability to deliver a higher concentration of DOX with better internalization was achieved. Moreover, SLNs were established for the aim of decreasing breast cancer metastasis. Prepared via thin-film hydration method, these nanoparticles were coated with d-(alpha)-tocopheryl PEG 1000 succinate and phosphatidylcholine, and were loaded with Silibirin—an antimigratory agent for invasive tumors (29,30). According to the \textit{in vivo} model results, 67% less pulmonary metastases formation and 39% less blood vessel metastases was reported compared to the saline-treated group. This can be attributed to the high efficiency and accumulation of silibinin-loaded SLNs taken-up by the MDA-MB-231 breast cancer cells.

1.2.3 Noble metallic nanoparticles

Although lately research is employing noble metal nanoparticles as drug carriers in cancer therapy, its applications in the biomedical field existed since ancient time (31). Their effectiveness against numerous microorganisms and their efficacy in drug delivery was evident in several studies (32,33). One property that makes them attractive is surface stability. This allows their surface to be decorated with countless organic polymers and biological molecules serving as targeting agents and improving their efficiency. Dating many hundreds years back till today, gold, silver, and platinum nanoparticles are the most common. Interestingly, green silver nanoparticles (AgNPs) synthesized though leaf extract of \textit{Podophyllum hexandrum Royle} under optimized conditions was reported (31,34). They have demonstrated the ability to selectively damage DNA and induce caspase-mediated cell death in breast and cervical carcinoma cells. Similar results regarding the effect of AgNPs on breast cancer cells were found in many studies (35,36). On another note, colloidal gold nanoparticles have been used widely due to their optical-electronic properties and high electron density, which can be applied in both diagnostic and therapy of breast cancer (37). Banu \textit{et al.} (38) engineered folate conjugated polyethylene glycol gold nanoparticles (GNPs) loaded with DOX for folate receptor overexpressing breast cancers targeted treatment and combined it with photo-thermal therapy using laser. The efficacy of these particles was validated \textit{in vitro} by their high internalization in MDA-MB-231 breast cancer cells with improved therapeutic
effects compared to plain DOX. However, it had lower effect on MCF-7 cell line since it expresses low levels of folate receptor compared to that of MDA-MB-231.

1.2.4 Magnetic nanoparticles

Magnetic nanoparticle are not only a well known category of nano-enabled drug delivery systems, they are also FDA approved and have been applied in many medical fields such as immunoassay, drug delivery, gene therapy, magnetic resonance imaging (MRI), tissue repair, cellular repairing, and biosensors (39). These particles posses a promising site-directed treatment using an external magnetic field. Decades ago, Frenkel and Dorfman speculated the ability of ferromagnetic particles to behave as super-paramagnetic at the nano-scale (40). This means that when the size of the particles are reduced below a critical point, they do not preserve any magnetism once the magnetic field is removed (41). This is the crucial factor that makes magnetic nanoparticles so distinctive. Theoretically, Superparamagnetic nanoparticles have no hysteresis, zero coercivity, zero remanence, much stronger magnetization, low particle agglomeration, and the ability to remain in the systemic circulation for long periods of time without being filtered out by natural mechanisms such as the immune system or though the liver. Beside particles size, chemical composition and the method of synthesis strongly affects the particles’ magnetic properties. Great efforts have been made to alter the chemical composition of nanoparticles’ core for the purpose of enhancing its magnetic properties. Metallic cores including Fe, Fept, FeCo alloys are the first to be investigated; however, their high toxicity and oxidation sensitivity in-vivo shifted the interest to ceramic cores, and specifically to metal oxides. Magnetic metal oxides provide boundless opportunities for super-paramagnetic nanoparticles design with anticipated properties (42). Among the different types of metal oxides, magnetite (Fe₃O₄), is the most attractive owing to its high magnetic saturation, chemical stability, biodegradability, biocompatibility, ease of synthesis, non-toxicity, relatively ease of functionalization, and low surface oxidation (43–46). Comprehensive literature had reviewed the different methods of iron oxide synthesis (47–49). They are categorized into three types: physical such as electron beam lithography and gas-phase deposition; chemical such as chemical co-precipitation, hydrothermal reaction or thermal Deposition; and synthesis through microbial process (47). The method selected is based on the desired product and where it is going to be applied, as each method produce distinctive crystalline phase, shape, and size distribution of iron oxide nanoparticles (IONPs).
As it is fundamental to choose the appropriate core type and characterization of magnetic nanoparticle, coating its surface is equally important, yet challenging. Providing a suitable coating later is a key aspect to promote the chemical and biological functionalization needed for bioselectivity and biocompatibility, consequently enhancing tissue and cell targeting effect. IONPs can be coated with organic materials using polymers, inorganic materials as gold and silica, or metal oxides such as aluminum oxide and titanium oxide (39). Also, they can be further functionalized using antibodies, small molecules, aptamer or peptieds, all of which are targeting ligands serving to decrease nonspecific distribution and to extend their blood circulation time in-vivo.

Wide-ranging investigations on IONPs’ biological outcome and ability to target carcinoma cells, breast cancer more specifically, both in vitro and in vivo studies were performed. Among many examples, Marcu et al. (50) used laser pyrolysis method to synthesize IONPs in the range of 8–10 nm and found it to be better internalized in MCF-7 tumor cells’ cytoplasm and had lower anti-proliferation effects compared to commercial pure 20 nm IONPs. After further coating using antracyclin antibiotic Violamycine B1(VB1), IONPs demonstrated a much effective VB1 delivery and cellular uptake verses free administrated VB1 and commercial IONPs, respectively. In another study, IONPs functionalized with luteinizing hormone releasing hormone (LHRH) was demonstrated as promising tool for breast cancer cells targeting, as well as acting as contrast agent in MRI of breast cancer xenografts (51). Moreover, IONPs was applied and tested for detection of Sentinel lymph nodes (SLN) in breast cancer patients. SLN biopsy is a standard procedure used for the purpose of staging and diagnosis of breast cancer, whereas the combination of radioisotope and blue dye breast injection via gamma probe (GP) is commonly applied nowadays. Development of novel non-radioactive method using IONPs and a manual magnetometer (MS) would be very promising. According to Piñero-Madrona et al. (52), the detection efficiency in 181 breast cancer patients were significantly indifferent for GP and MS methods, verifying IONPs diagnostic effectiveness in clinical trials. On another note, it was demonstrated that IONPs could be used as an effective candidate for separating circulating cancer cells in fresh whole blood. Human breast cancer cell SK-BR3 (HER2 positive) was used as a model cell by Xu et al. (53) to be captured by IONPs in fresh human blood. HER2 is a protein that is overexpressed in many types of cancer cells including breast cancer. The 30nm IONPs were coated with antibodies
against human epithelial growth receptor 2. These nanoparticles were able to separate 73.6% of SK-BR3 cells with an enrichment factor of 1:10,000,000 under magnetic field (cancer over normal cells).

1.3 Breast cancer treatment via hormonal therapy

The use of adjuvant hormonal therapy have assisted in increasing the survival rates in breast cancer women patients (54). An adjuvant therapy is a systematic anti-cancer therapy that is used often after surgery to destroy any remaining microscopic cancerous cells that might have been left behind. A adjuvant hormonal treatment of five to ten years was proven to reduce cancer recurrence risk by 50% in hormone responsive early breast cancer (55,56). About 80% of breast cancers are estrogen-receptors (ER) positive. In other words, they need estrogen to grow (57). This type of breast cancer produce either estrogen receptors or progesterone receptors or both, thus are named hormone-responsive. Currently, hormone therapies, or so-called endocrine therapy, are effective in clinical use such as Tmx (an estrogen receptors blocker) (58). It is applied as adjuvant, non-adjuvant or in combination with other therapeutics, chemotherapy for example, depending on patient’s health circumstances. In a recent study (59), it was shown that the use of progesterone had suppressed estrogen-mediated growth of ER positive cell line and early ER positive breast cancer explants. It was also demonstrated that progesterone increased the anti-proliferation of these cells when combined with an ER antagonist.

1.5 Thesis hypothesis and Objectives

The main objective of this thesis was to develop superparamagnetic iron oxide nanoparticles (SPIONs) coated with novel soluble and biodegradable polymer (glycol chitosan) for application in hormonal therapy. Development of the controlled delivery system will be suitable for encapsulation of hydrophobic drug, progesterone. The fundamental hypothesis of this thesis is that the designed coated magnetic nanoparticles will be a promising drug carrier with enhanced bioavailability of progesterone to desired cells with sustained release depending on the concentration of glycol chitosan. This is achieved through studying the release kinetics of proposed surface modified SPIONs. Also, the release of progesterone mechanism is investigated using empirical mathematical under different reaction conditions.
Lastly, *in-vitro* studies were carried out to examine the biocompatibility of the synthesized nanoparticles.

### 1.6 Thesis outline

An investigation on a unique approach for designing magnetic nanoparticles is presented in this thesis. The proposed work focuses on progesterone as the therapeutic agent. Chapter one is an introduction to the thesis, where the motivation of research is presented. The common types of nanoparticle materials are discussed with a touch on the role of hormonal therapy in breast cancer treatment.

Chapter two is a comprehensive review, which elaborates on the current studies performed on magnetic nanoparticles. It describes their physiochemical properties that allow their success as nano-drug carriers. This chapter discusses in detail the reasons of surface modification and the main important polymers used in current research. Their central applications in drug delivery are also presented with the commonly used mathematical models of drug release.

Chapter three contains the research results described in detail. The magnetite (Fe₃O₄) nanoparticles were prepared for controlled delivery of progesterone through simple precipitation technique and then coated with new chitosan derivative that have never been used as a coat for IONPs nanoparticles in hormonal therapy applications. Progesterone encapsulation and release was examined and mathematically modeled to study its release mechanism/kinetics.

The conclusion and future prospects are outlined in chapter 4.
1.7 References


CHAPTER 2

2 Bioactivity of hybrid polymeric-magnetic nanoparticles and their application in drug delivery

2.1 Abstract

Engineered magnetic nanoparticles (MNPs) possess unique properties and hold great potential in biomedicine and clinical applications. With their magnetic properties and their ability to work at the cellular and molecular level, MNPs have been applied both in-vitro and in-vivo in targeted drug delivery and imaging. Focusing on Iron Oxide Superparamagnetic nanoparticles (SPIONs), this paper elaborates on the recent advances in the development of hybrid polymeric-magnetic nanoparticles. Their main applications in drug delivery include Chemotherapeutics, Hyperthermia treatment, Radio-therapeutics, Gene delivery, and Biotherapeutics. Physiochemical properties such as size, shape, surface and magnetic properties are key factors in determining their behavior. Additionally, tailoring SPIONs surface is often vital for desired cell targeting and improved efficiency. Polymer coating is specifically reviewed with a brief discussion of SPIONs administration routes. Commonly used drug release models for describing release mechanisms and the nanotoxicity aspects are also discussed.

Keywords: Hybrid- magnetic nanoparticles; Iron oxide; Superparamagnetic; Drug delivery; Polymer coating; Biomedical applications; Mathematical modeling.

2.2 Introduction

“At the atomic level, we have new kinds of forces and new kinds of possibilities, new kinds of effects. The problems of manufacture and reproduction of materials will be quite different” (1). The father of nanotechnology, Richard Feynman, addressed these words more than five decades ago. Since then, the inspiration of merging nanotechnology into clinical medicine had evolved. Nevertheless, recently nanotechnology research and development has been exponentially expanding, in both breadth and depth, like never before (2).
The interest in nanoscale materials as drug vehicles is mainly due to the need for change in conventional therapeutic strategies, particularly in delivery of highly toxic drugs such as in cancer therapy. Currently, the most commonly used conventional treatments are molecular or so called “free” drugs with systematic biodistribution. This encompasses many problems and adverse side effects primarily because of lack of specificity (1). Chemotherapeutics, for instance, attack both target and healthy cells due to its relatively poor specificity. Additional undesirable pharmacokinetics are present in molecular drugs including but not limited to: high dose admiration because of their rapid degradation in vivo, precipitation in aqueous solution as a result of their hydrophobic character, rapid clearance in biological body, and poor selectivity to target tissues (1). These issues along with other unfavorable properties existent in current treatments display great thrust to develop restricted drug delivery mechanism with high control at locoregional therapeutic level (3). The essence of optimizing drug nanocarriers is for i) directing drug to disease site with minimal side effects via reduction in systematic biodistribution of the cytotoxic drug and ii) reducing the dosage required through more localized and efficient targeting (4). Rising number of publications (5–7) have investigated how to engineer drug delivery system (DDS) nanocarriers which ideally should propose the following characteristics: 1) long body circulation, 2) specific targeting of disease site, 3) response to local stimuli in the pathological site such as abnormality in temperature, pH change or external magnetic field and/or heat, 4) enriched intracellular delivery of drugs or genes as required, 5) real time information of target accumulation and DDS biodistribution by carrying a contrast component (6).

Among the enormous types of nanomaterial, MNPs are the most attractive due to their amazing physical characteristics and ability to function at both cellular and molecular level (1,2). The unique properties that magnetic colloids hold make them very suitable for biomedical applications. They have the capability of being visual under magnetic resonance imaging (MRI) as a mean for noninvasive imaging modality with high-resolution imaging and as contrast agents; and they have the promising means of transporting and maintaining at target site as therapeutic vehicle (8,9). There are two main methods in which magnetic DDS can function. First, through magnetic drug targeting (MDT) where an external magnetic field gradient is applied at target tissue. This mechanism is what makes MNPs distinctive. Second,
delivering through active targeting using ligand attachment of high affinity. Detailed explanation of delivery techniques is summarized later in this review.

A major class of MNPs is iron oxides. They exist in sixteen pure phases in nature such as the oxides (magnetite and hematite), iron oxide beta phase and maghemite, hydroxides (iron (III) hydroxide or bernalite), oxy-hydroxides (geothite, akaganetite, lepidocrocite, feroxyhyte) and many others (10). One of their distinct characteristics includes low solubility, unique colors and having trivalent state (11). The most interesting and extensively studied type is the magnetite (Fe₃O₄). It is ferromagnetic black color iron oxide of both Fe (II) and Fe (III). Magnetite is the preferred type because of the presence of Fe²⁺ state with the potential of acting as electron donor. Also, it is relatively the most stable form in biological environment, as other forms like maghmeite and hematite produce free radicals which result in cell viability losses as more electron deficient Fe³⁺ are present (12,13). The structure of magnetite is an inverse spinel crystal with a face-centered cubic unit with edge length of 0.839 nm Fe²⁺ and half of the Fe³⁺ dominate the octahedral sites while the other half of Fe³⁺ dominates the tetrahedral sites and having 23 oxygen atoms (14).

A unique type of magnetite is the superparamagnetic iron oxide nanoparticles (SPION). They gained most of research focus because of their many desirable features, most importantly: biocompatibility, biodegradability and ease of synthesis (2). The human body has a large iron pool (3 to 5 g) and a daily intake requirement of 20 to 25 mg (15). The amount of injection of treatment per person is comparable to the amount of daily intake (approximately 0.5 mg/kg) (15–17). Thus, biodegradable iron can blend with present body iron and participate in physiological iron homeostasis after drug release is accomplished at target cite (2,18). This biosafety represent one of the major advantages of SPION. In addition, their superparamagnetic nature makes them the most suitable type of MNPs for biomedical application. SPION leaves behind zero residual magnetization after an external magnetic field is removed (19). This property assists in avoiding coagulation, which consequently lowers the possibility of agglomeration in vivo compared to other MNPs (19).
2.3 Physiochemical properties of magnetic nanoparticles

Physiochemical properties are extremely significant in determining the efficiency of targeted delivery. Same MNPs type with different physiochemical properties can potentially have completely altered pharmacokinetics, thus behaving differently in-vivo. Typically, MNPs have to overcome two biological barriers to reach the aimed target: physiological barrier and cellular barrier (1,15). The main properties that dictate their behavior in-vivo are size, shape, surface characteristic and its unique magnetic properties. SPIONs usually have two structural configurations: i) magnetic core (normally magnetite or Maghemite) with a biocompatible polymer coating on surface or ii) precipitate of SPIONs inside the pores of a highly porous biocompatible polymer (27). The coating act as a shield for SPIONs from surrounding environment where it aids to enhance targeting yield though improved properties and further surface functionalization (28,29). There are many SPIONs applications in biomedicine, the most known are considered in MRI as contrast agents (2, 30–33), and magnetic drug targeting or drug delivery as carriers of promising therapeutics (34–38). Table 2-1 summarizes the recent advances in hybrid polymeric decorated magnetic nanoparticles and their potential biomedical applications.

This review will focus on superparamagnetic nanoparticles coated with different types of polymers. It will start with the key physiochemical features that dominate their behavior. The importance of surface modification will be addressed. Subsequently, the major classes of polymer modified iron oxide nanoparticles is demonstrated according to their clinical use and application. Clinically approved nanoparticles with a touch on scale-up and industrial applications are then addressed and the different routes of administration are mentioned. Lastly, mathematical models of drug release profile of the common used nanoparticles are addressed.
### Table 2-1: Summary of the recent advances in hybrid polymeric decorated magnetic nanoparticles and their potential biomedical applications.

<table>
<thead>
<tr>
<th>Type of hybrid polymeric-magnetic nanoparticles</th>
<th>Biomedical applications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-methacryloxypropyl trimethoxy silane coated magnetic nanoparticles polymerized with glycidylmethacrylate-grafted-maleated cyclodextrin</td>
<td>Controlled delivery of anticancer drug 5-fluorouracil</td>
<td>(19)</td>
</tr>
<tr>
<td>Chitosan functionalized magnetite doped luminescent rare earth nanoparticles (Fe$_3$O$_4$@LaF$_3$:Ce$^{3+}$,Tb$^{3+}$/chitosan nanoparticles)</td>
<td>Targeted delivery of paclitaxel for lung cancer</td>
<td>(20)</td>
</tr>
<tr>
<td>Magnetic-Gold hybrid nanoparticles</td>
<td>Targeted delivery of the anti-cancer drug doxorubicin</td>
<td>(21)</td>
</tr>
<tr>
<td>Glyco-polymer modified magnetic mesoporous silica nanoparticles</td>
<td>Magnetic resonance imaging and controlled drug delivery</td>
<td>(22)</td>
</tr>
<tr>
<td>RGD-functionalized PEG-coated magnetic hydrogel</td>
<td>Targeted delivery of the anti-cancer drug doxorubicin</td>
<td>(23)</td>
</tr>
<tr>
<td>Hybrid Fe$_3$O$_4$- carboxymethyl chitosan nanoparticles</td>
<td>Tumor targeted delivery of rapamycin</td>
<td>(24)</td>
</tr>
<tr>
<td>N-glycyrrhetinic acid-polyethylene glycol (PEG)-chitosan-magnetic nanoparticles NGPC-Fe$_3$O$_4$</td>
<td>Hepatocyte-mitochondrial targeted delivery of brucine (natural anticancer drug)</td>
<td>(25)</td>
</tr>
</tbody>
</table>

#### 2.3.1 Particle Size

Particle size is the key determinant of the half-life of drug clearance in tissues (15). The main idea of using nanoscale versus macro-molecular drugs is to achieve a higher control residence time of drug release. Controlling the size is considered one of the most, if not the most, significant property in deciding particles fate. It must be chosen and formed with utmost care to ensure that it is small enough to avoid short blood circulation time via prompt splenic and liver filtration (smaller than 200nm), yet large enough to evade kidney filtration and rapid penetration (larger than 10 nm) (2,19–22). Predictably, particle size increases with surface modification as in coating and functionalization as it works hand in hand with MNPs chemical
composition. As blood vessels pore gaps can enlarge up to 400 to 800 nm during angiogenesis, size range for intervention administration of core MNPs should lie between 10 to 100 nm, preferably between 20 to 60 nm for maximal cellular uptake (19,22,23).

Size and size distribution can be controlled with method of synthesis. In coprecipitation, the most commonly used method, adjusting the pH and ionic strength of precipitation medium, particles mean size can be controlled over one order of magnitude (24,25). As pH and the ionic strength increases, the size decreases due to their effect on the surface chemical composition and thus particles electrostatic surface charge (24). Also, it has been shown that high-temperature decomposition of organometallic precursors can achieve higher control of size and size distribution (26,27). Changing the metal precursor or changing the reaction temperature can control nanoparticles size precisely. Using this method, Sun et al. showed that highly spherical Fe$_3$O$_4$ nanoparticles could be synthesized in the range of 4 to 20 nm with size variation of only 2nm. However, this process requires the use of oleic acid and oleylamine surfactants, which result in creating a hydrophobic coating on the nanoparticles. This demands an additional modification step to make it soluble in aqueous body fluid such as adding an amphiphilic polymer coating (28). Mostly SPIONs are applied as dispersed particles in aqueous medium. Even if mono-dispersed particles were achieved in synthesis, it can lead to greatly poly-dispersed aggregates in suspension due to hydrophobic-hydrophilic interactions, which creates new challenge of hydrodynamic size control (29,30). This is dealt with through critical choice of surface modification (30,31).

2.3.2 Particles morphology

Many studies have addressed MNPs shape and their magnetic properties and its effect on biodistribution and blood circulation time. However, most of the studies focused on spherical particles, while comparative investigations of non-spherical particles are lacking specially with regards to anisotropic configurations such as rode morphologies (32,33). This is due to the formation of one-dimensional (1-D) nano-ferrites, since spinel structure of iron oxide is highly symmetric (34). Nonetheless, recently there have been successful studies on synthesis of non-spherical particles such as cubic (35,36), rod (37,38), and hexagonal (39) shapes.
Alteration of chemical precipitation and coprecipitation method, nanoparticles shapes can be varied, yet it lacks shape control and size distribution. Yan et al. prepared nanosheets and nanowires with the addition of sodium acetate in co-precipitation of sulfate salts (40). Thermal process had shown to produce a more precise and fine size control. Hyeon et al. (41) achieved a perfect cubic MNPs morphology by using Fe(acac)_3 as precursor in benzyl ether and using oleic acid as a surfactant at 290 °C for 30 minutes. The reduction in benzyl ether allowed the alteration of truncated cubes and octahedral to very well defined cubic structure. Interestingly, it is shown that when chloride or bromide ions are lacking in the reaction, only spherical MNPs will be formed (42). In fact, it has been demonstrated that high control of shape towards cubic morphology is easily achieved by the presence of chlorine ions instead of controlling reaction thermo-kinetics (42,43). Recent report had described the effectiveness of these iron oxide nano-cubes in hyperthermia treatment (44). Moreover, it has been shown that non-spherical nanoparticles avoid bio-elimination more efficiency than spherical ones. Several studies demonstrated the relationship between the increase in length to width ratio to longer the blood circulation times (45–48). Although these findings are promising, there is still a need for more studies on the effect of morphology on pharmacokinetics.

2.3.3 Surface properties

Surface property such as charge, hydrophobicity/hydrophilicity, smoothness/roughness are vital factors in determining nanoparticles capability as drug delivery vehicles, not only for biocompatibility and toxicity, but in determining particles biodistribution (49), cell adhesion on biomaterials (50–52), cellular interaction especially in endocytosis and phagocytosis (53), and blood half-life (54). In-vivo, MNPs surface interact with many elements including the immune system, extracellular matrices, plasma proteins and non-targeted cells. Positively charged MNPs can bind with non-targeted cells resulting in non-specific internalization (54). Osaka et al. studied the effect of surface charge on internalization on different cell lines (55). Compared to negatively charged surface, SPIONs with positive charge showed higher cellular uptake efficiency into breast cancer cells, yet no effect on Human Umbilical Vein Endothelial Cells (HUVECs). Therefore, SPIONs uptake efficiency depend not only on their surface properties but also on the body cell type. Specifically, hydrophobic and charged particles tend to be recognized by reticuloendothelial system (RES) quicker as a result of plasma protein
adsorption (opsonization), thus shorter circulation time (56). Moreover, hydrophobic particles are more susceptible to agglomeration leading to prompt RES removal (54). To limit MNPs host interactions, development of MNPs surface became essential. Many reports discussed the benefits of surface modification, which will be addressed in later sections.

2.3.4 Magnetic properties

Iron atom has a strong magnetic moment due to four unpaired electrons in its 3d orbitals. Different magnetic states occur when crystallization of iron occurs (Figure 2-1) (57,58). The Paramagnetic crystal produces randomly aligned magnetic moments and overall structure has zero net magnetization. When paramagnetic state is subjected to an external magnetic field, moments will align producing a small net crystal magnetization. At the ferromagnetic and antiferromagnetic states, the individual moments are aligned parallel and antiparallel respectfully without an external magnetic field. Ferromagnetic state however differ from antiferromagnetic in having two different types of atoms of different strengths (57).

<table>
<thead>
<tr>
<th>Paramagnetism</th>
<th>Ferromagnetism</th>
<th>Antiferromagnetism</th>
<th>Ferrimagnetism</th>
</tr>
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</table>

Figure 2-1: Alignment of magnetic moment of individual atoms of iron
Bulk ferromagnets contain several domains in which uniformly magnetized regions exist. Each domain is separated by non-uniform magnetization distributions (domain walls) with different magnetization vector. Since the vectors of each domain are not aligned, the net magnetization is lowered. As the size of material decreases, the number of domain decreases until there is one domain left below critical size diameter (usually <15-20 nm) which are superparamagnetic nanoparticles (57,59,60).

Magnetization curve (Figure 2-2) illustrates the relation between applied external magnetic field with strength \( H \), and magnetic strength \( M \), which increases until a saturation value \( M_s \) is reached. Hysteresis loop is produced because the domains do not return to their original orientation when external field is removed and a saturation magnetization value (coercivity) is attained. This coercivity only vanishes after applying coercive field of strength \( H_c \) in the opposite direction. In the case Superparamagnetic particles, no hysteresis is produced; a much stronger magnetization is present which is zeroed after the removal of external magnetic field (coercivity=0) (19,50,61,62). Curie temperature terms the transition temperature \( T_C \) for a material to lose its permanent magnetic properties, while blocking temperature \( T_B \) describes the temperature where usually superparamagnetic ordering exist (57). Particle size is very crucial for

Figure 2-2: magnetization curve of magnetic strength \( M \) verses applied magnetic
superparamagnetic property to appear. **Figure 2-3** shows the effect of particle size on magnetic property and is described by **Eq. 2-1** (50).

\[
 r = \frac{3}{2} \frac{\sqrt[3]{6kT}}{K_u}
\]  

(Eq. 2-1)

Where, \( r \) is particles radius, \( k \), Boltzmann constant, \( T \), temperature, and \( K_u \) the universal axial anisotropy.

\[
\begin{aligned}
&\geq 1 \, \mu m \\
&\text{spontaneous magnetization below } T_c, \text{ nonzero magnetic moment formation of domain structure}
\end{aligned}
\]

\[
\begin{aligned}
&50 - 1000 \, \text{nm} \\
&\text{sample, synthesis and processing method dependent}
\end{aligned}
\]

\[
\begin{aligned}
&1 - 30 \, \text{nm} \\
&T < T_B \text{ magnetic hysteresis} \\
&T > T_B \text{ superparamagnetization} \\
&T < T < T_c \text{ nonzero net magnetic moment}
\end{aligned}
\]

\[
\begin{aligned}
&\sim 0.2 \, \text{ nm} \\
&\text{usual paramagnetic properties}
\end{aligned}
\]

**Figure 2-3- magnetic properties of ferromagnets dependence on particles' size**

\( \text{Fe}_3\text{O}_4 \) is ferromagnetic and has a \( T_c \) of 850 K (63) and becomes superparamagnetic below critical diameter. It is important to note that magnetic properties depends strongly on the method of synthesis (64,65) and on crystal structure (66) even when particle size is similar. The presence of impurities disturbing the crystal structure during synthesis as well as the effect of surface properties described earlier result in magnetization alteration (2). The coercivity is strongest in octahedral shape followed by cubes and spherical morphology due to the increase in the number of magnetic axes of these shapes order (57,67).
2.4 Surface modification Exigency

Since the properties SPIONs are mostly surface dependent, surface chemistry plays a main role in changing its biological and physiochemical properties to a desired in-vivo application. There are two main methodologies in which surface modification is employed: ligand addition and ligand exchange (68,69). In ligand addition, polymers adsorb to the surface of particles physically due to hydrophobic interactions, electrostatic and/or by hydrogen bonding. Polymers containing functional groups such as hydroxyl and amine, are adsorbed to the SPIONs surfaces readily (70). In ligand exchange, replacing original surface with functional groups such as thiol, carboxylic acid, amine, diol, customize surface properties better (71). There are other commonly available methods of surface alteration including lipid and micelle, biomolecular conjugation, inorganic core-shell formation, use of organic surfactants and biofabrication (72). Oligonucleotides, peptides, or enzymes are also important for specific cell/tissue targeting and its attachment is done via addition of the specific functional group on the coating layer (73). The selection of specific surface modification technique is made according to the end use of SPIONs in biomedical field.

2.4.1 Colloidal Stability

Three principles control the colloidal stability of SPIONs in aqueous suspension i) hydrophobic-hydrophilic interaction, ii) magnetic forces, and iii) Van der Waals forces (4). Due to their dominant characteristic of high surface energy and large surface area to volume ratio, nano-sized SPIONs tend to aggregate to micro-size clusters (74–76). Van der Waals attraction also plays a role to cluster nanoparticles in order to minimize the total surface and/or interfacial energy. Microns further agglomerate due to the magnetic dipole–dipole moments between them. When an external magnetic field is applied, additional magnetization is added which increase their aggregation (74). Using electrostatic stabilization through repulsion of charges is typically not sufficient to prevent agglomeration in biological solutions because electrolytes such as salts, neutralize the charges (50). Subsequently, these aggregations affects the efficacy of SPIONs negatively in drug delivery as larger particles with lower surface area limits drug loading and release. It may also result in capillary blockage when injected in the body (73). Coating provides steric barrier between the particles, which diminish aggregation.
2.4.2 Blood half-life and uptake by the reticulo-endothelial system (RES)

Blood half-life indicates the circulation time the particles can exist in blood before they are removed by RES (77). When nanoparticles are recognized as foreign bodies, elimination by phagocytic cells occurs (78,79). When particles are coated, resistance of non-specific proteins adsorption improves, which will in return, lowers the probability of phagocytic cells elimination and thus increases blood half-life (76,80,81). In Molday and Mackenzie (82), blood circulation was proven to increase when MNPs were coated with dextran in-situ while Liu et al. (83) and Woodle et al. (84) showed that coating with a polymer PEGylated improved pharmacokinetics and extended blood half-life. Longevity in half-life helps in achieving enhanced drug release as well as more time for interaction with target site (6).

2.4.3 Nano-cytotoxicity of hybrid polymeric-magnetic nanoparticles

Although SPIONs are considered the most biocompatible compared to other nanoparticles such as carbon nanotubes, gold and silica-based nanoparticles bimetallic or magnetic alloys such as FePt, metal oxides, such as cobalt and nickel ferrites, its cytotoxicity and genotoxicity remain not clearly understood (85,86). Toxicity is evaluated on how nanoparticles interact with the body during its functional lifetime, and how the components affect the body during biodegradation and liver processing (87). At longer duration times and high concentration, bare iron oxide nanoparticles exert some toxic effects while coated SPIONs are relatively nontoxic. Gupta et al. found that using PEG-coated SPION, cells remained > 99% viable at a concentration of 1 mg/mL. However, using bare SPION, 25–50% loss in fibroblast viability was noticed at only 250 microg/mL concentration (88). Similar studies indicated that iron oxide nanoparticles with different coating exerts very low to no toxicity in different cell lines (89–92). However, it has been shown that coated-SPION can propose some levels of toxicity at elevated concentrations (93,94). Therefore, more research is needed in terms of coating types and particles exposure timing in-vivo so that their undesirable effects can be avoided.

2.4.4 Oxidation resistance properties of hybrid polymeric- magnetic nanoparticles

Iron oxides have relatively high oxidation resistance, however, they could be oxidized and converted to $\alpha$-Fe$_2$O$_3$ due to of the presence of exposed Fe$^{2+}$ in Fe$_3$O$_4$ MNPs (50). $\alpha$-Fe$_2$O$_3$ demonstrates no magnetic properties, which affects nanoparticles drug delivery efficiency.
Hybrid polymeric-magnetic nanoparticles provide much better stability against corrosion and oxidation and allows particles to maintain their single domain structure (95,96).

2.5 Polymer coating

Bare magnetic nanoparticles generally, and SPIONs specifically, proposes many challenges in vivo such as lack of biodegradability in the physiological environment, chemical instability and poor biocompatibility. Coating is an extremely essential factor in achieving successful drug nano-vehicles. The fundamental reasons are to attain colloidal stability, longer blood circulation, evade cell toxicity and prevent oxidation. Polymer-based surface modification of SPIONs is classified into two categories: surface exchange and polymer encapsulation (22,97). In surface exchange, polymers replace the original nanoparticles surface similar to ligand surface addition except that a polymer substitutes the ligand. In encapsulation, a polymer encapsulates the nanoparticle by hydrophobic interactions between the original hydrophobic MNPs surface and the hydrophobic section of the polymer chains where the hydrophilic section of the polymer faces the media.

Polymer coating provides a much-enhanced colloidal stability compared to other surface modification types due to abundance of chains that provide electrostatic and steric dispersion. Aspects that influence polymeric SPIONs performance include, molecular weight or molecule length, chemical structure, chemical conformation and the approach in which the coating is attached to the MNPs, e.g. covalently bonded or electrostatic attraction (2). The polymer molecular weight and configuration contribute strongly to the
way the coating is accomplished on the particles surface. Different coating schemes are formed depending on the type of polymer such as end-grafted, trains, loops, tails or fully coated polymer shells (2,98). Polymer coating is classified into two types: natural and synthetic. The most widely studied natural polymer in SPIONs are dextran, chitosan, gelatin, and starch; while the synthetic ones include poly (ethylene glycol) (PEG), poly (vinyl pyrrolidone) (PVP), poly (vinyl alcohol) (PVA), and poly (lactic-co-glycolic acid) (PLGA) (99,100). Figure 2-4 and Figure 2-5 represents different polymers used as primary coating or stabilizer for SPIONs sorted by their functional groups, and the polymers chemical structures, respectively.

2.5.1 Natural polymers
2.5.1.1 Dextran
Dextran is a biodegradable and biocompatible neutral polysaccharide composed of α-D-1,6-glucose-linked repeating glucan units, and sometimes has side-chains 1–3 attached to its backbone (101). It is considered one of the most extensively studied coating polymer for ISPONs and is successfully implemented in vivo applications (69,102). Its main advantage is
the ability to increase blood circulation time of particles as it stabilizes the colloidal solution (82,103). Often Dextran is susceptible to dislocate from SPION cores and thus modification of coating layer by functional group addition and covalent bonding is often desirable. Li et al. improved stability of coating by addition of carboxymethyl group and cross-linking with epichlorohydrin forming cross-linked iron oxide (CLIOs) (82,103). CLIOs can be prepared by physical adsorption followed by dextran cross-linking to provide tighter attachment to the core, yet CLIOs without a cross-linking step still showed great stability and biocompatibility (73). Enhanced functionality was observed when CLIOs was treated with amine group, which then can bond to proteins and peptides (104). Alternatively (104), covalent conjugation of partially oxidized dextran though secondary amine group was performed by Sonvico et al. to enhance its stability after a modification to SPIONs surface with aminopropylsilane (105).

Dextran multivalent nature permits attachment of various molecules at many sites along its chain. As a result, further functionalization is often performed by addition of hydroxyl group, carboxylate, or aldehyde (73). Sodium periodate can be used to oxidize dextran polymer by cleaving the carbon between the two adjacent diols to create an aldehyde. This form of dextran is highly reactive and can combine with various amine-containing molecules. For instance, modification of oxidized dextran was done by Cen et. al. (106) in order to generate folate and fluorescein isothiocyanate (FITC). Also, TAT-crossed linked dextran coated SPIONs (TAT-CLIOs) were synthesized and their effect on cellular localization in human lymphocytes were studied (107). TAT-CLIOs was made by epichlorohydrin treatment and The TAT peptide is attached via a disulfide linkage and FITC-labeling was performed. Before localizing the TAT-CLIOs in the cells and the sytoplasm, cells were treated with anti-dextran antibody to ensure that both the CLIOs and the peptide reach the nucleus and not only disulfide cross-linked peptide. Immunohistochemical staining showed that CLIOs had indeed researched the nucleus and were detectable by MRI as they were highly magnetic. Moreover, the study showed the ability dextran coated particles to characterize the nodal stages during cancer with an MRI.

2.5.1.2 Chitosan

Chitosan is a natural, hydrophilic, biocompatible, bio-degradable, and non-toxic copolymer (poly-aminosaccharide): β-(1 → 4)-linked 2-acetamido- 2-deoxy-d-glucopyranose and 2-
amino-2-deoxy-d-glucopyranose (50,99). Chitosan has repeated hexosaminide residues where each unit is made of two hydroxyl groups and an amino group. Chitosan attaches firmly and stably to SPIONs surface due to these groups that form complexes with the surface (108). The negatively charged nucleic acid in chitosan, on the other hand, can interact with the positively charged amine groups for gene delivery therapeutics with MRI (109). This also makes chitosan bio-adhesive resulting in increasing retention time at tissue site (110). Chitosan is commonly used for coating SPIONs for enhanced quality as contrast agent in MRI (111–113) and is known to ease particles movement across cellular barriers and quickly opens the tight junctions in epithelial cells (114,115). Synthesis of fluorescence dye modified magnetic iron oxide nanoparticles improves cellular imaging and can serve as both magnetic resonance contrast agents for MRI and as an optical probe for intravital fluorescence microscopy. Chitosan was attached non-covalently to particles surface after modifying it with covalently attached fluorescent dye. Fluorescent modified chitosan allowed for direct imaging and localization of particles, which were visible inside the living cells, mostly in late endosomes or lysosomes, as well as on cell surfaces via confocal laser scanning microscopy (CLSM) and Transmission electron microscopy (TEM). Approximately, 85% of cells contained the FITC-labeled particles (labeled-cells) 30 min after incubation and within the first two hours. The T2 relaxivity of the labeled cells in-vitro detected 104 cells with 1.5 TMR Imager and the prepared FITC-labeled particles were biocompatible as demonstrated by a cytotoxicity test. However, SPIONs still exhibited aggregation that possesses a safety concern in clinical application.

### 2.5.1.3 Gelatin

Gelatin is an organic protein derived from collagen. Some of its properties include solubility, biocompatibility, biodegradability, and pH-induced surface charge and thus it is used in many pharmaceutical and biomedical applications (116,117). Doxorubicin (DXR) creates a drug–polymer conjugate due to the presence of multifunctional groups in the gelatin chain such as COOH and NH₂ (118). Gelatin can be fabricated as micro and nano-spheres depending on synthesis technique, and is found to enhance phagocytosis in tumor cells (119). Although Gelatin seems to be a suitable candidate as a coating material for SPIONs, yet only few studies have been performed on it (120). Intorasoot et al. used Gelatin-coated MNPs for bacterial genomic DNA isolation (121). DNA extraction using coated MNPs demonstrated
much more efficient results compared to commercially available extraction kit and phenol-
chloroform extraction, where double the amount of DNA was recovered with significantly
higher yield. Gaihre et al. investigated drug-loading efficiency on two types of gelatin-coated
iron oxide nanoparticles using DXR as the model drug (116). Two gelatine types (from
porcine skin vs. bovine skin) were used and drug loading was performed using two different
methods: 1) adsorption (charge induced of negative coated particles to positive doxorubicin)
which was done by incubating MNPs and drug in different drug concentrations, pH levels,
gelatin type, and DXR to coated particles ratios; 2) desolvation/cross-linking method where
acetone and glutaraldehyde (GTA) where added to the DXR and coated particles mixture.
Regardless of gelatin type, desolvation/cross-linking technique had higher drug loading
efficiency. This indicates drug-particles interaction in the loading phenomenon where the
encapsulation efficiency changes according to surface charge of the gelatin-coated particles.
Also, loaded particles were pH responsive in drug release with higher release at pH 4
compared to 7.4.

2.5.1.4 Starch
Starch is a natural long D-glucose chain that has good biodegradability and stability as
SPIONs coating material. Starch-coated SPIONs with fairly low agglomeration can be
synthesized by alkaline coprecipitation of iron salt in a starch matrix (122) rat brains for in-
vivo MRI and monitoring. The starch-coated SPIONs showed great biocompatibility and
feasibility as MR contrast agents in brain scans (123). Also, carboxymethyl starch (CMS-
SPIONs) has been investigated extensively for controlled-release system and analyzed with
many kinetic studies. Saboktakin et al. (118) showed that carboxymethyl starch is a reliable
coating for SPIONs to achieve stable, spherical and relatively mono-dispersed particles for
drug delivery of ferrofluid. Core-shell structure was prepared via hydrogen bonding CMS
alcohol groups with the hydroxylated and protonated SPIONs surface. The study also
demonstrated that CMS- SPIONs has the capacity to be delivered in cancer tissue, and high
potential to be used for cancer diagnosis as contrast agents.
2.5.2 Synthetic Polymers

2.5.2.1 Poly (ethylene glycol) (PEG)

PEG is a linear water-soluble synthetic polyether that is mostly used to enhance solubility of hydrophobic drugs (124,125). One of the main reasons for use of this polymer for preparation of many bio-conjugates is its tendency to exclude macromolecules, proteins and other particulates from its surrounding. It shows no adverse effect on enzyme activities or protein conformation in aqueous solution when used as coating material (126) and causes no immune interactions while increasing circulation time when MNPs are coated with PEG (99,127–129).

PEG enhances steric stabilization in vivo, thus prolongs blood circulation. It is highly internalized by cells with no toxicity due to its high solubility and fluid phase (130,131). PEG molecule has only one hydroxyl group at the end of its chain, which results in reduced further functionalization of coated SPIONs-PEG (73). Nevertheless, the coating of PEG acts as a good spacer, which allow for various biomolecules attachments (132,133). Novel SPIONs coated with polyethylene glycolylated bilayer was reported for synthesizing small (60-100nm) and ultra-small (20-35 nm) particles with narrow size distribution (134).

A simple method for preparing PEG-IONPs by hydrolysis was described by Kumagai et al. (135). Hydrolysis using FeCl₃·6H2O in water with addition of PEG and poly (aspartic acid) block copolymer. These particles expressed great stability and solubility in aqueous media and in physiological saline. Recently, PEG coated IONPs are mostly loaded with cancer drugs for in-vivo applications. Hałupka-Bryl et al. (136), synthesized PEG coated IONs loaded with DOX via coprecipitation method and presented their in-vivo use possibility though analysis of their physical and magnetic properties. Interestingly, Nazli et al. showed that PEG coated SPIONs can be taken by cancer cells 11 times more efficiently than bare nanoparticles. The study reveals the use of IONPs coated with integrin-targeted and matrix-metalloproteinase (MMP) which is a sensitive PEG hydrogel. The PEG hydrogel coating with functionality was designed for active targeting and intra-cellular of DOX.

2.5.2.2 Poly (vinyl pyrrolidone) (PVP)

Poly (vinyl pyrrolidone) (PVP), made from the monomer N-vinylpyrrolidone, is an an is a non-ionic, soluble, chemically inert synthetic linear polymer (137). PVP, or sometimes referred to as povidone or polyvidone, is synthesized in a range of molecular weights, from
approximately 1 to $10^3$ kg/mol by radical polymerization of vinylpyrrolidone, and are classified based on their $K$-value. PVP is used widely in pharmaceutical industry as a binder and in many cosmetics and as food additive. PVP coated SPIONs synthesized with narrow size distribution of 10-20 nm was shown to enhance the blood circulation time and increased in colloidal solution stabilization in drug delivery. It was also investigated for its effect as contest agent, where IONPs-PVP was synthesized by thermal decomposition to study its efficiency in MRI (138). The produced particles of 8–10 nm had macrophage uptake similar to Fexidex with magnetization of 110 emu/g Fe, compared to that of 70 emu/g Fe for Feridex. Huang et al. (139) studied the effect of particle size on PVP-coated iron oxide nanoparticles in the range of 30-120 nm on cellular uptake and Liver MRI. It was found that contrast enhancement and RES sequestration of PVP-IONs was strongly size dependent with particles having 37nm core and 100nm hydrodynamic size demonstrated the best enhancement. Interestingly, Lu et al. (140) investigated the dispersibility of PVP-Fe$_3$O$_4$ nanocrystaled synthesized via “one-pot” method. Ten types of aqueous solutions and organic solvents were used with varying pH values. It was shown that PVP-Fe$_3$O$_4$ is super-dispersing in all mediums forming stable colloidal mixture.

2.5.2.3 Poly (vinyl alcohol) (PVA)

PVA is a very attractive hydrophilic synthetic polymer that is extensively used in many industries including pharmaceutical industry and in biomedical research. PVA exhibits important properties such as biocompatibility, excellent film/gel forming, and adhesive properties (141). PVA coated magnetic nanoparticles synthesized with narrow size distribution of 10–50 nm was studied for In-vivo imaging and drug delivery applications (142,143). PVA impressively assisted in preventing coagulation by steric hindrance mechanism, providing colloidal stabilization, and consequently increasing mono-dispersed particles. Applying proper amount of PVA is vital in achieving the desired properties. It is reported that high amount of PVA polymer to iron mass ratio greater than 3, results in magnetic bead formation which reduces SPIONs crystallinity and affects cytotoxicity profile negatively (142,144). Mahmoudi et al. showed that the critical mass ratio was approximately 3, where the highest levels of magnetic saturation and the permeability of SPIONs was detected (90). Lower ratio values had no significant outcome on magnetic
saturation while higher than 3 ratios resulted in the formation of magnetic beads. PVA-coated SPIONs was investigated for cytotoxicity evaluation on mice fibroblast cells (L929), showed that particles with ratio r=3 coating had good biocompatibility (95% cell viability). Moreover, Petri-Fink et al. (145) studied the effect of PVA and the thiol, carboxylate, and amino-functionalized derivatives of PVA coated SPIONs on Melanoma cancer cells. The PVA-SPIONs interacted with the tumor cells and was shown to be dependent on the chemical structure of polymeric coating.

2.5.2.4 Poly (lactic-co-glycolic acid) (PLGA)
PLGA is used in a large crowd of FDA approved products for its advantages in biomedical engineering generally and in drug delivery specifically. It is a biocompatible, hydrophilic, biodegradable and low toxic copolymer. PLGA is made of two monomer, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. When degraded, these two byproducts are carbon sources for Krebs cycle or the tricarboxylic acid cycle, thus are eliminated safely from the body in the form of water and carbon dioxide (146). PLGA crystallinity varies strongly from fully amorphous to fully crystalline depending on LA/GA monomer ratio. Subsequently, PLGA affords a controlled drug release profile when coated on SPIONs as degree of degradation can be adjusted though its crystallinity (147). Iron oxide coated the PLGA nanoparticles are synthesized by emulsification–diffusion procedure in most reported literature (148–150).

PLGA is the most used polymer coating to improve the labeling efficiency of SPIONs to cancer cells (151). Hajikarimi et al. (151) investigated the uptake and cytotoxic effects of PLGA-coated-IONs as a drug carrier of 5-fluorouracil (5-FU) on prostate cancer cells monolayer culture. Cells where treated with free 5-FU as a control and 5- FU loaded nanoparticles with combination of X-ray radiation. It was found that these carriers were excellent in penetrating into the cell with effective drug delivery, as reduction in colony number was significantly higher in 5-FU-loaded IONs. Also, PLGA encapsulated Oleic acid coated magnetite was synthesized for the purpose of studying its toxicity effect on MCF-7 cells after loading them with DOX, an anti-cancer drug. Cell death was drastically higher in DOX loaded nanoparticles compared to drug free ones, indicating that these DOX-PLGA-MNP are appropriate for targeted drug delivery purposes. Cheng et al. (152) prepared various
sizes with narrow distribution of iron oxide PLGA-coated NPs via nanoprecipitation method. These particles were fluorescein isothiocyanate encapsulated and conjugated with quantum dots. They showed a controlled release pattern and yielded a high efficiency in relaxivities, demonstrating their effectiveness as contrast agents. Also, Hwang et al. (153) examined their detection as contrast agents in MRI and optical imaging systems after associating them with transplanted pancreatic islets in-vivo. PGAL-IONs containing cyanide dye were successful in labeling the transplanted islets in both imaging systems, where docyanine green fluorescence appeared after 2 and 4 days of transplantation in optical imaging and scans were monitored in-vivo MR after 4 weeks. Figure 4 shows the chemical structures of main natural and synthetic polymers used in SPIONs coating.

<table>
<thead>
<tr>
<th>Chitosan</th>
<th>Dextran</th>
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<tr>
<td>Poly (vinyl pyrrolidone) (PVP)</td>
<td>Polyethylene glycol (PEG)</td>
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2.6 MNPs in drug delivery and therapeutic platforms

2.6.1 Chemotherapeutics agents
Cancer is one of the major medical research challenges and most difficult to treat because of difficulty in timely diagnoses and limited treatment options. The treatment options available can be very devastating to human body (surgery, chemotherapy and radiation) and have enormous adverse side effects. There has been extensive research done to find innovative ways to solve cancer treatment limitations. Most chemotherapy remedies rely on cytotoxic and cytostatic effects with no specific cell-targeting capabilities (54). Fortunately, MNPs have the capability to be engineered for target-specific cell internalization carrying chemotherapeutic payload and the subsequent drug release to cell cytoplasm for desired actions to be carried out. Studied mechanisms on magnetic nanoparticles uptake by the cells include internalization by caveolae structures (154) and receptor-mediated endocytosis (155). As previously stated; particles shape, size, surface properties affect cellular uptake efficiency drastically. Also, permeation enhancers can be attached to MNPs to ease its delivery to cytoplasm (156). Koch et al. (157) showed the effective use of tat-CLIO nanoparticle in cell tracking in drug delivery applications. They demonstrated rapid internalization, 100% labeling cells in less than an hour, and slow excretion of nanoparticles, in a period longer than 144 h from the adopted cytoplasm. Upon MNPs merging into targeted cells, the challenge of drug release arises.

Nanoparticles must be able to deliver desired chemotherapeutic to subcellular organelles such as the nucleolus and mitochondria before being ejected from the system by lysosomes. Strategies for release includes enzymatic cleavage, physiological conditions alteration such as

\[ \text{Poly (vinyl alcohol) (PVA)} \]
\[ \text{Poly (lactic-co-glycolic acid) (PLGA)} \]

**Figure 2-5: chemical structures of the main natural and synthetic polymers used in SPIONs coating**
change in pH, temperature and osmolality, or “proton sponge” effect which is the incorporation of cationic polymers which produce osmotic swelling (73,158). There are many cancer drug formulations associated with MNPs such as doxorubicin, paclitaxel and methotrexate (MTX), epirubicin (206,209,221). Incorporating these drugs into nanoparticles have been explored extensively to be loaded and protected until reaching cancer cells, where cytotoxic drug molecules will deliver its therapeutic effect. Most hydrophobic drugs are loaded via physical adsorption, in which encapsulation are done within MNPs coating restricting non-specific cell interface (54). This technique can be applied to drugs causing severe damage to non-targeted cells. On the other hand, other cancer drugs that hold affinity to aimed cells through presence of chemically active functional group, MTX for example; it is directly attached to surface of MNPs through mild chemistry or strategic crosslinking. MTX loaded into polymeric IONPs were studied in many trials. Amine terminated PEGylated saline was used to modify IONPs, and MTX was consequently loaded via EDC/MHS mediated coupling as reported by Kohlar et al. (159). The particles were examined in glioma cell uptake in-vitro. Proceeding to the cells internalization, MTX was detached due to presence of proteases and low pH in the lysosome. The unique superior magnetic property makes MNPs responsive, aggregately and easily controlled once an external magnetic field is applied. This method or so called Magnetic drug targeting (MDT) is advantageous for transporting cancer drug-loaded MNPs to the area of interest at the desired rate (160,161). Many studies have been performed to investigate the concentration of MNPs upon addition of external field in-vivo. Lubbe et al. (162) conducted a study on rats where epirubicin loaded 100 nm IONPs coated with polymeric anhydroglucose partially functionalized, were injected into their femoral vein (estimated to 0.5% of rat blood volume). Upon magnetic field influence of 0.2 T for 5, 10 and 30 minutes, irreversible thrombus was observed after 10 minutes with display of microcirculation in the capillary bed. This demonstrates the ability of loaded SPIONs for use in mechanical tumor embolization. Similar experiments were conducted with same observations (163). In a different study also prepared by Lubbe et al., full tumor regression was achieved of starch coated SPIONs loaded with epirubicin for severely metastasizing adenocarcinomas and hypernephroma implanted in mice (164). Encouragingly, human clinical trials have also been reported. Using the same loaded anhydroglucose-SPIONs from Lubbe et al., seven breast cancer patients in the fourth stage were injected with the same
anhydroglucose-SPIONs. Vain injection was positioned contralateral to the tumor and the magnetic field placed on the tumor was applied for 45-105 minutes, and successful regression and direction to tumor site was reported. No apparent clinical symptoms were observed except for skin discoloration in both animal and human trials which appeared 7-14 days latter (160,162). Doxorubicin release on-demand \textit{in-vivo} was assessed by Lee et al. (165). Significant tumor volume suppression was shown through injection of magneto-thermally responsive nanoparticle system after 15 days of treatment. Comparably, trials with groups given double injections of Dox\_NPs exhibited continued and improved tumor growth inhibition. Interestingly, however, studies have shown that application of external stimuli in drug release, magnetic field in this case, results in a drug release reduction because of aggregation of the magnetic nano-hybrid particles (166,167).

Combination of more than one cancer therapeutic agents loaded nanoparticles to multiple targets has also been examined. A novel approach of sequential release of two drugs endocrine and chemotherapy, tamoxifen and diosgenin respectively, in advanced breast cancer cells was recently evaluated by Kumar et al. (34). A multi-layered polyvinyl alcohol hollow manganese ferrite nanocarriers were designed and synthesized with very unique features: having sensitivity towards tumor acidic milieu, compact, high encapsulation efficiency and monodispersed. Mediating drug(s)-induced cell apoptosis \textit{in-vitro and in-vivo}, it was shown that tumor suppressor protein P53 was stimulated while antiapoptotic Bcl2 protein level was decreased as confirmed by protein profiling, immunohistochemical, and mitochondrial membrane potential investigations. This result shows the success of multi-drug nanoparticle delivery system and may provide promising treatment approach for advanced cancer. Fang et al. (168) highlights the effectiveness of using dual drug-delivery using nanocapsules. 100-150 nm core-shell nanostructures made of PVA, polyvinyl alcohol (PVA), polyacrylic acid (PAA) and iron oxide and loaded with doxorubicin and curcumin were designed. High accumulation at brain tumor cells in bearing mice was observed after intravenous administration and magnetic field application at targeted site. Impressively, cancer growth suppressing \textit{in vivo} was more efficient than the delivery of either drug alone.
2.6.2 Hyperthermia treatment

As explained earlier, the advantage of superparamagnetic in contrast with ferromagnets nanoparticles is the absence of magnetic remanence after application of magnetic field, in which problems with particles aggregation is prevented (169). However, the magnetic dynamics of remanence recovery is relatively slow. Applying a high frequency alternating magnetic field can better control it resulting in release of energy in a form of heat, termed as magnetic hyperthermia. Extensively studied lately, this effect provides a promising cancer therapy through raising cells temperature abnormally (41–45 °C) (170). The damage of normal cells in this temperature range is reversible whereas it is irreversible to cancer cells (171). The detection of the effect of heat generation on cancer cells was first studied in the 19\textsuperscript{th} century (172), where administration of living bacteria to cancer patients was carried out causing an infection associated with a fever. It was observed that fractional tumor regression has occurred, but also neighboring healthy tissue was effected significantly. Enormous studies have been done in attempt to find alternative mechanism to control heat generation (104,173,174). Magnetic fluid hyperthermia (MFH) is a possible solution to that problem, where it is based on colloidal suspension MNPs injection at tumor cells through passive or active targeting, then MNPs are used as mediators of hyperthermia for localized treatment (175).

MFH opens many doors to new cancer therapy that is non-toxic, non-invasive with relatively no damage to normal cells with high efficiency of killing cancer cells by heat diffusion. Additionally, MNPs used in Hyperthermia can be engineered to target tumor sites via specific agents or antibodies and can also serve for diagnostic purposes in MRI before heating to detect particles distribution (104). Recent trials in mice models demonstrated the success of MFH in many types of cancers such as brain cancer (176) and pancreatic tumors (177). Furthermore, combination of MFH and targeting ligands maximized tumor targeting and improved treatment efficacy. Ito et al. (178) used Anti-HER2 antibody functionalized immunoliposomes containing magnetite nanoparticles with hyperthermia therapy at 42.5°C. This system showed great potential in treating HER2-overexpressing cancer. In another study by Tsiapa et al. (179), peptide RGD derivate (cRGDfK-Orn3-CGG) coated MNPs was used accompanying heat therapy of approximately 39°C. High targeting ability in U87MG
glioblastoma tumor-bearing mice was observed while accumulation of MNPs in neighboring organs was negligible. Tumor volume reduction was confirmed by dissection of tumor following hyperthermia treatment.

2.6.3 Radio-therapeutic agent

Radionuclides, mainly β-emitters, have localized decay in target cells, which promotes DNA to damage free radicals that result in apoptosis, hence, they can function as cancer therapeutic agents. Recently, SPIONs and some other nanocarriers were recognized as radionuclides (180). Similar to chemotherapeutics, there are no clear guidelines on targeting strategy in place yet for radio-therapeutics to direct them away from healthy cells towards cancer cells. Unlike chemotherapeutics, however, engineering SPIONs in radiotherapy is uniquely challenging since radionuclides are continuously decaying making it very difficult to avoid healthy cells. Thus, even after cell uptake, SPION-radionuclide complex has to be kept intact throughout radiation decay to avoid interaction with non-targeting cells. The most used radioactive isotope to develop radionuclide-SPIONs is $^{188}$Re with half-life of 17 hours, which showed ability to induce cell death in specific targeted liver cells in vivo (181–183).

There are several studies present that address the combination of radiation and hyperthermia (184,185). The higher the formation of free radicals to DNA damage ratio, the better efficacy of radiation therapy. When Hyperthermia is added, it increases local oxygen levels due to increase in blood supply to tumor cells. This causes more formation of free radicals resulting in enhancing radiation therapy. In one clinical study (186), 59 patients with recurring glioblastoma were injected with IONPs followed by 30 of heating at 43 °C. A radiation dose of 30 Gy was then given immediately after or before hyperthermia therapy. The combination of therapy raised the median overall survival rate from 14.6 to 23.2 months since the primary cancer diagnosis and 7.2 month from recurrence date. The increase in survival is believed to be due to the duel thermo-radiation therapy.

2.6.4 Gene Therapy and Magnetofection (MF)

Gene Therapy is the use of exogenous DNA to correct genetic defects or to produce proteins/peptides that enhance the immune system (73). Antisense RNA can also be used in gene therapy for expression silencing of defective genes (54). This major advancement in
modern medicine can be applied in wide range of applications including but not limited to: genetic disorders, cardiovascular disease, cancers or neuro-degenerated disease. Development of effective gene delivery system that distribute plasmid DNA and insert it in specific DNA sites is the curial aspect determining gene therapy success (187). Regardless of the substantial research conducted, no ideal effects of gene therapy are present due to many challenges. Transfection efficiency is greatly lacking in terms of targeting desired sites with no toxic or hostile side effects due to lack of specificity, genes short life time in vivo and poor diffusion across cell membrane (188,189). Using MNPs as carriers for antisense oligodeoxynucleotides (ODNs), termed as Magnetofection, may minimize many of these problems. MNPs and gene therapeutics have been integrated as a gene vectors to protect the nucleic acids from enzymatic degradation and enable endosomal release after cellular internalization (190). There are many advantages of MNPs-based transfection including low vector dose to achieve high transfection yield, potential use as viral and non-viral vectors, ability to attain high efficiency and short incubation time transfection/transduction, ability to deliver genes to non-permissive cells and the possibility of precise targeting in-vivo (73,191). Particularly, polymeric MNPs have an advantage since polymers, such as PEI and chitosan, can enrich endosomal release via acidification induction of endosomal vesicles (192). The efficiency of this system has been proven in vitro and still under studies for in vivo applications. Gene vector attached to PEI coated SPIONs, named tranMAG\textsuperscript{PEI} was tested in vitro were a strong magnet was placed beneath the cell monolayer. After 10 minutes of incubation, peak transfection levels were observed, in comparison to 2-3 hours of incubation required to other vectors such as cationic lipids. High concentration of MNPs vectors at the surface results in noticeably improved dose-response profile (193). Pan et al. (194) develop a dendrimer-modified MNPs for delivering antisense survivin oligodeoxynucleotide to liver and breast cancer cells. Results indicated that the particles were successfully delivered within 15 minutes among cell growth inhibition in time and dose dependent manner. Other magnetofection studies on liver cancer cells showed also similar results (195,196).

2.6.5 Peptides/antibodies therapeutics
As MNPs facilitate as drug molecules carrier, so as it can be carrier of peptides or anti-bodies. These biotherapeutics serve as a great potential for therapeutic agents due to their ability to manipulate cell-specific desired mechanism. They control their remedial effects through
stimulation or inhibition of particular cell activity involving immune system stimulation, activation of apoptosis, or function blocking such as angiogenesis, cell adhesion prevention, interfering with protease/kinase action and others (197). Therefore, engineered MNPs must have appropriate size, reported as 25–50 nm hydrodynamic size (198), and coating, such as PEI which enables internalization for example, in order fit with most biological pathways. RGD (Arg-Gly-Asp) is a frequently used peptide with SPIONs. It contains the receptor integrin αvβ3 in which tumor blood vessels and some melanoma cells has over-expressed amount of it (199). Recently, chlorotoxin (CTX) peptide is examined due to its high affinity to many cancer types. In addition to being targeting agent, it suppresses tumor growth through inhibition of cell invasion mechanism. Veiseh et al. (200), studied the potency of CTX peptide on SPIONs conjugated with an amine-functionalized poly (ethylene glycol) silane. CTX has high attraction to lipid raft-anchored that includes both membrane-bound matrix metalloproteinase 2 (MMP-2) and chloride ion channels, which are important elements in glioma cancer survival and cell invasion. Through cells receptor-mediator endocytosis, MNPs has greatly enhanced cellular uptake, thus prohibiting targeted cells ability to affect neighboring healthy tissue. SPION-CTX had an improved internalization and invasion inhibition rate up to 98%, compared to free CTX particles of 45% only. Moreover, antibodies had also shown great effect with SPIONs as targeting agents. Their efficiency is proven since most of the commercially available magnetic nanoparticle systems are labeled with antibodies against bacteria, epitopes of cells or other antigens (201). One example is Herceptin, marketed as Trastuzumab, is incorporated with SPIONs targeting HER2/neu receptor, which is responsible for cell proliferation and serves in cell growth. Herceptin™-magnetite nanoparticle were proven to anti-proliferate breast cancer cells (202,203). Furthermore, other studies have collaborated anti-HER2 antibody therapy with hyperthermia using IONPs had a much higher therapy efficiency with increasing cytotoxic effect (202,204).

2.6.6 Hybrid magnetic nanoparticles for mitochondrial targeted anticancer drug delivery

The mitochondrion plays an important role in controlling the translocation of pro-apoptotic proteins to the cytosol. In other words, mitochondria have a direct impact on the non-apoptotic cell death (205). There is increasing evidence about the correlation between mitochondrial
dysfunction and a variety of neurodegenerative diseases, diabetes and cancer. Therefore, mitochondrial targeted compounds represent a promising approach in cancer treatment.

One of the major differences between the normal and malignant cells is the presence of negative potential (20-60 mV) on the inner mitochondrial membrane. However, difficulties remain in mitochondrial-targeted therapy due to the possible degradation of drugs before getting access to its target points. So far, there are only few publications on the successful mitochondrial targeted drug delivery systems (206,207). Surface modified chitosan has drawn great interests for the development of mitochondrial targeted drug delivery systems. Chen et al. (208) have investigated two chitosan derivatives (N-glycyrrhetinic acid-polyethylene glycol (PEG)-chitosan (NGPC) and N-quaternary ammonium-chitosan) as possible drug carriers for targeting the mitochondrial inner membrane. The presence of quaternary amine group in N-quaternary ammonium chitosan and PEG in the NGPC helped in pH cleavage of the surface modified chitosan. Smart Fe₃O₄-quaternary ammonium chitosan or Fe₃O₄-NGPC hybrid nanoparticles have shown a good promise in targeting cancer cells because of the endosomal escape and mitochondrial targeting. Typically, magnetic nanoparticles approach the tumor site by the effect of the tumor targeting N-glycyrrhetinic acid. Following the step of endocytosis, the pH cleavable functional group creates a strong positive potential on the surface of magnetic nanoparticles; which facilitates the proton influx to endolysosomes. The increased proton influx leads to endolysosomal bursting, thus creates favorable conditions for magnetic nanoparticles to escape back to the cytoplasm. The positive charge on the surface of magnetic nanoparticles facilitates their attachment into the inner mitochondrial membrane and results in an efficient drug targeting.

2.7 Routes of administration

As stated earlier, the fate of MNPs and its bio-distribution are dependent on countless factors such as size, protein adsorption, surface characteristics, stability, molecular weight, drug loading and release kinetics. Nevertheless, their destiny, and possible toxicity effect, is strongly reliant on the dose and route of administration. This section will discuss briefly the most commonly used ways of administrations for MNPs.
2.7.1 Parenteral administration

Generally for parenteral applications, the nanoparticles must meet three conditions: i) non-toxic, ii) suitable size to be delivered to target site and iii) non-immunogenic. Once MNPs reach the bloodstream, filtrations start via RES system response. Large particles will be cleared out by cells capable of phagocytosis (dendritic or macrophages cells), and small particles are removed by cells capable of endocytosis (B and T lymphocytes), while biodegradable MNPs are taken by any type of cells via pinocytosis. Particles smaller than 4 microns are cleared mostly in liver (60 - 90%) and spleen (3-10%). Particles 200nm and larger are filtered by spleen, whereas particles 100nm and smaller are phagocytised by liver cell (209). Parenteral administration can be performed by intravenous, subcutaneous or intra-arterial (Carotid artery) administration. Recent study demonstrated that magnetic targeting for tumor via intravenous administration is effective by passive and active targeting (210). Intra-arterial have the advantage in that the vasculature of tissue receives higher plasma concentration, especially under low blood flow rate condition and is proven through mathematical analysis (211, 212). Intra-arterial administration was tested for chemotherapy agent delivery for brain tumor and it validated higher tumor exposure for fast eliminating drugs (213, 214). Subcutaneous administration, on the other hand, is a local injection of colloidal carriers of 60 nm or smaller (215). They infiltrate around the injection site and then are absorbed gradually by lymphatic capillary system (216). It is used as a tool for lymphatic tumors but not as commonly studied because it is not a suitable method of administration for metastatic tumors.

2.7.2 Oral administration

Oral delivery demonstrates one of the easiest routes for patients for its painless, non-invasive and ability of self-administration (217). However, the main problem of this route is that nanocarriers surface modification, peptides and proteins, degrade in the gastrointestinal acid, which leads to low or no absorption and burst release of drug initially (218). Thus, MNPs must be engineered in a way to have mucoadhesive features. NPs coated with chitosan have shown to increase the retention time in the gastrointestinal tract (GIT) and improve therapeutic efficiency (219). Typically, the smaller the particles size the higher the mucosal interaction and the more the half-life and drug bioavailability. Feng et al. (220) explained the fate of chemotherapeutical NPs in oral administration. Study states that particles smaller than 5 µm
are removed by lymphatic drainage, particles 500-50 nm are capable of crossing the epithelial cells and particles 50 nm or less can pass through intestinal epithelial cells.

2.7.3 Inhalatory administration

Airway is a great route in providing large absorption area for treatment of many pulmonary diseases (asthma) and non-pulmonary diseases (i.e. infectious diseases) with low systematic delivery and contrary effects (221). Various drug categories such as proteins, hormones and peptides display an enhanced therapeutic efficiency when are pulmonary administered (222). It provides the ability to maximize deposition level and the dose administered and offers a much more compliance to patients since it is non-invasiveness drug delivery and can allocate both local and systemic therapy (223). Pharmaceutical Products for inhalation purposes have specific features that must be met in order to penetrate deeply in the lungs (224). This includes specific mean aerodynamic diameter, low size distribution, high drug release efficiency, permeability, effective adhesion to lining mucosa and high permeability (225). Polymeric micro- and nanoparticles delivered though pulmonary track showed to improve therapeutic index of drug though modification of its bioavailability, in which both drug absorption rate and drug metabolism reduction is achieved (223).

**Table 2-2: the main clinically approved SPIONs in drug delivery.** Data collected from (15,226–230]
Most of MNPs in trials and approved nanoparticles are focused towards cancer nano-therapy due to physical and chemical magnetic property which allow use as therapeutic vehicle and contrast agents (226). Current work toward clinically approved magnetic nanotherapeutics aims
to develop personalized medicine with real time monitoring of biological response to the
given therapy (226). Most clinical trials performed on MNPs and/or approved ones are SPION
are based on inorganic iron oxide cores coated with hydrophilic polymers (15). Some serve as
bowel contrast agent such as Gastromark and Lumiren; others are for liver and spleen imaging
such as Endorem and Feridex; and Lymph node metastases detection such as Combidex
(15,226). To date, 5 SPIONs based contrast agents are approved clinically (Table 2-2).

Ferumoxytol is the most recent FDA approved for the treatment of iron deficiency anemia
(IDA), used as iron replacement for adults with chronic kidney disease (CKD) (15). Currently,
14 out of 23 cases of SPION FDA approved clinical underway trials uses Ferumoxytol, ten of
these cases uses Ferumoxytol as MRI contrast agent in early stage tumor, lymph node cancer
metastasis, multiple sclerosis and for cardiovascular diseases applications; while the other four
are in phase IV for DIA (231). Ferumoxytol is more effective than conventional MRI, which
doesn’t provide detail information on the tumor margin of aggressive tumors such as
pancreatic carcinoma. Patients having Ferumoxytol-enhanced scans can offer better primary
tumor allocation and assist in achieving tumor-free margin during surgery of pancreatic
carcinomas (232). Ferumoxides, on the other hand, is used for detection of liver lesions and
had been communalized since approval. However, it was withdrawn from the market by the
manufacturer company, AMAG Pharma, due to its limited use by radiologists in 2008, yet, its
application in active cell MRI tracking is still documented (233). Presently, four FDA
approved trials with Endorem for tracking inflammation cell or monocyte, and a phase II stage
study using Feridex for tracking bone marrow derived stromal cells (MSC) in severe cases in
adults (234). Ferucarbotran is an organ-specific contrast agent for small liver lesions and was
shown to be very safe in clinical trials (15). Due to the trials successes, further investigations
on Ferucarbotran derivative, Supravist, are conducted by Bayer Schering and are now at phase
III study to be used as a positive improving blood pool agent (226). Furthermore, Ferumoxsil
and Ferristene are oral admiration SPIONs imaging agents coated with silicone and
polystyrene, respectively. They are applied in gastrointestinal to differentiate between bowel
loops and other abdominal organs as it darkens the bowel when ingested (226). Nevertheless,
these two agents were eliminated from the market as they suffered from negative profit
although they are safe and effective. In addition, Ferumoxtran-10/Combidex® and
Clariscan™ are under clinical trials with promising results especially Ferumoxtran-10. It is approved in some European countries but still at testing phase for detection of brain and pancreatic tumors and metastatic disease in lymph nodes in the USA (227). Other products that are present in the market are CellSearch® and NanoDX™, both of which are FDA approved used for magnetic detection of cells in vitro (226). CellSearch® (Veridex LLC/Johnson & Johnson) is a diagnostic device that uses SPION-bound antibodies to capture and then quantify the circulating colorectal, breast or prostate cancer cells in blood samples (235). On the other hand, it is worth to note that polymer micelle based SPION are also greatly expanding in research towards clinical approval. They are composed of four components: i) hydrophilic shell surface, ii) hydrophobic SPION crystallites with hydrophobic polymer segments, iii) therapeutic drug or gene agents, and iv) targeting ligands on shell. However, micelles are beyond the scope of this review.

2.9 Industrial applications and scale up

Since the extent of SPIONs applications in nanomedicine is increasing and already existent in clinical use, larger amounts and higher production rate at reasonable prices is indispensable. Scale up does not only requires production process to be safe, cost effective, and simple, but also requires the nanoparticles to be dispersible in water, biocompatible and holds the desired physiochemical properties. Attempts for scale up synthesis of SPIONs were reported. Kolen’ko et al. worked on SPIONs scale up via co-precipitation method using iron precursors of FeCl₃·4H₂O and FeCl₃·6H₂O (236). Although yield was considered on the low side (approximately 68%) with poor mono-dispersity of nanoparticles, these particles did have good particle size (20 nm), exhibited superparamagnetic behavior with high magnetization (up to 84 emu/g), and its performance in magnetic hyperthermia was excellent. Also, Scale-up synthesis of MNPs using thermal decomposition method of iron (III) acetylacetonate in 1-octadecene was studied by Ibarra-Sánchez et al. (237). The produced nanoparticles were shown to be sensitive to stirring rate, reaction temperature and stirring time. Although these parameters could be optimized to obtain desired nanoparticles size and size distribution, the produced nanoparticles are hydrophobic and need ligand exchange procedure prior to being applicable in-vivo. Gonzalez-Moragas et al. (238) produced SPIONs with well-established magnetic properties and colloidal stability by microwave-assisted reaction. With a yield of
around 80%, 3 grams of SPIONs were approximately gathered compared to the average 22 mg per batch in normal lab-scale procedure. Interestingly, From a 30 L of bacterial fermentation process, over 1 kg of good mono-dispersited Zn-substituted magnetite was recovered by moon et al. (239) Microbial process possesses high yield, reproducibility at relatively low cost and energy, However, the production rate is much slower compared to most of synthesis methods (up to several weeks) which maybe inconvenient at the industrial level. Overall, steps toward the scale-up of MNPs synthesis using different production procedures is improved. However, all have limitations and future investigation in that field is desperately needed.

2.10 Mathematical models of drug release from hybrid polymeric-magnetic nanoparticles

A variety of techniques are used in the drug loading into SPIONs: i) the drug molecules can be loaded onto the polymeric matrix surrounding SPIONs, ii) encapsulated into a core-shell stimuli responsive hydrogel, iii) Covalently bonded to an activated surface SPIONs and iv) trapped in magneto-liposomes. Figure 2-6 graphically illustrates the different alternatives for drug encapsulation into SPOINs.

The main objective of drug release control in nanocarriers is to preserve drug concentration in blood and/or target site at the effective level. Release kinetics aims to maintain the release profile at the therapeutic window via achieving the balance between the minimum effective concentration (MEC) and the minimum toxic concentration (MTC). At initial administration of a single large dose, drug level is elevated above the MTC resulting in toxic side effects then it rapidly drops below the MEC. This gives very minimal timing to have the dosing at the effective operational levels. Within a certain interval, multiple dosing can decrease these fluctuations, but it proposes many incompliance concerns to the patient (240). Thus, it is of great importance to achieve optimal design of new system by developing carriers having sustained release profiles with low dosing frequency.
Mathematical modeling aids in predicting the temporal release of molecules through understanding the release mechanisms and ensuring proper system design. Drug release mechanisms can be classified into four categories: i) diffusion-controlled, ii) chemically controlled, iii) solvent-controlled, and iv) stimuli-controlled release (240,241). Most frequently, models are built according to the diffusion mechanism (241), which is demonstrated as a reservoir system in which the drug is dispersed in a core surrounded by polymeric membrane (225). When no membrane is present, a matrix type system is followed where diffusion mechanism can also apply with high initial release followed by slower rate (243). Fick's law of diffusion describes the drug transport and can be used as the bases for diffusion models (Eq. 2-2 and 2-3) (244).

Figure 2-6: Graphical illustration of the various techniques of drug encapsulation in magnetic nanoparticles for targeted magnetic delivery: a) loaded into polymeric matrix surrounding SPIONs, b) trapped in magneto-liposomes, c) Encapsulated into a core-shell stimuli responsive hydrogel, and d) Covalently bonded to an activated surface SPIONs.
\[ j_i = -D_{ip} \frac{dc_i}{dx} \]  
\[ \frac{\partial c_i}{\partial t} = D_{ip} \frac{\partial^2 c_i}{\partial x^2} \]  
(Eq. 2-2)  
(Eq. 2-3)

Where, \( c_i \) is the concentration, \( j_i \) is the mass flux of species \( i \). \( D_{ip} \) represent the diffusion coefficient of species \( i \) in the polymer, \( t \) and \( x \) symbolize time and position, respectively (244). These above equations represent a planar geometry of one-dimensional diffusion. Similar equations are also available for thick slabs, cylinders, and spheres (243,244).

Chemically controlled mechanism includes both erodible and pendant chain systems. Erodible system is controlled by polymer degradation such as PLGA, PLA, and polycaprolactone (PCL). These polymers display simultaneous degradation of entire matrix or could erode from surface through core as in the case of polymers made of poly (orthoesters) and polyanhydrides (bulk versus surface degradation). It must be noted that in nano-matrices such as MNPs, the domain size of crystallization is restricted and water diffusion distance is limited, thus polymer degradation is accelerated (245). On the other hand, in pendant chain systems, the release is controlled by the enzymatic and hydrolytic degradation of chemical bonds between drug molecules and polymeric carrier (241). Biodegradable polymers such as polyesters, poly (amino acids), polyamides and polysaccharides discharge drug by degradation of bonds (amide, hydrazine and ester) in their backbones (211,212).

The solvent-controlled release consists of two types: osmosis-controlled and swelling-controlled release (243). Osmosis-controlled release usually occurs with highly soluble drugs that are enclosed by semi-permeable membrane. This mechanism is similar to diffusion except that water flows from outside the polymeric carrier to the core (low to high drug concentration) causing a build-up of osmotic pressure, which results in rupture of the system, and consequently drug release is accomplished (240). At constant concentration gradient, this mechanism illustrates zero-order release (205), and the models are anticipated from an irreversible thermodynamics and the Kedem–Katchalsky analysis (249). The rate of drug release can be written as:
\[
\frac{dM}{dt} = \frac{A}{\delta} L_p \sigma c \Delta \pi s
\]  
(Eq. 2-4)

Where, \( A \) is the cross sectional area, \( \delta \) thickness of the device, \( L_p \), a permeability coefficient, \( \sigma \), a reflection coefficient, \( c \), drug concentration, and \( \Delta \pi s \) is the osmotic pressure of water. The second type of solvent control release, swelling-controlled, occurs when glassy hydrophilic polymer systems are surrounded with aqueous solution. Movement of water leads to volume expansion. Two moving interfaces are accompanied in the expansion: swelling interface moving inwards and polymer interface moving outwards via the contacting water (241). Both diffusion and chain relaxation rate of polymers determine the degree of release (Fickian or non-Fickian diffusion). This system is indicated as Stefan or moving boundary problem, where diffusion (Eq. 2-3) can be solved with moving boundary conditions at two fronts. Refer to (241,250,251) for further details on polymer swelling-controlled modeling.

The Stimuli-controlled drug delivery system uses stimulus or pathological changes occurring in target site as triggers. Stimuli could be either intrinsic - local within the targeted area such as changes in pH, up-regulated enzymes or redox potential; or extrinsic-induced externally at desired region such as temperature, magnetic field, ultrasonic waves, or light (252,253). These carriers are known for target-specific drug delivery. Solid tumour tissues, for instance, exhibit weaker acidic pH and higher redox potential than normal tissues (71,254) pH-sensitive linkers where developed as a result for more accurate controlled release.

Although one mechanism may be more dominant to a specific nanocarriers system, multiple mechanisms may contribute to the drug release. The relationship between geometry on release patterns and drug dissolution is the key in shaping the mathematical Model (207). Enormous models have been developed and applied to describe the release mechanism of various nano vehicles systems. For polymeric coated MNPs, five main models will be discussed which are believed to be most applicable from literature.
2.10.1 Korsmeyer–Peppas model
Korsmeyer et al. derived a relationship describing drug release from a polymeric system (Eq. 2-5) (256).

\[
\frac{M_t}{M_\infty} = Kt^n
\]  

(Eq. 2-5)

Where, \( \frac{M_t}{M_\infty} \) is a fraction of drug released at time \( t \), \( K \) is the release rate constant which incorporate the geometric and the structural characteristics of the drug form, \( n \) is the release exponent. The model was widely accepted due to its simplicity and applicability to different release kinetics. It is used when release mechanism is not fully known or if multiple types of mechanisms are involved in the system (257). Drug Release data were fitted to the Korsmeyer-Peppas model to observe the fitted mechanism of the system and the release exponent became the indicative of system mechanism for cylindrical shaped matrices as described in Table 2-3.

**Table 2-3: Diffusional release mechanisms interpreted from polymeric films according to exponent of release (255)**

<table>
<thead>
<tr>
<th>Exponent of release (n)</th>
<th>Mechanism of drug transport</th>
<th>Rate as a function of time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Fickian diffusion</td>
<td>( t^{-0.5} )</td>
</tr>
<tr>
<td>0.45 &lt; n = 0.89</td>
<td>Non-Fickian drug transport</td>
<td>( t^{n-1} )</td>
</tr>
<tr>
<td>n = 0.89</td>
<td>Case II transport release</td>
<td></td>
</tr>
<tr>
<td>n &gt; 0.89</td>
<td>Super case II transport</td>
<td>( t^{n-1} )</td>
</tr>
</tbody>
</table>

2.10.2 The Huguchi model
The first mathematical model designed to explain drug release from a matrix system came into existence in 1961 by Huguchi (258). It’s a geometry dependent model initially used to simply fit release data, then the so-called Huguchi equation expanded to include different geometrics and porous systems (259,260). The classical model expression (Eq. 2-6) is obtained with the following assumptions (241,255): i) drug solubility is much less than the initial drug concentration which allow using pseudo steady state approach, ii) the drug diffusion is one
dimensional, meaning edge effects are negligible, iii) particles size of drug is much smaller than the system’s thickness, iv) the polymer swelling and/or dissolution is neglected, v) coefficient of drug diffusion is constant, and lastly vi) perfect sink condition exist and are maintained in the release environment.

\[ Q = A \sqrt{D C_s (2C_0 - C_s)} t \]  \hspace{1cm} (Eq. 2-6)

Where, \( Q \) is cumulative amount of drug released at time \( t \) per unit of surface area \( A \), \( D \) is the drug diffusion coefficient (diffusivity of drug in matrix), \( C_0 \) and \( C_s \) are the initial concentration and the solubility of drug in polymer matrix, respectively. Although equation 5 cannot be used for most controlled drug release systems, it can be used to analyze the release profiles to provide conclusion about the mechanism and it can sometimes be modified accordingly. For instance, when the cumulative depletion of drug in the system is reached (voiding first assumption with drug solubility being higher than its concentration), and for planner matrix with release occurring through pores in the system, the dissolution rate can be studied via the adjusted Huguchi expression (Eq. 2-7) (255)

\[ Q = A \sqrt{\frac{D \delta}{\tau}} C_s (2C_0 - \delta C_s) t \]  \hspace{1cm} (Eq. 2-7)

Here, \( \delta \) represents porosity of the matrix; \( \tau \) is the tortuosity, while \( Q \), \( A \), \( C_s \), and \( C_0 \) denote the same meaning described above. The Huguchi model and its adjusted forms can be used to describe to many types of pharmaceutical dosage forms such as matrix tablets with water-soluble drugs and transdermal systems (259).

2.10.3 Hixson–Crowell model

Hixson-Crowell model describes the release from a system in which surface area and diameter of the matrix is changing (261). The tablet or the particle’s surface area is proportional to the cube root of its volume assuming uniformly sized particles as recognized by Hixson and Corwell in 1931. Their derived equation is as follows:

\[ \frac{1}{3} W_o^\frac{1}{3} - W_t^\frac{1}{3} = \kappa t \]  \hspace{1cm} (Eq. 2-8)
Where, \( W_0 \) and \( W_t \) are the initial and the remaining amount of drug in the pharmaceutical dosage form at time \( t \), respectively. \( \kappa \) is the rate constant for Hixson-Crowell rate containing the surface-volume relation (255,261,262). After dividing by \( W_0^{1/3} \), \( F=1–(W_t/W_0) \) represents the drug dissolved fraction at time \( t \) and \( k \) is the release constant. Hixson-Crowell model can be applied to dosage forms where the dissolution happens in planes that are parallel to the drug surface in such a way that the initial geometry is constant throughout process time (263).

2.10.4 First order model

Noyes and Whitney first proposed this model in 1897 (264), as the following equation:

\[
\frac{dc}{dt} = K(C_s - Ct) \tag{Eq. 2-9}
\]

It describes the absorption and elimination of drug, where \( dc/dt \) represent the rate of change in drug concentration, \( k \) is the rate constant applied to the concentration gradient \( (C_s – Ct) \) between the liquid layer close to the solid membrane and the surrounding bulk liquid (262). This equation as explained by Noyes and Whitney have the concept similar to the diffusion model in the case that there is no change in of the solid shape. Meaning the surface area are constant throughout the dissolution process. This might be not the case for degraded polymer surfaces. As the size of the particles decrease to nano-range, polymer degradation becomes less significant in release mechanism due to the short diffusional path (265). Eq. 2-9 can be re-written as:

\[
\log C = \log C_0 - \frac{kt}{2.303} \tag{Eq. 2-10}
\]

Where, \( C_0 \) is the initial concentration of loaded drug and \( k \) is the first order constant.

Plotting the data according to Eq. 2-10 for log cumulative of drug remaining verses time reveals a straight-line relationship (first order) with a slope of \( -k/2.303 \) (255).

Application of this model appears in pharmaceutical dosages having porous matrices loaded with highly soluble drugs (266).
2.10.5 Baker and Lonsdale

This model was derived from the Higuchi model by Baker and Lonsdale in 1974. It portrays drug release from spherical matrices as presented by the following expression (267):

\[
\frac{3}{2} \left[ 1 - \left( 1 - \frac{M_t}{M_\infty} \right)^\frac{2}{3} \right] - \frac{M_t}{M_\infty} = \frac{3 D_m C_{ms} t}{r_0^2 C_0} 
\]  
(Eq. 2-11)

Where, \(M_t\) is the amount of drug released at time \(t\), whereas \(M_\infty\) is amount released at infinite time. \(D_m\), \(C_{ms}\) are the diffusion coefficient and drug solubility in the polymer matrix, respectively. While \(r_0\) represent the radius of the matrix and \(C_0\) is the initial concentration of drug (267). When the matrix is not homogenous, meaning factures or capillaries are existent and contribute significantly to the release profile, Seki et al. (268) modified Eq. 2-11 to Eq.2-12 where \(D_f\) and \(C_{fm}\) are the diffusion coefficient and the drug solubility in the liquid surrounding the matrix, respectively. The added terms \(\tau\) signify the tortuosity factor in the capillary system and the \(\varepsilon\) is matrix porosity which can be found by Eq. 2-13 where \(\varepsilon_0\) is the initial porosity and \(K\) is drug specific volume (269).

\[
\frac{3}{2} \left[ 1 - \left( 1 - \frac{M_t}{M_\infty} \right)^\frac{2}{3} \right] - \frac{M_t}{M_\infty} = \frac{3 D_f C_{fs} \varepsilon}{r_0^2 C_0 \tau} \cdot t 
\]  
(Eq. 2-12)

\[
\varepsilon = \varepsilon_0 + KC_0
\]  
(Eq. 2-13)

When the established conditions are met, the equation on left side will be in linear relationship to time as the following:

\[
\frac{3}{2} \left[ 1 - \left( 1 - \frac{M_t}{M_\infty} \right)^\frac{2}{3} \right] - \frac{M_t}{M_\infty} = k \cdot t 
\]  
(Eq. 2-14)

On a graphic representation \(k\) corresponds to the slope (270) and this Baker and Lonsdale model can be applied in linearizing release data from microcapsules and microspheres pharmaceutical formulations (255,271,272).
2.10.6 Weibull model

Weibull model is an empirical model developed by Weibull in 1951 (272). It successfully befits many dissolution/release curves and had been applied in many dissolution processes (272,273). When it is used in dissolution from pharmaceutical dosage forms, it is expressed by:

\[
m = 1 - \exp\left(\frac{-(a-T_i)^b}{a}\right) \quad \text{(Eq. 2-15)}
\]

Where, \(m\) is the accumulated released drug in media at time \(t\) and the scale parameter, \(a\), describes the time scale of the process. The location parameter, \(T_i\), is the lag time before dissolution/release is started, and is zero in most of curves fitting. The shape parameter, \(b\), donates the shape of the dissolution progression curve in terms of three cases. When \(b=1\) (case 1), the curve is a normal exponential. When \(b>1\) (case 2) the graph represent a sigmoid, S-shaped, with upward curvature and a turning point is followed. Lastly, graph would have a parabolic shape with a steeper initial increase than \(b=1\) followed by a consistent exponential curve when \(b<1\) (case 3) (272). Weibull equation can be rearranged to fit release data as a linear function for a log-log of \(-\ln(1-m)\) verses time plot as follows:

\[
\log[-\ln(1-m)] = b \log(t - T_i) - \log a \quad \text{(Eq. 2-16)}
\]

Here, the \(b\) is found from graph slope and is obtained from the ordinate value (1/a) at \(t=1\). This model has been criticised due to the fact that it’s an empirical and not a fundamental equation (274,275). Some deficiencies include that it doesn’t incorporate any parameter related to the intrinsic dissolution rate of drug, its inability to characterize the kinetic properties of dissolution, and its limitation of use in vivo and vitro correlations.
2.11 Conclusion

MNPs emerging in recent medicine are notably accelerated in the past decade. The development of these nanoparticles with variety of formulations is fascinating for both imaging and therapeutics. Particularly in this paper, we reviewed the use of MNPs as drug carriers for their great potential in targeted delivery and cancer treatment. Targeting ability and biocompatibility can be improved though surface coating and have been investigated enormously in recent studies. Coating provides a mean to alter the surface features of MNPs including physical characteristics and chemical functionality. Coating is chosen with extreme care to fit the desired surface characteristics for a specific biomedical application. More particularly, the use of biocompatible polymers to coat magnetic cores have great advantages such as preventing aggregation, increase colloidal stability, evades nanoparticles uptake by RES, and can provide a surface for conjugation of targeting ligands such as peptide and biomolecules with high affinity to target cells. Great efforts to bring MNPs from lab testing stage to clinic are needed to understand their physicochemical properties and how they behave \textit{in-vivo}, which resulted in few of them to exist in the market today. Although magnetic nanoparticles have not yet fully reached their optimal safety and efficiency due to the challenges they face \textit{in-vivo}, their shortcomings can be overcome through improvement of magnetic-targeted carrier by pre-clinical trials and continuous studies.
2.12 References


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CHAPTER 3

3 Synthesis and Characterization of Dual Stimuli Responsive Glycol Chitosan-Fe$_3$O$_4$ Core-Shell Magnetic Nanoparticles for Controlled Delivery of Progesterone

3.1 Graphical Abstract
3.2 Abstract

Magnetic nanoparticle, such as superparamagnetic iron oxide nanoparticles (SPIONs), have been extensively studied as therapeutic and diagnostic agents due to their remarkable characteristics including biocompatibility, bioselectivity, prolonged circulation, and chemical stability. The aim of this study was to develop an effective polymeric-metallic hybrid nanoparticles coated with novel glycol chitosan polymer (GC) and loaded with progesterone. The crystalline nanoparticles were characterized by X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), thermo-gravimetric analysis (TGA), and vibrating sample magnetometer (VSM). Spherical-like superparamagnetic nanoparticles, in the size range of 10-20 nm, with properly designed GC coating were fabricated. Progesterone release from glycol chitosan hybrid magnetic nanoparticles was examined and investigated through several mathematical models. Progesterone release kinetics was demonstrated to differ significantly with small changes in the pH environment, where glycol chitosan-altered magnetic nanoparticles (SPION-GC) exhibited swelling at pH 6.5 and shrinking at pH 7.4. Moreover, MTT assay of C3H10T1/2 cell line cultured with SPION-GC indicated biocompatibility of the magnetic nanoparticles. Polymeric-metallic hybrid nanoparticles have shown to be a promising potential nanocarriers system for controlled drug delivery applications.
3.3 Introduction

Magnetic nanoparticles are rapidly growing in expansive fields of biomedical applications, particularly in drug delivery, magnetic resonance imaging (MRI), gene delivery and tissue engineering. They have gained great attention in recent years mainly due to their magnetic properties and their ability to function at both cellular and molecular levels (1–3). Magnetic nanoparticles (MNPs) have many particular physical and biological features including, but not limited to: biocompatibility, injectability, high magnetic flux density, and small particle size distribution (1,4,5). In drug delivery, some of the main advantages of MNPs are their capability to integrate drug payloads with different solubility, the improvement in the longevity and stability of the therapeutics in blood circulation, their ability to be modified according to their surface chemistry for specific cell targeting, and the ability to regulate therapeutic release through unique mechanisms (3,6,7). In addition to the utilization of MNPs as drug vehicles, their properties allow them for multifunctional therapeutics. Magnetic fluid hyperthermia (MFH), for instance, is a heat treatment for cancer that takes the advantage of the fact that cancerous cells are more sensitive to heat than normal cells and the unique ability of MNPs to self-heat in an external magnetic field (3,8). This key feature of hyperthermia combined with their high specific absorption rate due to the nano-size of the drug delivery system particles and high cellular selectivity through particle surface modification present a promising treatment for cancer and other diseases with minimized systemic side effects existing in current treatments such as chemotherapy and radiation (8,9).

Superparamagnetic iron oxide nanoparticles (SPIONs), a main class of MNPs, were developed in this study for their attractive properties and promising outcomes. Their high magnetization in an AC magnetic field, which demolish completely once the field is removed provides them with opportunities not only in targeted drug delivery and hyperthermia, but also in MRI applications (4,10). Their main advantages are biodegradability, ease of synthesis, chemical stability, less sensitivity to oxidation, and biocompatibility, recyclability by iron metabolism through normal biochemical pathways and their enhanced permeability and retention (EPR) effect (1,2,11). SPIONs effectiveness is strongly dependent on the particle dimensions, thus controlling a monodisperse size distribution of the particles is very crucial (12–14). A size between 10 to 100 nm was reported to be the most efficient for SPIONs parenteral administration (13,14). Nevertheless, due to magnetic dipole-dipole interactions, high surface
energy and large surface area-to-volume ratio, MNPs tend to agglomerate. Obtaining stable and resistant-to-aggregation magnetic colloidal suspension can be achieved through surface modification, which creates an electrostatic repulsion between the particles in an effort to attain close to equilibrium condition between attractive and repulsive forces (2). Numerous studies on surface coating of SPIONs have been performed such as inorganic materials (silica (15,16), gold (17,18)), liposomes (19), polymers (Dextran (20–22), polyethylene glycol (PEG) (23,24), alginate (25), chitosan (26–28), and others polymers (14)). Moreover, these coatings provide further functionalization by attaching various functional groups (carboxylates, sulfates and phosphates (29)) and/or targeting biological ligands.

Chitosan, deacetylated chitin, is a naturally existing polymer with abundant functional groups in its backbone structure (30). Although it has been a suitable coating material for iron oxide nanoparticles, its application is limited because of its poor solubility. Its deacetylated form is only soluble in organic acids at low pH (31) or by modifying the degree of its acetylation to about 50%, in which the free amine-containing residues are reduced (30,32). In this study a very novel polymer derivative is used, glycol chitosan (GC), as the coating material for SPIONs of ferrite nanocrystals of magnetite (Fe₃O₄). GC is a self-assembled chitosan derivative conjugated with ethylene glycol branches. It is fully soluble in neutral and acidic pH values due to the glycol branches that aid in increasing the steric stabilization and the aqueous solubility (32). Glycol Chitosan (poly β(1-4)-glucopyranosamine) is from the family of cationic hydrophilic polysaccharides, which consist of Nacetyl D-glucosamine and D-glucosamine residues joined by αβ (1-4) (glycosidic) linkage (33). This polymer captured a lot of attention in biomedicine applications such as wound dressings, scaffolds for tissue engineering, and drug delivery carriers due to its favourable features, which are believed to be because of its free amine groups present in its backbone (33,34). Scheme 3-1 illustrates the structure for GC in comparison with Chitosan. The amine group is left unaffected after the modification of chitosan to glycol chitosan unlike other chitosan derivatives. This amine group allows for alteration to GC, and attaching functionality groups (succinyl, dicarboxymethyl, polyethylene glycol and carboxymethyl) for targeted drug delivery, while preserving the favourable biological interactions (33,35). Previous studies used GC as the core material for nanoparticle self-assembled fabrication. It is widely used as anti-cancer drug carrier (36–38) and as a carrier for many hydrophobic drugs and genes (38–40). However,
research has rarely touched on its application as a coating material for SPIONs. In only one study, Inbaraj et al. assessed the antibacterial activity of GC-coated iron oxide nanoparticles (41).

Scheme 3-1: Chemical structure of chitosan (a) and glycol chitosan (b)

Progesterone is used in this study as the model active pharmaceutical ingredient (API) for encapsulation in the uniquely modified GC-SPIONs nanoparticles. It is a 21 carbon hydrophobic steroid hormone with water solubility of $3.79 \times 10^{-5}$ M, produced in the ovaries, adrenal glands and Leydig's cells (42–44). Progesterone, known as the female hormone, plays an important role in the menstrual cycle and during pregnancy and it has vital functions, such as being anti-mineralocorticoid and anti-androgenic agent (43). Progesterone structure is presented in Scheme 3-2 (45). Interestingly, progesterone showed promising results in many studies as a treatment for prostate hyperplasia, which leads to cancer in men (46), it affects sleep patterns and erectile function (47), and it has positive effects in the neurotransmission system and brain injuries recovery (48–50).
The objective of this paper is to comprehensively examine the potential application of GC-coated SPIONs as a carrier for controlled delivery of progesterone. The suggested inorganic-polymeric hybrid nanoparticles were developed for the aim of designing a dual responsive drug carrier system. The effects of pH and magnetic field were investigated due to the presence of magnetic core as well as the pH-responsive glycol chitosan. The kinetics of progesterone release was mathematically modeled at different environmental conditions for optimization of the drug release rate.

3.4 Materials and Methods

3.4.1 Materials

The chemicals used in this study were analytical grade and used as received without further purification. The ferrous (II) sulfate hepta-hydrate (FeSO$_4$.7H$_2$O) was purchased from Sigma-Aldrich (St. Louis, MO), MW =278.01, Reagent Plus®, ≥99%). Progesterone (MW= 314.46, ≥99%, mp=128-132 °C), phosphate buffered saline tablets, dialysis kit (Pur-A-Lyzer Mega 12000) with a membrane type of regenerated cellulose, and Glycol Chitosan (MW = 250,000, ≥60% (titration) with degree of polymerization ≥400, from crystalline) were purchased from Sigma (St. Louis, MO). Phosphate buffer saline tablets were dissolved in 200 mL of deionized water to yield 0.01M phosphate buffer, 0.137 M sodium chloride, and 0.0027 M potassium chloride, pH 7.4 at 25°C. Ammonium hydroxide (MW: 35.05 g/mol, 28 - 30% assay) was obtained from VWR (2360 Argentia Rd.). An Incubating Mini Shaker (120 V, 5 amps, 450 watts) and Ceramic hot plate Stirrer were bought from VWR international. Deionized water and essential glassware and apparatus were used from the laboratory.
3.4.2 Synthesis of bare and GC-coated SPIONs

The synthesis technique used to prepare the magnetic nanoparticles is a modification of the co-precipitation method described by Xia et al. (51, 52), which was reported by Ragab et al. (45). Instead of using two iron salts which are ferrous and ferric chloride, the commonly used in co-precipitation synthesis, only one type of salt was used and nitrogen purging is substituted with atmospheric conditions. These modifications provide easier and less expensive method of synthesis while maintaining the magnetic nanoparticles’ properties unchanged (45). The synthesis ran according to the following chemical reactions:

\[
\text{Fe}^{2+} + 2 \text{OH}^- \rightarrow \text{Fe(OH)}_2 \quad \text{Eq. 3-1}
\]

\[
4 \text{Fe(OH)}_2 + \text{O}_2 \rightarrow 4 \text{FeOOH} + 2 \text{H}_2\text{O} \quad \text{Eq. 3-2}
\]

\[
\text{FeOOH} + 2 \text{Fe(OH)}_2 + 5 \text{OH} \rightarrow \text{Fe}_3\text{O}_4 + 5 \text{H}_2\text{O} + \text{O}_2 \quad \text{Eq. 3-3}
\]

Batch synthesis of 1.396 g of the iron precursor FeSO\(_4\)·7H\(_2\)O were dissolved in 50 mL distilled water under continuous magnetic stirring using hot plate stirrer (VWR International, Mississauga, ON) for 30 minutes at 40 °C, where color is changed from clear solution to yellow/green. Alkaline solution (20 ml of Ammonium hydroxide) is then added slowly to the mixture, where a dark green/black solution is obtained. At this point stirring is continued and the temperature is immediately increased to 90 °C and is covered for additional 90 minutes. After the mixture has cooled to room temperature, the prepared precipitate of SPIONs was separated from the supernatant using an external strong magnet and the supernatant was removed with a pipette. Ten milliliters of distilled water was added and mixed well with the SPIONs precipitate. The water was removed using a magnet for separation and the supernatant was removed using a pipette as well. SPIONs were washed three times with distilled water (10 ml each) and a fourth time with ethanol (5ml wash) using the same procedure. The precipitate was then manually collected from the reaction mixture with the external magnet and is left to dry at room temperature for 24 hours or by vacuum oven at 80 °C for one hour.
In preparing GC-coated SPIONs, same procedure of bare-SPION synthesis was performed followed by a post coating step of glycol chitosan polymer. Different amounts of GC were dissolved in 100 ml of distilled water by magnetic stirring. The dried SPIONs are then added to the mixture at room temperature with continuous stirring for 24 hours.

3.4.3 Preparation of progesterone loaded GC-coated SPIONs

The progesterone loaded magnetic nano-aggregates coated with glycol chitosan were prepared with different concentrations of progesterone solution (0.25x10^{-3} mmol, 0.50x10^{-3} mmol, and 0.75x10^{-3} mmol). The progesterone solution was prepared by dissolving different amounts of progesterone in 1 mL of acetone in a glass vial. SPIONs dispersion was prepared by mixing 100 mg of SPION in 100 mL of distilled water with continuous mixing for half an hour. Progesterone solution was then added to the SPIONs solution and vigorously mixed for 24 hours at room temperature. The precipitated magnetic nano-structures were washed with ethanol using same procedure described above and then dried at room temperature.

3.4.4 Magnetic and Structural characterization of bare and GC-coated SPIONs

*X-ray Diffraction (XRD) Analysis:* The crystallite structure of Fe₃O₄ nanoparticles was investigated using XRD powder analysis (Rigaku-Miniflex, The Woodlands, TX,) and the samples were exposed to radiation CuKα, 40 KV, 20 mA at a wavelength of 1.54 Å. The diffracting angle 2-theta covered from 15° to 65° with a 0.02° step size (45, 53, 54).

*Fourier Transform Infrared (FTIR) Spectroscopy:* A Bruker vector 22 spectrometer controlled by OPUS 5.1 analytical software was used to obtain the FTIR spectra of the SPIONs samples in a powder state. The powder was scanned by an attenuated total reflectance (ATR) with resolution of 4 cm⁻¹ and total scans of 32. The samples were scanned between 4000 -500 cm⁻¹.

*Transmission Electron Microscopy (TEM):* Philips CM10 Transmission microscope was used with magnification range of 18× to 450,000×; Resolution (objective lens): 0.5nm/5.0Å (point), 0.34nm/3.4Å (line); and accelerating voltage range of 40kV to 100kV. ImageJ software was used to analyze the particle size. A total of 200 particle diameters for each sample were measured to obtain the particle size distribution and. There samples for each GC coated SPIONs was analyzed by ImageJ for accuracy.
**Scanning Electron Microscopy (SEM):** The surface morphology was examined using SEM (Hitachi High-Technologies GmbH, Germany). Before examining the samples, they were prepared on aluminum stabs then sputtered with gold and measured at an accelerating voltage of 20 kV coupled with energy dispersive X-ray spectroscopy (EDX) for elemental analysis.

**Thermo-Gravimetric Analysis (TGA):** The weight percentage of GC attached to the SPIONs surface was analyzed by TGA with a range of 25 to 600 °C in air at a ramp rate of 10 °C min⁻¹. The original and the first derivative results were obtained.

**Powder Magnetization (Vibrating Sample Magnetometer, VSM):** The magnetic properties were measured using a vibrating sample magnetometer (LakeShore cryotronics 7407, Westerville, OH). The magnetic properties of nano-aggregates samples were studied at moment measure range of 10⁻⁷ to 10⁻⁵ emu, 0.05% full scale with field accuracy. All the magnetization measurements were carried out at room temperature under a maximum field of 10 kOe.

### 3.4.5 Drug loading and encapsulation efficiency

Drug loading is defined as the amount of drug encapsulated inside the nanoparticles per unit mass (45). SPIONs were loaded with various amounts of progesterone, which was predetermined according to its solubility in aqueous solution (5, 10, 15, 25, 50, 75mg). The concentration of progesterone encapsulated was determined by dissolving a known amount of nano-aggregate samples in ethanol. The supernatant taken after 24 hours of magnetic stirring with SPIONs was measured with UV spectrometer (manufacturer) to obtain a quantitative amount of the drug discharged from the nanoparticles. The ratio of progesterone recovered in SPIONs to the total amount of drug loaded is the encapsulation efficiency. Each measurement was repeated in triplicate per sample. **Eq. 3-4** and **Eq. 3-5** were used in the calculation (51, 52).

\[
\text{Encapsulation efficiency} = \frac{\text{amount of drug encapsulated}}{\text{total amount of drug}} \times 100
\]  

Eq. 3-4
Drug loading = \frac{\text{weight of drug encapsulated}}{\text{Weight of dry nanoparticles}} \times 100 \quad \text{Eq.3-5}

Table 3-1: Effect of the initial progesterone concentration (w/w) on the drug loading and encapsulation efficiency

<table>
<thead>
<tr>
<th>Initial drug loaded concentration (mg / ml)</th>
<th>Progesterone concentration (w/w, mg drug / mg nanoparticles)</th>
<th>Drug loading (%)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1</td>
<td>12.40±0.35</td>
<td>34.02±3.54</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>15.90±0.26</td>
<td>24.52±1.29</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>19.25±0.52</td>
<td>19.25±1.73</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
<td>19.77±0.71</td>
<td>18.51±1.43</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>27.17±0.98</td>
<td>15.17±0.98</td>
</tr>
<tr>
<td>7.5</td>
<td>30</td>
<td>29.54±2.03</td>
<td>9.34±1.35</td>
</tr>
</tbody>
</table>

3.4.6 In-vitro cytotoxicity study of GC-coated SPIONs
The cytotoxicity of the progesterone-loaded GC-SPIONs was evaluated using C3H10T1/2 mouse mesenchymal cell line, where metabolic cell activity was measured by MTT assay; a colorimetric measure of the mitochondrial activity. Two independent cell culture studies were performed: time-course and dose-course studies. In the dose-course study, cells were pre-cultured for 24 hours after seeding at a density of 8,000 cells/well in 96-well plates. Afterwards, C3H10T1/2 were treated with various concentrations of progesterone encapsulated nanoparticles (25, 50, 75, and 100 µg/mL) with various coating concentrations of GC in the presence of 10% FBS. After 48 hours of incubation at 37°C, MTT solution (5 mg mL⁻¹ MTT in phosphate buffer solution, pH 7.4) was added to each well and absorbance
was measured at 570 nm. The same procedure was used in the time-course study. However, one concentration (50 µg/mL) was chosen and cells were treated with progesterone loaded SPIONs with different GC coatings concentrations and without GC coating (as a control). Then, MTT absorbance values were measured following 48 hours and 96 hours of culture.

3.4.7 In-vitro release study of GC-coated SPIONs

*In-vitro* progesterone release was carried out in a dialysis bag (Pur-A-Lyzer Mega 12000). The dialysis bag insured that diffusion occurred only for drug molecules without passage of the SPIONs. **Scheme 3-3** shows the floating dialyzer setup. For the in-vitro release experiment, 15 mg of GC coated SPIONs at different GC coating concentrations (62.6, 125, or 178.5 mg GC) loaded with 5mg progesterone in 10 mL of the release media was introduced into the inner tube of the dialyzer. Progesterone is freely soluble in the media with an addition of 0.5ml of acetone and 0.1% of Tween 20. The dialyzer was placed into a 60 mL beaker containing the release media. Two release media were used: distilled water or phosphate-buffered saline (PBS) to compare the release at different pH values, 6.5 and 7.4, respectively. The setup was placed at 37°C incubating shaker at 300 rpm in order to prevent the formation of unstirred water layer at membrane/outer media interface. Diffusion was measured by sampling 5mL of the outer solution at predetermined time intervals. Fresh solution of distilled water or PBS solution was replaced in the breaker. The samples were measured with UV visible spectroscopy at 450 nm maximum wavelength.

3.5 Statistical analysis

The experiments were performed in multiple replicates. The data from each replicate was calculated independently. The experimental data was presented as mean ± standard deviation (SD) and was analyzed statistically by a one-way analysis of variance and the level of significance was determined at $p < 0.05$. 
3.6 Results and Discussion

3.6.1 X-ray Diffraction (XRD)

XRD provides information about the crystalline structure of the synthesized particles, where the degree of structure order is identified (53). It was performed in this paper to prove that magnetite was in fact produced and that the polymer coating did not affect the magnetite crystalline phase. In Figure 1a, The characteristic peaks of inverse cubic spinal structure is observed (54,55). According to the literature, iron oxide nanoparticles show sharp prominent peaks at 30.5 (220), 35.84 (311), 43.46 (400), 53.90 (422), 57.38 (511) and 62.90 (440) with 311 peak having the highest intensity (55–57). Figure 1b, shows the standard XRD pattern for magnetite obtained from ICCD with card number 00-019-0629. These peaks with similar intensities and with the corresponding angles were present in all of the tested samples. This indicates that the prepared Fe$_3$O$_4$ by alkaline precipitation corresponded to the pure phase of Fe$_3$O$_4$, and that the used synthesis technique was feasible (55). It was also noted that the addition of different weight concentrations of GC polymer did not alter the XRD spectrum, where the same peaks were present indicating that the spinal structure of Fe$_3$O$_4$ NPs was...
retained (56). However, the peak intensity of the coated SPIONs appeared to be lower compared to the naked Fe₃O₄, since the amount of naked particles is lower (56). The width of the diffraction peak is related to the size of the crystalline particles; the narrower the diffraction peak, the larger the particle size (53). The average particle size of SPIONs (D) can be calculated using Scherrer’s equation (Eq.3-6).

\[
D = \frac{K\lambda}{(\beta \cos \theta)}
\]

Eq. 3 - 6

Where, K is a constant, \(\lambda\) is X-ray wavelength, \(\beta\) is the peak width of half-maximum and \(\theta\) is the Bragg diffraction angle (54). In this work, the strongest diffraction peak was chosen for calculating the diameter of SPIONs. The size was determined using TEM as well for precision as presented in Table 3-2. It is clear that particle size increased with polymer coating. TEM and XRD results were in good agreement with each other.
Table 3-2: Effect of GC concentration on the measured particle size data (TEM) and the calculated values based on XRD

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Concentration of glycol chitosan (mmol)</th>
<th>Concentration of Fe₃O₄ (mmol)</th>
<th>Particle size (nm, Estimated from the XRD pattern)</th>
<th>Particle size (nm, measured using TEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated Fe₃O₄</td>
<td>0</td>
<td>1.6x10⁻³</td>
<td>10.31</td>
<td>8.76±2.00</td>
</tr>
<tr>
<td>Fe₃O₄ - GC 1</td>
<td>0.25x10⁻³</td>
<td>1.6x10⁻³</td>
<td>12.71</td>
<td>11.87±3.20</td>
</tr>
<tr>
<td>Fe₃O₄ - GC 2</td>
<td>0.5x10⁻³</td>
<td>1.6x10⁻³</td>
<td>15.68</td>
<td>12.20±2.61</td>
</tr>
<tr>
<td>Fe₃O₄ - GC 3</td>
<td>0.75x10⁻³</td>
<td>1.6x10⁻³</td>
<td>18.41</td>
<td>20.40±3.24</td>
</tr>
</tbody>
</table>

Figure 3-1: XRD pattern of Magnetite: a) the effect of GC coating on the crystalline structure of SPIONs, b) standard XRD pattern of Magnetite (58).
3.6.2 Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was performed to confirm that GC had truly coated the Fe₃O₄ nanoparticles. The FTIR spectra in Figure 3-2 prove the interaction between CG and the Fe₃O₄ nanoparticles. The clear broad peak at absorption band 3353 cm⁻¹ could be attributed to the stretching vibration of O-H and N-H (58). Also, C-H stretching vibration could be assigned to the peak at 2925 cm⁻¹. The absorption band at around 1630 cm⁻¹ in the pure GC and the coated samples corresponded to the amide bond of the undeacetylated section of GC whereas; this peak was absent in the bare SPIONs (data not shown). The small peak at 1544 cm⁻¹ is an indication of the N–H bending vibration of the free amine group of the deacetylated section of GC, which was more apparent in the pure GC spectrum (56,59). The glycosidic linkage (ether bond) and C–N vibrations were illustrated in the absorption bands at 1110 and 1062 cm⁻¹, respectively (56,60). The peak at 586 cm⁻¹ only appeared in the coated samples and was absent in pure GC. This peak could be assigned to Fe–O stretching vibration of Fe₃O₄ (45,58,61). The FTIR of bare SPIONs contained peaks at 578 cm⁻¹ and at 3401 cm⁻¹ that were attributed to Fe-O and O-H vibrations, respectively. Shifting in the amide bond peak was observed from 1677 to 1630 cm⁻¹ in GC/Fe₃O₄. This shift from a higher energy level to a lower one could indicate that the interaction between GC and SPIONs was through a nitrogen atom as suggested by literature (62,63). Altogether, these data has proved the coating of SPIONs with GC.
Figure 3-2: FTIR spectra of Glycol chitosan (GC), GC-coated magnetic nanoparticles, and uncoated magnetite

3.6.3 Transmission electron microscopy (TEM)

Figure 3-3 shows the representative TEM images of coated Fe$_3$O$_4$/GC with varied concentrations as specified in Table 3-2 and uncoated SPIONs as a control. The corresponding histogram of particle size distribution for uncoated and GC coating concentration of (0.5 $\times$ 103 mmol), determined by image analysis using the count of 200 particles, was illustrated in Figure 3-4a and b. Spherical-like agglomerated SPIONs was
clearly observed in Figure 3-3a as expected, due to the magnetic dipolar forces and van der Waals forces between the nanoparticles. As shown, Fe₃O₄/GC maintained nearly spherical shape similar to that of bare SPIONs with wider separation of particles compared to uncoated particles. This means that the forces between the particles had likely decreased due to the surface modification with GC coating. Particle size was found to increase with higher coating concentration starting at 8.76 nm for uncoated and ending at 20.4 nm for Fe₃O₄/GC-3 as detailed in Table 3-2. Particle size measurements by TEM corresponded well with particle size analysis by XRD. The increase in particle size may indicate that GC was evenly surrounding the core SPIONs in a core/shell structure. Moreover, the particle size distribution had increased as GC layer was added. Uncoated SPIONs tend to show poor distribution (skewed to the right wide distribution), while Fe₃O₄/GC shows better distribution (normal distribution with narrower distribution range). This may reflect a lower degree of aggregation with GC coating in comparison to bare SPIONs.
Figure 3-3: TEM images illustrating the effect of polymeric GC coating on bare SPIONs: (a) uncoated SPIONs (b) Fe3O4/GC-1 (c) Fe3O4/GC-2 (d) Fe3O4/GC-3
Figure 3-4: Histogram of particle size distribution: A) for uncoated SPIONs with average size diameter of 8.76nm and B) coated SPIONs with the middle used concentration of GC

3.6.4 Scanning electron microscopy (SEM)

SEM images of magnetic SPIONs nano-aggregates are presented in Figure 3-5. Samples of uncoated SPIONs (Figure 3-5a) and three different GC-SPIONs coating concentrations were examined. The presented images unveiled that the prepared iron oxide powder encompassed uniformly aggregated spherical primary nanoparticles. According to literature, spherical drug vehicles are much more favored in drug delivery application, especially in the nanoscale (64). Bare SPIONs tend to be clustered in groups. The addition of GC layer on the nanoaggregates did not affect the particle morphology; however, the clustering tends to loosen up as GC
coating was introduced and decreased gradually as thicker GC coating was used. In fact, it is apparent that with Fe$_3$O$_4$/GC-3 (Figure 3-5d), the nanoparticles were much more separated and homogenously distributed. In addition, it can be observed that the individual particles were enlarged because of the GC coating of the SPIONs starting with their particle size of 8.76 nm. Moreover, SEM images had confirmed that the overall particle layout became denser as the coating concentration increased.

**Figure 3-5:** Scanning electron micrograph (SEM) images for different magnetic nanoaggregates. Effect of polymeric composition of GC on their morphology: a) uncoated SPIONs b) Fe$_3$O$_4$/GC-1 c) Fe$_3$O$_4$/GC-2 d) Fe$_3$O$_4$/GC-3

3.6.5 Thermo-Gravimetric Analysis (TGA)

TGA graphs in Figure 3-6 illustrate the surface adsorption of GC on SPIONs. TGA measurement was preformed to confirm the GC coating formation and determine the amount of polymer associated with the SPIONs. It could be observed that the coating rates for uncoated, Fe$_3$O$_4$/GC-1, and Fe$_3$O$_4$/GC-3 were 96.02%, 89.17%, and 90.46%, respectively. The results clearly showed that the coated nanoaggregates were significantly different from the
bare SPIONs, and both Fe₃O₄/GC-1 and Fe₃O₄/GC-3 displayed a similar weight loss profile below 400°C. The initial stage of weight loss was around 2% for all samples within the first 200°C, this is most likely related to the removal of adsorbed water, because of surface hydroxyl groups, or both as expected in systems containing polymer-coated magnetite nanoparticles (65,66). The slight weight loss noticed in bare SPIONs after 200°C is probably attributed to the decomposition of amorphous iron hydroxides as confirmed by literature (41).

A significant gradual weight loss for both of the Fe₃O₄/GC tested samples was observed above 180°C with maximum temperature at 252.5°C. This weight loss is likely due to the evaporation and the decomposition of the GC coating layer surrounding the nanoaggregates, which may demonstrate that a substantial amount of GC, estimated as approximately 10% of the SPION weight, was successfully coated on the surface of the Fe₃O₄ nanoparticles.
3.6.6 Powder magnetization

Power magnetization was studied to determine how the magnetic properties are affected after surface modification via GC surface coating. Vibrating sample magnetometer (VSM) is used to measure the magnetic properties for uncoated SPIONs and the three coating samples of GC. Figure 3-7 exhibits a typical magnetization pattern of bare nanoparticles and GC-Fe3O4. Superparamagnetic property is demonstrated, where the hysteresis loops for all samples indicates a single-domain magnetic nanoparticle. Remanence is a measure of the remaining magnetization after the driving field is dropped to zero while coercivity is the measure of the reverse field needed to drive the magnetization to zero after it had reached saturation. When these two values are low, it shows better superparamagnetic property. This is a standard property for maghemite and magnetite with diameters between 10-20 nm (67,68). This attractive feature allows for their reuse by their ability to demagnetize once the external magnetic field is removed. Fe3O4/GC-2 shows a particular superparamagnetic characteristic that may surpass that of naked SPIONs as Table 3-3 displays. Fe3O4-GC 1 and Fe3O4-GC 3 have similar retentivity, yet higher coercivity compared to Fe3O4-GC 2, but not significantly. Particles having less than 20 Oe coercitivity are part of the superparamagnetic family as termed by literature (69). Thus, the three GC coated samples are confirmed to be superparamagnetic. The decrease in superparamagnetic property in Fe3O4/GC-1 and Fe3O4/GC-
3 may be due to the increase in particle size because of the thicker GC layer incorporated. This is also evident in the increase of retentivity compared to the naked SPIONs. The presence of GC on particles surface lessened their uniformity due to reducing the surface moment, which sequentially decreased the magnetic moment of these particles. The measured saturation magnetization value for bare SPIONs is 68.98 emu/g, which is closely similar to Fe$_3$O$_4$/GC-2 and Fe$_3$O$_4$/GC-3. These values are similar to the reported values in literature (66,70). Nevertheless, Fe$_3$O$_4$/GC-1 showed elevated saturation magnetization at value of 113.39 emu/g. Altogether, the prepared Fe$_3$O$_4$/GC can provide targeted delivery and ease of post-delivery separation.

Figure 3-7: Hysteresis curves at room temperature of bare and GC coated SPIONs
### Table 3-3: Magnetization parameters of glycol chitosan coated magnetic nanoparticles compared to the bare magnetic core

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Magnetization (emu/g)</th>
<th>Retentivity (emu/g)</th>
<th>Coercivity (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated Fe$_3$O$_4$</td>
<td>68.98</td>
<td>2.54</td>
<td>1.56</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ - GC 1</td>
<td>113.39</td>
<td>7.56</td>
<td>4.84</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ - GC 2</td>
<td>67.88</td>
<td>0.19</td>
<td>0.95</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ - GC 3</td>
<td>68.67</td>
<td>4.97</td>
<td>4.62</td>
</tr>
</tbody>
</table>

#### 3.7 Evaluation of GC-SPIONs cytotoxicity

**Figure 3-8** shows the dose-course metabolic activity of C3H 10T1/2 cells as determined by MTT assay, and values are presented as mean ± SD of triplicates. The uncoated nanoparticles did not show any significant difference in metabolic activity in all concentrations except for 100 µg/mL of progesterone-loaded nanoparticles. For coated nanoparticles, both 25 and 50 µg/mL demonstrated insignificant decrease, whereas 75 and 100 µg/mL had a significantly decreased metabolic activity in comparison to the startup rate but not significant compared to the bare nanoparticles. This suggests that the uncoated and unloaded SPIONs do not impose any cytotoxicity to the cells and same goes with GC-coated nanoparticles. Nevertheless, when the GC-SPIONS are loaded, progesterone could affect cellular activity at the highest tested concentrations. The significant decrease at 75 and 100 µg/mL in GC-SPIONs might be due to the higher encapsulation rate of progesterone in GC-coated nanoparticles verses the uncoated particles. By decreasing the progesterone initial loading, the level of cytotoxicity decreased as a function of concentration. Also, the time-course study (**Figure 3-9**) demonstrated the ability of the 10T1/2 cells to grow and function under the treatment of GC-SPIONs. Cells after 48h and 96 h were able to grow in a time-dependent manner showing the biocompatibility of the
synthesized particles *in-vitro*. Overall, results show that the prepared GC-SPIONs are well tolerated by C3H 10T1/2 cells.

**Figure 3-8:** Dose-course of metabolic activity of C3H 10T1/2 cells as determined by MTT assay

**Figure 3-9:** Time-course of the metabolic activity of C3H 10T1/2 cells as determined by MTT assay
3.8 Progesterone loading and *in-vitro* Release

The amount of 5 mg of progesterone (0.1 mg / mg nanoparticles) was chosen as the ideal concentration to be encapsulated in the coated SPIONs for all the proceeding studied samples, based on the predetermined encapsulation efficiency data. Encapsulation efficiency was calculated to be 34.02±3.54 for this loading ratio. Progesterone is likely to have placed itself into the SPIONs highly porous structure. Changing the concentration of glycol chitosan in the composite has significantly increased the drug encapsulation efficiencies as shown in Table 3-4.

In fact, Fe$_3$O$_4$/GC-3 encapsulation efficiency was almost doubled compared to naked Fe$_3$O$_4$ (34.02±3.54 add naked particle values to the table to 63.54±4.65). The increase in the efficiency and percentage loading had decreased when higher concentrations of GC were used. This may be due to the saturation of the GC coating, which may have prevented progesterone from penetrating though the cross-linked network of the GC structure. Also, it was indicated that progesterone-loading percentage had increased with increasing the surface coating (Table 3-4). Glycol chitosan is a self-assembled polymeric amphiphile, where the hydrophobic moieties are facing towards the core, and the hydrophilic moieties are facing towards the solution (30). For this reason GC is freely soluble in water at a wide pH range (71). When GC meets the hydrophobic progesterone, they are more prone to form hydrogen bonding with other weaker interactions such as hydrophilic/hydrophobic and electrostatic interactions (72,73). These bonds are expected to form at the internal surface of GC. Meaning, progesterone will be encapsulated into SPIONs and GC core. The more binding sites are available, the higher the chance for progesterone molecules to attach to GC (Scheme 3-4). This explains why the loading percentage of progesterone has increased with higher concentrations of GC coating.

The release profiles of progesterone from Fe$_3$O$_4$/GC SPIONs at pH 6.5 were depicted in Figure 3-10. For all surface modified magnetic nanoparticles, the initial burst release was observed within the first 3h with a maximum cumulative release of 5%. This strongly corresponds to the release of drug present on the surface of the nanoparticles. Initial burst was much better suppressed compared to the uncoated nanoparticles. Fe$_3$O$_4$/GC exhibited sustained release behavior for 15 days at cumulative percentages of 64.82%, 30.00%, and 25.50% for
FeO\textsubscript{3}/GC-1, FeO\textsubscript{3}/GC-2 and FeO\textsubscript{3}/GC-3, respectively. The release after day 15 had an insignificant release rate increase and had reached equilibrium state at day 16.

In nanoparticles drug delivery systems, polymer degradation plays an important role in the release profile. Since GC is soluble in water it tends to deteriorate faster in solution compared to other non-soluble polymers in a shear thinning behaviour. Also, the type of bonds and cross-linking of polymer to drug/nanoparticles greatly influence release rates (73). Moreover, combination of both drug diffusion and polymer degradation can play an important role in influencing release rates of progesterone remarkably (74).

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Concentration of glycol chitosan (mmol)</th>
<th>Progesterone encapsulation efficiency (%)</th>
<th>Progesterone loading percentage (%)</th>
<th>Release rate constant at pH 6.5 (k, day\textsuperscript{-1})</th>
<th>Release rate constant at pH 7.4 (k, day\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeO\textsubscript{3} - GC 1</td>
<td>0.25x10\textsuperscript{-3}</td>
<td>35.49±1.84</td>
<td>16.51±2.89</td>
<td>11.2615±0.32</td>
<td>-</td>
</tr>
<tr>
<td>FeO\textsubscript{3} - GC 2</td>
<td>0.5x10\textsuperscript{-3}</td>
<td>46.82±5.78</td>
<td>31.76±6.21</td>
<td>9.0170±0.58</td>
<td>-</td>
</tr>
<tr>
<td>FeO\textsubscript{3} - GC 3</td>
<td>0.75x10\textsuperscript{-3}</td>
<td>63.54±4.65</td>
<td>38.23±5.7</td>
<td>9.0170±0.58</td>
<td>14.0973±1.15</td>
</tr>
</tbody>
</table>

Table 3-4: Release data containing encapsulation efficiency, loading rates and release rate constants for coated and uncoated SPIONs
Scheme 3-4: likelihood positions of H-bonding forming between glycol chitosan and progesterone

Figure 3-10: Release profiles of progesterone from variable GC coated SPIONs formulations: effect of increasing GC surface coating on Fe3O4 at 5mg progesterone
3.9 Study of the kinetics of progesterone release from glycol chitosan coated magnetic nanoparticles

The observed enhancement in cumulative release of progesterone as the concentration of GC is lowered can be explained by the hydrogen bonding and hydrophobic interactions present between GC and progesterone. At high GC concentration, progesterone tends to attach firmly to GC and consequently restrict its permeation through its polymeric networks. Besides, the incorporated negatively charged \( \text{Fe}_3\text{O}_4 \) was attached tighter to the positively charged GC upon additional GC concentration, constraining the release even further. Progesterone release kinetics was analyzed by fitting the data to the simplified semi-empirical Korsmeyer-Peppas model (\textbf{Eq. 3-7}) (73,75,76)

\[
\frac{M_t}{M_\infty} = K t^n
\]

\textbf{Eq. 3-7}

Where, \( \frac{M_t}{M_\infty} \) is the fraction of drug released at time (t), K is the release rate constant (integrates the geometric of the drug form), n is the release exponent that assists in determining the best fitted mechanism for the system. This equation is a simplified version of the Peppas model where diffusion is assumed to be the main drug release mechanism. Peppas et al. (75) explained the controlled delivery of drug release using different geometrics and drug vehicles structures. The value of release exponent n was emphasized to describe the release mechanism for non-swellable systems with the assumption of having mono-dispersed particles under one-dimensional diffusion (\textbf{Table 3-5}). For spherical particles with \( 0.5 < n < 1.0 \), the model indicates an anomalous diffusion or non-Fickian diffusion (coupled transport), as in the case of \( \text{Fe}_3\text{O}_4/\text{GC-1} \) detailed in \textbf{Table 3-6}, while \( \text{Fe}_3\text{O}_4/\text{GC-2} \) and \( \text{Fe}_3\text{O}_4/\text{GC-3} \) exhibited a Fickian diffusion. Although Peppas model provided an idea about the release mechanism, this system does not fully apply to the assumptions made by Peppas et al. For example, having a wide size distribution, this can cause significant diffusion acceleration in the early stage of release and considerable delay of release towards the end stage. Thus, further investigation on drug release behaviour must be conducted for more accurate explanation of progesterone release from the \( \text{Fe}_3\text{O}_4/\text{GC} \) nanoparticles.
Table 3-5: diffusional release mechanisms interpreted from polymeric films according to exponent of release

<table>
<thead>
<tr>
<th>Exponent of release (n)</th>
<th>Mechanism of drug transport</th>
<th>Rate as a function of time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Fickian diffusion</td>
<td>$t^{0.5}$</td>
</tr>
<tr>
<td>0.45 &lt; n = 0.89</td>
<td>Non-Fickian drug transport</td>
<td>$t^{n-1}$</td>
</tr>
<tr>
<td>n = 0.89</td>
<td>Case II transport release</td>
<td>Zero order drug release</td>
</tr>
<tr>
<td>n &gt; 0.89</td>
<td>Super case II transport</td>
<td>$t^{n-1}$</td>
</tr>
</tbody>
</table>

Table 3-6: Determination of the drug release mechanism based on the release exponent value of progesterone from SPIONs coated with different concentrations of glycol chitosan (GC)

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Peppas fitted equation</th>
<th>$R^2$</th>
<th>Release exponent</th>
<th>Drug release mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$_3$O$_4$ - GC 1</td>
<td>$Mt/M∞ = 10.587t^{0.5776}$</td>
<td>0.95104</td>
<td>$0.5 &lt; n &lt; 1.0$</td>
<td>Anomalous diffusion</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ - GC 2</td>
<td>$Mt/M∞ = 9.3258t^{0.4248}$</td>
<td>0.87711</td>
<td>$n \leq 0.5$</td>
<td>Fickian diffusion</td>
</tr>
<tr>
<td>Fe$_3$O$_4$ - GC 3</td>
<td>$Mt/M∞ = 7.927t^{0.4248}$</td>
<td>0.87711</td>
<td>$n \leq 0.5$</td>
<td>Fickian diffusion</td>
</tr>
</tbody>
</table>

3.9.1 Investigation of the release behavior though various mathematical models

Mathematical modeling of composite drug delivery has great value in deepening the understanding of the physical mechanisms governing drug release, by determining important parameters that impact the release rates. Mathematical investigation of progesterone release process was conducted by fitting various validated release models: Baker–Lonsdale,
Korsmeyer–Peppas, Hixon and crowell, Higuchi equation, and first-order equation to the manipulated experimental data. Equations of the empirical models are illustrated in Table 3-7. Correlation coefficients and simulated equations were calculated and compared. Determining the best fitting model was carried out though the comparison of the R² values shown in Table 3-8. It is clear that Baker–Lonsdale and Korsmeyer–Peppas models best describe the progesterone release from Fe₃O₄/GC nanoparticles as the correlation coefficient is greater than 0.9690 under all different GC concentrations. In Korsmeyer–Peppas model, the release mechanism showed via the n value had the same results compared to the semi-empirical Korsmeyer-Peppas found earlier. Fe₃O₄/GC-1 had an n value between 0.5 and 1.0 representing a non-fickian diffusion mechanism, while both Fe₃O₄/GC-2 and Fe₃O₄/GC-3 showed fickian diffusion mechanism with n ≤ 0.5. Increasing the coating concentration was associated with a decrease in the drug release rate for both Korsmeyer-Peppas and Baker–Lonsdale models. For example, Fe₃O₄/GC-1 had a Peppas release rate of 11.2615± 0.3150, whereas Fe₃O₄/GC-2 had a value of 9.0170± 0.5765.

Table 3-7: The empirical mathematical models used to fit progesterone release data

<table>
<thead>
<tr>
<th>Mathematical model</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker- Lonsdale model</td>
<td>( \frac{3}{2} \left[ 1 - \left( 1 - \frac{M_t}{M_\infty} \right)^{\frac{2}{3}} \right] - \frac{M_t}{M_\infty} = k )</td>
</tr>
<tr>
<td>Peppas model</td>
<td>( \frac{M_t}{M_\infty} = 2 \left( \frac{D \tau}{\delta^2} \right)^{1/2} = at^{1/2} )</td>
</tr>
<tr>
<td>Hixon and Crowell model</td>
<td>( \frac{1}{W_o^3} - \frac{1}{W_t^3} = \kappa t )</td>
</tr>
<tr>
<td>Higuchi model</td>
<td>( Q = A \sqrt[\frac{D \delta}{\tau}} \frac{C_s (2C_o - \delta C_s) t}{2} )</td>
</tr>
<tr>
<td>First Order Model</td>
<td>( \frac{dc}{dt} = K (C_s - Ct) )</td>
</tr>
</tbody>
</table>
Table 3-8: Correlation coefficients values of fitted kinetic models on cumulative release curves on Fe₃O₄/GC

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Concentration of glycol chitosan (mmol)</th>
<th>R²ᵃ</th>
<th>R²ᵇ</th>
<th>R²ᶜ</th>
<th>R²ᵈ</th>
<th>R²ᵉ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₃O₄ - GC 1</td>
<td>0.25x10⁻³</td>
<td>0.9939</td>
<td>0.9974</td>
<td>0.9362</td>
<td>0.9970</td>
<td>0.9483</td>
</tr>
<tr>
<td>Fe₃O₄ - GC 2</td>
<td>0.5x10⁻³</td>
<td>0.9690</td>
<td>0.9743</td>
<td>0.8498</td>
<td>0.9688</td>
<td>0.8601</td>
</tr>
<tr>
<td>Fe₃O₄ - GC 3</td>
<td>0.75x10⁻³</td>
<td>0.9690</td>
<td>0.9743</td>
<td>0.8498</td>
<td>0.9688</td>
<td>0.8709</td>
</tr>
</tbody>
</table>

a. Baker-Lonsdale model  
b. Peppas model,  
c. Hixon and Crowell model  
d. Higuchi model  
e. First Order Model

3.10 Effect of pH change on progesterone release profile

The release of progesterone from Fe₃O₄/GC-3 was investigated at two different values of pH release media (6.5 and 7.4). The maximum cumulative release amount of progesterone was 72.02% at pH 7.4, while the released amount decreased to 30.00% at pH 6.5. However, similar release trends were observed for both, where the samples exhibited an initial fast burst release followed by relatively slower release till equilibrium was reached (Figure 3-11). Nevertheless, for all of the examined samples, an increase in the pH yielded an increase in the rate of drug release. These data could be explained by the swelling nature of glycol chitosan in accordance with the change in the pH of release medium. Scheme 3-5 demonstrates the two possible mechanisms of progesterone permeation both through the pores between the GC shell and Fe₃O₄ core, and through the GC shell itself.
Figure 3-11: The effect of pH value on the release profile of the highest concentration of GC coated SPIONs (Fe3O4/GC-3)

Scheme 3-5: An illustration for the proposed pH-responsive mechanism of Fe3O4-glycol chitosan hybrid magnetic nanoparticles
The rigid Fe, core does not swell in accordance with the pH changes. However, the GC shell swells or shrinks according to the external pH condition (77). At relatively lower pH value (pH 6.5), the GC shell is protonated and swelled to a greater extent. The swollen GC shell was expected to block the pores in the GC-Fe,O, network structure; which explains the lower rate of drug permeation at slightly lower pH value. On the other hand, the enhanced rate of drug release at pH value 7.4 was attributed to the shrunken GC; which was expected for less pore blockage in the GC-Fe,O, structure. The permeation of progesterone through the GC shell itself increased due to the protonation of free amino group at lower pH value. The slight change in the pH resulted in uncoiled and more elongated GC networks. In addition, the slight reduction in the pH value of the release medium resulted in an increase in the internal osmotic pressure and mutual repulsion of the charged amino groups, which yields the uncoiling of the GC networks (78) .

As mentioned above, the overall release rate of progesterone is dependent on its permeation through the pores between GC and Fe,O, and the permeation of progesterone through the GC shell itself. However, the overall release rate has been increased at pH value 7.4 because the permeation of progesterone through the pores between GC and Fe,O, is much larger than the permeation of progesterone through the GC shell itself.

3.11 Conclusions

Polymeric-metallic hybrid nanoparticles were prepared with different compositions of glycol chitosan. Generally, glycol chitosan-coated magnetic nanoparticles samples showed significant swelling at pH 6.5 and shrinking at pH 7.4. However, the results of progesterone release from glycol chitosan hybrid magnetic nanoparticles showed a reversible proportionality with the swelling ratio of Fe,O,- GC nanoparticles. In addition, progesterone diffusion through the hybrid nanoparticles was observed to change significantly as the environmental pH was slightly changed. Investigation of progesterone release kinetics demonstrated that a narrow range change in the pH value yielded an increase in the rate of drug diffusion. In addition, testing the metabolic cell activity under the treatment Fe,O,- GC showed that the particles have good biocompatibility. The suggested pH-responsive nanoparticles demonstrated a good candidate for controlled drug delivery. In addition, the
presence of a magnetic core gives a prospect that other stimuli, such as exposure to an externally applied magnetic field, could provide targeted delivery.
3.12 References


61. Inbaraj BS, Kao TH, Tsai TY, Chiu CP, Kumar R, Chen BH. The synthesis and characterization of poly(γ-glutamic acid)-coated magnetite nanoparticles and their effects on antibacterial activity and cytotoxicity. Nanotechnology. 2011 Feb 18;22(7):075101.


CHAPTER 4

Conclusion and recommendations

This thesis describes an innovative way to form hybrid polymeric-metallic superparamagnetic nanoparticles (SPIONS-GC). It addresses their great potential to become a controlled targeted drug delivery system in hormonal therapy for breast cancer patients. More specifically, magnetic magnetite nanoparticles were produced by a modified co-precipitation method. Particles were then loaded with progesterone and coated with a novel polymer, glycol chitosan. A comprehensive literature review is presented in chapter 2 with detailed focus on polymer types used to surface modify magnetic nanoparticles and their drug delivery applications.

The experimental paper in chapter three studied the preparation of SPION-GC with different polymer coating compositions. It presented an extensive study of its physical characterization techniques, cytotoxicity cell study, drug encapsulation for controlled progesterone release, and mathematical modeling. Drug encapsulation was optimized to control nano-carrier release and to prolong its action duration. Release kinetics was analyzed by mathematical fitting, where results showed that peppas model was the best-correlated model, indicating the diffusional drug release mechanism from the hybrid matrix. In addition, the study highlights the capability to control drug release through alteration of environmental pH. It demonstrated that swelling of GC at lower pH and its shrinking at higher pH in a small change range can significantly trigger progesterone release. It is important to note, however, that the release pattern was the same at both environments, where an initial burst effect is noticed followed by a sustained release profile for 15 days.

The promising results shown in this thesis open many doors for future research on SPION-GC. Regarding drug delivery for breast cancer, it would be ideal to include a chemotherapeutic drug in addition to the hormonal therapy in the produced polymeric-metallic nanoparticles to mimic clinical therapy. Merging two therapeutics will probably enhance cancer treatment and build more efficient nanocarriers. Moreover, active targeting can be incorporated to offer a much more controlled delivery. This can be accomplished by further surface modification via conjugation of mediated ligands such as folic acid, which can
selectively target folate receptors that are over expressed by breast cancer cells. Also, since the proposed nanoparticles exhibit superparamagnetic properties, adding an external stimuli could be beneficial, such an external magnetic field, to allow for magnet-induced guidance to cancer sites. With regards to the \textit{in vitro} drug release, it would be best to test the hybrid polymeric-metallic on breast cancer cell to prove that these particles can in fact suppress their growth, and this will be ideally tested before and after the addition of dual-drug therapy. Furthermore, the ability to better control the initial burst effect can be investigated on to optimize it according to therapeutic effectiveness and lag time for nanoparticles to reach the desired site. Additionally, it is suggested to conduct an \textit{in vivo} drug release test in buffer, and that will also investigate magnetic targeting more accurately. Generally, the developed biocompatible nano-carrier gives a great initiative for further research in controlled drug delivery and targeted localized delivery.
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