SPION-C595 NANOPROBE FOR MAGNETIC RESONANCE IMAGE CONTRAST ENHANCEMENT OF HORMONE DEPENDENT BREAST CANCER CELLS

by

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AAS	Atomic Absorption Spectroscopy
Ab	Antibody
ABC	Accelerated Blood Clearance
ALND	Axillary Lymph Node Dissection
ASR	Age Standardize Rate
ATTC	American Type Culture Collection
B	Magnetic induction
BSA	Bovin Serum Albumin
CGS	Gaussian System
CT	Computerized Tomography
DCIS	Ductal carcinoma in-situ
DIW	Deionised Water
DLS	Dynamic Light Scanning
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EM	Electromagnetic Unit
EPR	Enhanced Permeability and Retention
FA	Folic Acid
FMT	Flourescence Molecular Tomography
FTIR	Fourier Transform Infrared Spectroscopy
H	External magnetic fields
h	hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HNO_3	Nitric acid
i.p	intraperitoneally
IgG	Immunoglobulin G
IO	Iron Oxide
kBr	Potassium Bromide
KSCN	Potassium Thiocyanate
LCIS	Lobular carcinoma in-situ
M	Magnetization
MAb	Monoclonal antibodies
MCF 7	Human hormone sensitive and invasive breast cancer cell line

MI	Molecular Imaging
MPS	Phagocytosing System
MRI	Magnetic Resonance Imaging
MTT	Methylthiazolydiphenyl-tetrazolium bromide
MUC1	Mucin 1
NEX	Number of Excitation
NP	Nanoparticle
NT	Néel temperature
OD	Optical Density
OI	Optical Imaging
PBS	Phosphate Buffer Salin
PdI	Polydispersity
PET	Positron Emission Tomography
PFA	Paraformaldehyde
R_1	Longitudinal relaxation rate
R_2	Transverse relaxation rate
RES	Reticuloendothelial System
ROI	Region of Interest
S	Standard
S	Spin quantum
SD	Standard Deviation
SEM/EDEX	Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy
SI	System International
SI	Signal Intensity
SPECT	Single-Photon Emission Computed Tomography
SPIO	Super Paramagnetic Iron Oxide
T	Tesla
T_1	Longitudinal relaxation time
T_2	Transverse relaxation time
TAA	Tumor Associated Antigen
T_E	Echo time
TEM	Transmission Electron Microscopy
T_R	Repetition time
USPIO	Ultrasmall Superparamagnetic Iron Oxide
Vs	Versus
VSPIO	Very Small Superparamagnetic Iron Oxide
VTA	Vascular Targeting Agent
VTF	Vascular Volume Fraction
XRD	X-ray Powder Diffraction

NANOPROB SPION-C595 UNTUK PENINGKATAN KONTRAS IMEJ RESONANS MAGNET BAGI SEL KANSER PAYUDARA BERSANDAR HORMON

ABSTRAK

Kini pengimejan diagnostik berkesan dan khusus kanser payudara pada peringkat awal merupakan suatu cabaran yang besar. Nanoperubatan memainkan peranan penting dengan cara menyampaikan agen kontras disasarkan kepada sel-sel tumor tertentu yang membawa kepada penambahbaikan dalam ketepatan diagnostik dengan visualisasi yang baik dan demonstrasi tertentu sel-sel tumor. Kajian ini menyelidiki fabrikasi, pencirian dan penggunaan (*in vitro* dan *in vivo*) agen kontras magnetic resonan kanser payudara tertentu. Nanozarah C595 ferum oksida superparamagnetik antibodi terkonjugat monoklonal telah dihasilkan melalui kaedah EDC, untuk mengesan tumor payudara peringkat awal dengan ungkapan MUC1 yang melebih. Selain itu, antibodi terkonjugat monoklonal ke atas ferum oksida superparamagnetik monoklonal telah disahkan menggunakan teknik FTIR, XRD, TEM, SEM-EDAX serta zetasizer. Lebih daripada 84% dan 98% peningkatan isyarat diperhatikan untuk imej wajaran T_1 dan T_2, masing-masing pada dos 100 μg Fe/ml Spion-C595. Untuk menilai keberkesanan SPION-C595, beberapa kajian *in vitro* telah dijalankan. Kajian saitotoksisiti sel *in vitro* telah dijalankan ke atas sebilangan sel MCF 7, mendapati bahawa SPION-C595 tidak menunjukkan ketoksikan yang ketara. Tambahan pula, inkubasi berpanjangan sel MCF 7 dengan SPION-C595 tidak menjejaskan morfologi sel serta keupayaan percambahanya.

Pemilihan nanoprob untuk jenis kanser payudara ini telah diperhatikan pada sel MCF 7 dengan biru Prusia dan AAS. Lebih-lebih lagi, kaedah novel 3D ketul barah Prusia biru, telah dibangunkan untuk menunjukkan pengikatan nanoprob pada ketul barah *in vitro*. Keputusan menunjukkan pengikatan signifikan nanoprob 200 μg Fe/ml terhadap tumor payudara MCF 7. Pengimejan *in vitro* MR menunjukkan perubahan yang jelas imej T2-ditimbang pada pengurangan 76% berbanding dengan sel-sel yang tidak dirawat pada dos 200 μg Fe/ml. Selain itu, imej *in vivo* MR menunjukkan peningkatan signifikan masa relaksasi T_1 dan T_2 tumor payudara selepas administrasi SPION-C595. Peningkatan kontras yang signifikan kanser payudara masih boleh dilihat dengan jelas walaupun 24 jam selepas suntikan. Serapan tumor yang signifikan telah diperhatikan pada pepejal payudara dalam haiwan yang diuji. Dengan demikian dapat disimpulkan bahawa nanopartikel magnetik terkonjugat dengan C595 mempamerkan keupayaan kontras MR yang tinggi (T_1 dan T_2), dan dapat digunakan sebagai agen kontras kanser payudara tertentu.

SPION-C595 NANOPROBE FOR MAGNETIC RESONANCE IMAGE CONTRAST ENHANCEMENT OF HORMONE DEPENDENT BREAST CANCER CELLS

ABSTRACT

Currently, effective and specific diagnostic imaging of breast cancer in early stages is a major challenge. Nanomedicine plays an essential role by delivering the contrast agent in a targeted manner to specific tumor cells, leading to improvement in accurate diagnostic by good visualization and specific demonstration of tumor cells. This study investigated the fabrication, characterization and application (*in vitro* and *in vivo*) of a specific breast cancer MR contrast agent. C595 monoclonal antibody-conjugated superparamagnetic iron oxide nanoparticles (SPION-C595) was developed by EDC method, for early stage breast tumor detection with MUC1 over-expression. Moreover, monoclonal antibody conjugation on superparamagnetic iron oxide was confirmed using FTIR, XRD, TEM, SEM-EDAX and zetasizer techniques. More than 84% and 98% signal enhancement was observed for T_1 and T_2 weighted images, respectively at doses of 100 μg Fe/ml of SPION-C595. To evaluate the efficacy of SPION-C595 several *in vitro* studies were conducted. *In vitro* cell cytotoxicity studies were conducted on the number of viable MCF 7 cells. It was found that SPION-C595 did not exhibit significant toxicity. Furthermore, prolonged incubation of the MCF 7 cells with SPION-C595 did not affect the cell morphology and its proliferation ability. Selectivity of the nanoprobe for this type of breast cancer is observed on MCF 7 cells by

Prussian blue and AAS. Moreover, 3D solid tumor Prussian blue, a novel method was established to demonstrate the binding of the nanoprobe on solid tumor *in vitro*. The results show significant binding of 200 µg Fe/ml nanoprobe towards MCF 7 breast tumor. The MR *in vitro* imaging shows the obvious change of T_2-weighed images at a 76% reduction compared with untreated cells at doses of 200 µg Fe/ml. Moreover, MR *in vivo* images shows significant enhancement of T_1 and T_2 relaxation times of breast tumor after administration of SPION-C595. Significant contrast enhancement of breast cancer could still be clearly seen even 24 hours post-injection. A significant tumor uptake was observed in solid breast in the tested animals. It thus can be concluded that the magnetic nanoparticles conjugated with C595 exhibit high dual (T_1 and T_2) MR contrast potential, and can be applied as specific breast cancer contrast agent.

CHAPTER ONE: INTRODUCTION

1.1 Background of the Study

Breast cancer is a major global health problem and the leading cause of death among women of all ethnic backgrounds. In Malaysia, breast cancer is the most common form of cancer where one in 19 Malaysian women will be diagnosed with cancer at the age of 85 ("Facts & Figures about Breast Cancer," 2014). The commonest cancer among females in Penang was reported to be breast cancer with 912 cases from 1994 till 1998. The Chinese had highest incidence of 648 cases, followed by Indians and Malays with 89 and 171 cases, respectively (Zarihah et al., 2003). The number of cases increased to 1087 cases among females during the period 1999 to 2003, when breast cancer was again mentioned as the most common form of cancer type among females. At that time, Malays had the lowest age standard rate (ASR) 25.8 in comparison with the Chinese and Indians rate (ASR 45.6 and 32.4), respectively (Rai et al., 2005). During the period 2004-2008, 1699 cases (ASR 48) were reported. Chinese females had the highest incidence compared to Indians and Malays, who had 612 more cases compared to the period 1999 to 2003 (Manan et al., 2010).

Breast cancer mortality has decreased due to hormone replacement therapy, mammography screening, and complete axillary lymph node dissection (ALND) (Giuliano et al., 2011; Njor et al., 2012; Olsen et al., 2005; and Zahl et al., 2005). Nevertheless, new diagnostic methods and treatments are needed. The surgical procedure normally performed is the removal of the whole breast or that part of the breast which contains cancer cells. This is followed removal of the lymph nodes

(positive cancer receptors), lining over the chest muscle or part of this section. After surgery the oncologist decides whether the patient will be given radiotherapy, chemotherapy or both to kill any remaining cancer cells. Chemotherapy and radiotherapy will decrease the chance of mortality, but they are damaging normal tissues and putting organs at risk. It has been suggested to dose the normal tissues with antibody-nanoparticle conjugates to minimize off-target effects (Brannon-Peppas et al., 2012; Fay et al., 2011).

Imaging has actually been preferred in scientific and technological applications due to its visual as well as intuitional interface. Biological imaging has been used in fundamental biology and medical sciences. Therefore, advanced techniques are continuously being launched to meet a wide range of biomedical requirerments when there are many types of imaging devices available. Thus, by enhancing imaging techniques, causes exceeding the conditions of current techniques. Developing new imaging tools, or even upgrading the current tools, requires a lot of effort and resources before launching to the laboratories and the hospitals (Safriel, 2003). Due to this fact, with the development of imaging equipment, many researchers were trying to fabricate probs and contrast agents to increase the detectability and sensitivity of imaging instruments. Therefore, the interrelation of biological systems and contrast imaging is producing remarkable biological information in visual forms. Without applying contrast agents, achieving these detailed images is difficult. Thus, imaging probes and the contrast agents are important inquiries in biological and medical sciences that provide an imaginative and farsighted vision for the analysis of biological information and the diagnosis of diseases. Recently, molecular imaging enhanced the capability of

biomedical images with diagnostic tools at the cellular and molecular levels, attracting much attention. Molecular imaging combines the molecular and *in vivo* studies (Park et al., 2009).

As the number of cancer cases increases, it is necessary to find a way to detect cancer in early stages. According to Radermacher et al., (2009), early diagnosis has become easier with genetic testing and radiologic approaches such as mammography, thermography, computerized tomography scan (CT scan), ultrasound, magnetic resonance imaging (MRI) and positron emission tomography (PET) as well as biopsy. Although all of these diagnostic techniques have been utilized, still breast cancer was only diagnosed years later. Despite different types of imaging, molecular imaging has recently emerged enabling the discovery and identification of new molecular pathways of living organisms in a non-invasive fashion. This has opened up new horizons into diagnosis, which has attracted the attention of many researchers. Molecular imaging is useful for the early detection of cancer. It can also help accelerate research and development of new drug delivery techniques for better therapeutic agents.

Diagnostic tools have been useful to detect and distinguish various different types of diseases. Despite their useful applications, they have acted as impediments as well. For instance, ultrasound has been used for detecting cancer in patients, however, for patients at higher risk, this tool is not suitable due to the many false-positive and also false-negative data produced. The CT scan is now commonly used to diagnose the inner mammary node and to examine the chest and axilla after mastectomy. However, the use of the CT scan can be harmful due to the high intensity of ionising radiation which can lead to cancer. The dye utilized in the CT scan can also cause allergic reactions in

certain individuals. The PET scan is another effective tool to diagnose and detect cancer. This imaging tool provides an anatomical and functional view of the tested cells. However, it is not able to detect tumors which are less than 5-10 mm. Furthermore, sensitivity of the mammography is inversely proportional to the breast density. Increase in breast density will reduce the sensitivity of detection. However, Kolb et al., (2002) reported that mammography is also not successful in young women who do not have breast density. It is noteworthy to mention that, the MRI is a non-invasive and very highly sensitive imaging instrument which can identify the primary site of breast cancer with a painless examination, by producing cross-sectional images of internal organs and full body inner structures just by using external magnetic fields and radio waves. Based upon the water content of each tissue and the magnetic properties of a certain lesion, various tissues or even organs of the body can be distinguished from each other by detecting different signals from images. The MRI has characteristics, but this can be compensated by using a special contrast agent with great relaxivity. Generally, a magnetically active material, which is known as a contrast agent, is applied to obtain clear images of internal structures or abnormalities. The MRI technique does not deal with ionizing radiation however, unlike other diagnosis tools such as CT, PET, and single-photon emission computed tomography (SPECT) which depend on ionizing radiations. The high energy radiations can damage the deoxyribonucleic acid (DNA).

Besides the diagnosis tools and techniques, antibodies and monoclonal antibodies are widely used in cancer diagnosis *in vitro* and *in vivo.* One of the targets of the breast tumor is the breast specific membrane antigen (MUC1). The capability of MUC1 for causing a tumour is related to cellular transformation, which is used as a

diagnostic marker in cancer that is accompanied with antibodies against the tumor associated antigen (TAA). The epithelial mucin generated by the MUC1 gene is present in ductal epithelial cells and is more than 90% due to the presence of estrogen receptors in breast cancers. The expression of the MUC1 protein is considerably unregulated on tumors, which undergoes alteration in glycosylation as well as distribution, leading to exposure of the core protein of the tandem repeat region. Overexpressions of mucin together with distribution on the cell surface are believed to affect the biological behavior of the tumor cells at the time of malignant transformation. MUC1 in cancerous cells are different from the one in normal cells in terms of light O- linked glycosylation. Moreover, mucins are found at the epithelial surface of the mammary gland, kidney, uterus, prostate and testis (Hollingsworth et al., 2004, and Mcguckin et al., 1995). Therefore, the MUC1 antigen may be a useful diagnostic target to minimize the growth of incurable cancers (Hattrup & Gendler, 2006 ; Wang et al., 2007).

Bon et al., (1999) and Rahn et al., (2001) actually found that the presence of any MUC1 in most of the tumor cells is associated with an improved prognosis. However, a significant relationship between expanding amounts of positivity and improved recurrence free survival or overall survival has been reported.

Previous studies showed that several methods in MR imaging have been carried out in the form of non-contrast enhancing techniques as well as enhancing techniques. In 1988, Gd-DTPA, the first MRI contrast agent was developed, and it opened a door into an exciting research area that focuses on the contrast-enhanced method. The MRI contrast agents consist of two categories; T_1 and T_2 contrast agents. They differ in their magnetic properties as well as relaxation mechanism (Hengerer et al., 2006; Wang,

2011; Zhou et al., 2013). MR imaging methods are, (a) T_1-weighted, which improves the image contrast, (b) T_2-weighted contrast agent, mainly used for identifying the early stage tumors and malignancy, (c) diffusion-weighted magnetic resonance imaging, which has the potential to detect a malignant tumor by mapping the diffusion process of water molecules in a tumor, *in vivo* (Kuhl et al., 1999; Kuroki et al., 2004; and Sinha et al., 2002). The accuracy and reliability of MR imaging, using contrast agents is significantly better in high risk cancer women, because the contrast of the specific region in the tissue will be enhanced due to the affect the signal has on the surrounding tissue (Group., 2005; Kriege et al., 2004; and Kuhl et al., 2000). These contrast agents are used primarily to increase the sensitivity of the MRI to detect and characterise several pathologies. Therefore, they continue to be used as a new series of contrast agent or probes for clinical indications (Wang et al., 2001).

1.2 Statement of the Problem

The targeted delivery of superparamagnetic iron oxide nanoparticles (SPIONs) as a contrast agent may facilitate their accumulation in cancer cells and enhance the sensitivity of MR imaging. The MRI contrast media may perhaps improve its application for imaging of highly soft tissue interms of safty (Brannon & Blanchette, 2012).

Early detection of breast cancer is the key to designing effective treatment strategies and prolonging life span. By considering the usefulness of the MR imaging tecchnique, MRI techniques can be used to detect breast cancer through fabricated SPION-C595 nanopribe using a simplifed method. This might improve the sensitivity of the MRI for detecting early stage breast cancer tumors. The nanoprobe will be in the

category of MR contrast agents. Moreover, it must be noted that much research in nanotechnology and molecular imaging field have been done using different types of nanoparticles and antibodies which were conjugated. With regard to existing researches, the SPION-C595 fabricated nanoprobe will target MUC1 expression on the MCF 7 cells. However, the MR *in vivo* imaging study has not yet succeeded.

1.3 Research Objectives

The aim of this research is to develop a nanoprobe that consist of superparamagnetic iron oxide (SPIO) conjugated to a C595 Monoclonal antibody for early detection of breast cancer. The research objectives are as follows:

1. To functionalize and characterize SPION-C595 nanoprobe.
2. To determine the sinsitivity and selectivity of the nanoparobe to breast cancer cells (MCF 7).
3. To determine T_1 and T_2 relaxation times of a SPION-C595 in magnetic resonance imaging.
4. To characterize the biological distribution of the nanoprobe (SPION-C595) at optimal dose in xenograft tumor model.

1.4 Scope of Research

In this study, the nanomag-D-spio (20 nm) and the MUC1(C595) monoclonal antibody were used as the main compounds for the nanoprobe fabrication. Following physical and chemical characterization, *in vitro* and *in vivo* studies were performed to assess the effectiveness of the nanoprobe. The breast cancer cells (MCF 7) and

endothelial cells (EAhy.926) utilised throughout the study. To determine the properties of the nanoprobe *in vivo*, NCR NU/NU nude mice transplanted with the MCF 7 breast cancer were used. To determine the enhancement of T_1 and T_2 relaxation times, 1.5 T MRI machine was used. The T_1 and T_2 relaxation enhancement was optimized in digested breast tumors (MCF 7). The images of the samples were obtainted using spin-echo sequences. T_2^* relaxation is seen only with gradient-echo (GRE) imaging, nevertheless, the scop of MR imaging of this thesis focused on the spin-echo sequences. The SPION-C595 is designed to target overexpression of MUC1 receptor on breast cancer as the nanoparticle will be conjugate with the MUC1 antigen (C595 monoclonal antibody). Moreover, the biodistribution of new nanoprobe will be checked for liver, kidney, and spleen together with the breast tumor. One of the limitations in this study is the lack of accessibility to the MR imaging for *in vivo* studies. To overcome this problem, disgested tumor samples were sent for MR imaging. For distribution in addition to the tumor tissues, spleen, kidney and liver were also analyzed.

1.5 Significance of the Study

MRI has an important role in cancer prediction as well as diagnosis. Paramagnetic contrast agents are actually used to enhance the image contrast for better cancer detection as well as for the evaluation of treatment efficacy. Numerous efforts have already been made to fabricate better contrast agents with significant relaxivity, low toxicity, and also tumor specificity. This achievement helps to attain biological and functional details in an image as a result of the composition of the biological system coupled with the contrast agent. The ultimate goal of using SPIONs in diagnosis is to reduce patient suffering by applying selective treatments where efficiency is increased

through local concentrations, and general side effects are avoided. Targeted SPIONs might enhance the signal intensity as specific breast cancer contrast agents to recognize the breast cancer lesion in MR imaging. In addition, the metastasis of breast cancer cells will be limited. Early stage cancer diagnosis, that is, before it spread is more likely to be treated successfully. In cases where the cancer has spread, treatment becomes increasingly difficult, and generally a person's chance of survival decreases. Early detection means using an approach that enables breast cancer to be diagnosed before it occurrs. In contrast, breast cancer found during screening exams is more likely to be smaller and still confined to the breast. Doctors believe that early detection examination for breast cancer will save thousands of lives every year and that many more lifetimes could be saved in case if more, women and their health providers will take advantage of these types of tests.

1.6 Thesis Organization

This thesis consists of five chapters, starting with the introduction in Chapter one which consists of a review of breast cancer rate in Penang and Malaysia, the statement of the problem, research objectives, scope of the study, significance of study, and thesis organization. Chapter Two covers the theoretical background and literature review. It reviews the magnetization, classification of contrast agents, drug delivery, USPIO and antibody conjugation, molecular imaging, MUC1 and introduces breast cancer and the nanoprobe for breast cancer detection. In Chapter three, materials and equipment, characterization, *in vitro* and *in vivo* methods are explained. Chapter Four, displays all results in this study are reported followed by the discussion section. Chapter Five will summarizes the work followed by the conclusions and give suggestion for future work.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Two well-known forms of superparamagetic iron oxide (SPION) are magnetit (Fe_3O_4) and maghmetite (γ-Fe_2O_3). Ferrit substances that are mixed oxides of iron with other types of transition metal ions, for example Cu, Co, and Mn, are considered to be superparamagnetic. However, the scope of this research is just focused on the superparamagnetic properties of conjugated iron oxide nanoparticles with monoclonal antibodies. This chapter will cover the literature review on this subject and discuss topics such as magnetization, contrast agents and their classifications, superparamagnetic iron oxide nanoparticles that have been conjugated with different types of monoclonal antibodies as targeted nanoprobes and contrast agents, molecular imaging and drug delivery, breast cancer, and the chapter concludes with a summary.

2.2 Magnetism

The movement of an electron induces magnetism, which includes orbital motions and the spin of an electron. The electron is like a spinning sphere of charge, and its rotation causes a magnetic field around the spin. The orbital motion of the electron creates flow of charge, with a magnetic dipole generated by the flow. Then, all the magnetic dipoles in the molecular orbitals assemble into a net magnetization that generates currents loops around atoms. Virtually all materials naturally possess magnetic fields, where their magnetic properties are controlled by either spin or orbital motion (Beiser, 1986; Kittel et al., 1976).

- **Magnetization**

The homogeneous magnetic properties of ferri and ferro magnetic substances are noticeable. The alignment of ferromagnetic materials is similar to that of anti-ferromagnetic materials, which is in an anti-parallel order. Their unequal magnitude causes an impulse magnetic-field, where more than two interpenetrating sublattices are present. Ferrimagnetism occurs in ionic substances, for example, in magnetite (Fe_3O_4) which has two sublattices. The sublattices are an octahedral and a tetrahedral which are divided by oxygen.

To magnetize a substance, the substance should be placed in an external magnetic field (H). The intensity of the substance per unit volume is termed magnetization (M). The flux of the magnetic lines of forces exerted on the substance in a magnetic field is called magnetic induction (B), which is represented by as;

$$B= H + 4\pi M \tag{2.1}$$

The 4 π factor originates from the unit field generated by a unit polar on the surface of a sphere with 1 cm radius, which surrounds the pole with a surface area of 4 π^2 (Trout, 2000). The magnetism of a material is controlled by the various arrangements of magnetic moments and also their responses to an external magnetic field. The magnetic materials are classified into four groups as shown in Figure 2.1, which includes paramagnetism, ferromagnetism, antiferromagnetism, and ferrimagnetism.

Faraday's law of induction, due to 19th century physicist Michael Faraday. He explained electromagnetic induction using a concept he called lines of force. This is a

basic law of electromagnetism predicting how an electric current produces a magnetic field and, conversely, how a changing magnetic field generates an electric current in a conductor. The equation that expresses Faraday's law is defined as below,

$$\mathcal{E} = -\frac{dF}{dt} \tag{2.2}$$

Where $\mathcal{E}$ is the electromotive force (EMF), that refers to the potential difference across the unloaded loop, and

$$F(t) = \int B(x,t).dS. \tag{2.3}$$

In the magnetic flux through a surface S bonded by a contour C.

- **Magnetization units**

There are diverse sets of unit systems which have been used in magnetization measurement. They consist of the CGS (centimetres, grams, and seconds) system and the SI (system international) unit systems. In the Gussian system, magnetization (M) is represented as electromagnetic units per volume (emu/cm^3) or (emu/g). However, in SI units, magnetization (M) is defined as Tesla (T). Magnetic field (H) is measured in Oersted (Oe), while, in SI, magnetic field is measured in amper per meters (A/m).

2.3 Pinciple of Magnetic Resonance Imaging (MRI)

MRI is a non-invasive medical technique used to scan human body (soft tissue). The MRI phenomenon was discovered independently by Felix Block and Edward Purcell in 1946. Basically an MR device consists of (a) a big magnetc to generate the applied magnetic field B, (b) coils to make the magnetic field homogeneous, (c)

radiofrequency (RF) coil for transmission of a radio signal, (d) a receiver coil to sense the emitted RFsignals, (e) gradient coils for spatial localization of the signals, and (f) a computer for reconstruction of the received RF signals into image.

MRI images signal from atomic nuclie with a net spin. Spin is a vector quantity which comes in multiples of ½ and can be positively (+) or negatively (-) charge. Moreover, spin is an intrinsic property of materials which can be observed in the form of angular momentum in elementary particles, composite particles, electron and atomic nuclei. Atomic nucleus consists of nucleons (proton and neutron). Two or more nucleons with spins of opposite signs can pair up to eliminate the presence of a spin. However, unpaired nuclear with a net spin has intrinsic magnetic dipole moment μ which is the source of MRI signal. There are manu nuclei with net spin such as ^{13}C, ^{19}F, ^{31}P, ^{23}Na and 1H where MRI signal can be imaged. However, Hydrogen nuclei are abundant in the body tissue as water, fat, protein and macromolecules. Therefore, in MRI, the signal from Hydrogen nuclei which consists of a single positively charge proton is imaged. During MRI procedure the free hydrogen nuclei (proton) in human body align to the direction of the magnetic field with a net magnetic moment M which is parallel to B_0. Similar to gyroscopes, the nuclei precess by a phenomenon called Larmore precession about the magnetic field direction. The nuclei precess with a frequency known as Larmore frequency ω_0 which is defined as follows:

$$\omega_0 = \gamma B_0 \tag{2.4}$$

where γ is gyration ratio which equal to 42.58 MHz/T for hydrogen, and B_0 is the applied magnetic field strength. In a typical medical clinical application, the B_0 used is

between 1.5 or 3 T. at 1.5 T, the Larmore frequencies ω_0 for Hydrogen nuclei are 63.9 MHz. altening the B0 strength affects the Larmore frequency at which the protons precess. Consequently, increase the imaging period. Alignment of M (hydrogen proton) to B_0, a radio-frequency (RF) pulse with Larmor frequency value is introduced perpendicular to B0. This pulse causes proton turns over to the high energy state. The total M flipes away from B_0 (M_z orientation) with a flip agnle to M_{xy} axis. The longer the applied RF pulse the stronger and bigger the deflection of M and higher the angle (90 or 180 degrees). Therefore, when the frequency is turned off, the high energy hydrogen nuclei emitted is absorbed RF energy and turn to their low energy (ground) state. Thus, M realign themselves again and parallel to B0. The return of net magnetization to the equilibrium state is called relaxation. During relaxation, the emitted RF energy from the protons as they move to realign with the magnetic field, and fall out of phase with each other is picked up as MRI signal by RF COIL in the MRI system. The signal is measured as function of time.

However, not all the RF energy given off by the proton is detected as signal; some are observed as thermal energy which heat up the immediate tissue called lattice. Relaxation process in two forms, longitudinal (parallel) and transverse (perpendicular) to B_0. The time constants which describe how the relaxation processes take place are called T_1 and T_2 respectively.

Intracranial calcification refers to the deposition of crystalline calcium in the parenchyma in the brain. Calcification could appear in physiological as well as pathological conditions. However, calcium deposits can be associated with several intracranial pathologies including tumors. For diagnosing, the location and

characteristics of the calcification in these lesions plays an important role. In MRI, calcification appears with various signal intensities on T_1 or T_2-weighted images (Nu et al., 2009), which makes it difficult to identify definitively as calcium. However, in gradient-echo acquisitions, calcifications appear as hypointense. It has been recognized that using phase helps to discriminate between calcium and iron because calcifications tend to be diamagnetic and iron paramagnetic. Therefore, they appear with the opposite signal intensity in filtered phase images (Zhu et al., 2008). Dou et al., (2016) studied the diagnostic capabilities of susceptibility-weighted imaging (SWI) to detect prostate cancer and prostatic calcifications. According to the results, susceptibility-weighted imaging showed more sensitive and specific in compare to conventional magnetic resonance imaging, diffusion-weighted imaging, and computed tomography in detecting prostate cancer. Moreover, susceptibility-weighted imaging identified the prostatic calcifications similar to computed tomography. Bai et al., (2013) investigated on prostate cancer patient as well as the patients with benign prostatic hyperplasia by using 3 T MR and a 16-row CT scanner. CT demonstrated calcifications in 22 patients which were all detected by SWI whereas only 3 were detected by conventional MRI. Compared to CT, SWI demonstrated 100% in the diagnostic sensitivity, specificity, accuracy in detecting calcifications in prostate but conventional MRI demonstrated 13.6% in sensitivity, 100% in specificity, 75% in accuracy.

2.3.1 T_1 relaxation time

T_1 relaxation time measures how net magnetisation vector M recovers to its ground state (M_z orientation) in the direction of B_0. It's also known as spin-lattice relaxation time, due to the process whereby the excited protons (spins) released its

absorbed energy back into the surrounding lattice. Thus, thermal equilibrium is created between the spin (hydrogen proton) and the lattice. T_1 values are longer at higher field strengths. T_1 relaxation is an exponential process; the equation governing this behaviour is as follows:

$$M_z(t) = M_{max}[\,1 - e^{-t/T1}] \qquad (2.5)$$

Where M_z (t) is the magnetization at time equal t, M_{max} is the maximum magnetization at full recovery along the z orientation. The spins are completely relaxaed after t is 3-5 T_1 times. This recovery rate is a function of T_1 which is unique to every tissue. The Mz recovery rates ineach tissue permit MRI to differentiate between different types of tissue. Signial in MRI images is high or low (bright or dark). Therefore, fat appear bright in T_1 weighted image because it has long T_1. While water such as cerebrospinal fluid (CSF) is dark owning to its low T_1.

2.3.2 T_2 relaxation time

Immediately after a 90^o RF pulsed is applied the net magnetization M_0 flipped onto the XY plane. Thereby, there is gradual lost in phase movement of the spins. Consequently, there is a rapid decrease (decay) of the net magnetization between the spins in XY plane. This spin-spin relaxation time is termed T_2 which is an exponentioal function and its defined as follow:

$$M_{xy} = M_0 e^{-t/T2} \qquad (2.6)$$

Similar to radioactive decay, M_{XY} is the amount of magnetization that decayed at a time t and M_0 is the initial net magnetization. Both T_1 and T_2 processes occure

simultaneously. T_2 is less than or equal to T_1, the net magnetization in XY plane decay to zero and then the longitudinal magnetization recovers until there is no M_0 along Z plane. Due to short T_2 time in semi-solid tissues and tendons their image appears dark on T_2-weighted images. T_2 is long in water therefore urine and CFS appears bright on T_2-weighted images. However, there is a phenomenon called T_2^* which is due to magnetic inhomogeneity (non-uniformity in the scanner magnet itself) and magnetic susceptibility effects from the patient inside the field. T_2^* decay has greater magnitude than T_2 in tissues and this causes rapid signal loss. Moreover, in a perfectly uniform magnetic field and the patient without susceptibility effects, the T_2 and T_2^* would be equal.

2.4 MRI contrast agents and principels

Imaging has been widley used in scientific and technological application due to its visual ans intuitional interface. In particular, biological imaging has been a rapidly growing field, nor only in fundamental biology but also in medical science. An image must have the proper brightness and contrast for easy viewing. Brightness refers to the overall lightness or darkness of the image. Contrast is the difference in brightness between objects or regions (Na et al., 2009). Image consists of a collection discrete cells, that known as pixels (picture elements). Each of the pixels has a pixel value which describes how bright that pixel is, and/or what color it should be. For a grayscale images, the pixel value is a single number that represents the brightness of the pixel. Where for a particular portion of the image, if the pixel is a small block, it represents the amount of gray intensity to be displayed. For most images, pixel values are integers that range from 0 to 255 (black to white).

MRI is currently one of the most powerful diagnostic tools in medical science. It has been the preferred tool for imaging the brain and the central nerve systems, for assessing the cardiac function, and for detecting tumors. Because it can give anatomic images of soft tissued with high resolution, it is expected to become a very important tool for molecular and cellular imaging. Although MRI can give detailes images, making a diagnosis based purely on the resulting images may not b accurate, since normal tissues often show only small differences in relaxation time. MRI contrast agents, which can help clarify images, allow better interpretation in such cases (Caravan et al., 1999; Semelka et al., 2001)

The MRI contrast enhancement occurs as a result of the interaction between the contrast agents and neighboring water protons, which can be affected by many intrinsic and extrinsic factors such as proton density and MRI pulse sequences. The basic principale of MRI is based on nuclear magnetic resonance (NMR) together with the relaxation of proton spins in a magnetic field. When the nuclei of protons are exposed to a strong magnetic field, their spins align either parallel or antiparallel to the magnetic field. There are two different relaxation pathways (Fang & Zhang, 2009).

The first, called longitudinal or T_1 relaxation, involves the decreased net magnetization (M_z) recovering to the initial state. The second, called transverse or T_2 relaxation, involves the induced magnetization on the perpendicular plane (M_{XY}) disappearing by the dephasing of the spins. Based on their relaxation processes, the contrast agents are classified as T_1 and T_2 contrast agents. Commercially available T_1 contarst agents are usually paramagnetic complexes, while T_2 contrast agents are based

on iron oxide nanoparticles, which are most representative nanoparticle agents (Kim et al., 2009; Shokouhimehr, 2010).

2.5 Classification of contrast agents in MRI

Current diagnostic tools in the industry are as follows: CT, optical imaging (OI), MRI, PET, SPECT, fluoroscopy and, ultrasound. The purpose of utilizing these imaging tools is to study the cellular functions of living organisms with related diseases, by obtaining biological details as well as functionality statuses at an early clinical stage. Imaging techniques have been commonly applied in science and technology due to their visual and inherent interface. They are widely used in fundamental biology as well as in medical science, especially in cases of biological imaging. However, there are many imaging tools available and researchers are trying to improve and advance techniques for a variety of biomedical applications. Usually, new imaging tools are required to be tested through *in vitro* and *in vivo* experiments before being applied clinically (Brown et al., 2011).

The MRI is currently the most effective diagnostic tool in medical imaging. It has been the preferred tool for imaging the human brain along with the central nervous system, for assessing cardiac function, and for detecting tumor malignancy. It provides anatomical images of the soft tissues with higher resolution. Moreover, it has become an essential tool for molecular as well as cellular imaging. The MRI is based on assessing water molecules or the relaxation time of protons. The proton relaxation rate differs from others in different environments, because the property of water molecules vary according to the physical environment (Na et al., 2009; Park et al., 2009).

Accordingly, through the use of innovative imaging technologies, several studies have been carried out to design contrast agents to improve the sensitivity and detectability of such specific materials. Contrast agents help in obtaining biological and functional details in images through the composition of biological system compositions with the contrast agents (Koo et al., 2011). Such information from images would not be obtainable without the use of contrast agents. Imaging probes and contrast agents are important research tools in the field of disease diagnosis. Nowadays, among other non-invasive techniques, MR imaging has been launched in clinical diagnosis applications. To enhance this tool, innovative materials such as magneto-pharmaceuticals products are needed known as contrast agents. Contrast agents increase the contrast between normal and abnormal tissues in targeted body organs as well as the blood flow rate (Coroiu, 1999). Currently, biomedical imaging attracts the attention of researchers as a result of its enormous analytical and diagnostic capability at the molecular or cellular level. Hence, a cross of molecular biology and *in vivo* imaging which is a field called molecular imaging has emerged (Kumar, 2007). Paramagnetic contrast agents were categorized into two groups, which consists of gadolinium (III) chelates, representative of the T_1 (longitudinal relaxation time) agent and the SPIO nanoparticle, representative of the T_2 (transversal relaxation time) agent. T_1 agents are extremely toxic, while T_2 agents are nontoxic.

The SPIOs contrast agents (nanoprobes) are normally composed of one superparamagnetic iron oxide core and a shell (Babes et al., 1999). The characteristics of nanoprobes that can be used as contrast agents are: (a) the surface of nanoparticles must be modified for efficient attachment to biological materials; (b) the cellular uptake must

be easy; (c) there must be great distribution and function of the nanoparticles for cellular imaging; (d) they must cause very few side effects, and (e) they must be easy to deliver to the target (Kuo et al., 2006).

It was highlighted that dextran-coated iron oxides are safe for the body, and can be circulated in the blood and sequestered by phagocytic kupffer cells in the normal reticuloendothelial system (RES) of the liver to clear from the blood. Therefore, nano-sized iron oxide coated with dextran, has been utilized for liver contrasting (Wang &Yi-Xiang, 2011). The classification of SPIO contrast agents is done according to their size, as well as their physiochemical and pharmacokinetic properties. A nanoparticle (NP) is considered to be SPIO if it has a size greater than 50 nm and an USPIO if the nanoparticle is smaller than 50 nm (Berry, 2009, Bonnemain, 1998, and Pankhurst et al., 2003).

2.5.1 Positive contrast agents

When the nuclei of protons are exposed to a strong magnetic field, their spins alignment will either be parallel or antiparallel to the magnetic field. There are two relaxation pathways. The first one is called the T_1 longitudinal relaxation, which involves the decreased net magnetization recovering to the primary state (Zarihah et al., 2003). The second one, identified as transverse or T_2 relaxation, comes with the magnetization produced on the perpendicular plane (M_{xy}) which disappears by the dephasing of the spins. According to their relaxation procedures, the contrast agents are categorized as T_1 and T_2 contrast agents. Commercially existing T_1 contrast agents are

generally paramagnetic complexes, since T_2 contrast agents are based on iron oxide nanoparticles agents (Schwert et al., 2002).

A net magnetization (M) vector which is composed of M_z and M_{xy}, causes the spins to interrelate. When the spins receive an RF pulse, a transverse magnetization (M_{xy}) is generated on their xy-plane due to the RF pulse flipping exactly 90 degrees in the direction of the magnetic field. According to the theory, by transferring energy, Mz changes, and this causes M_{xy} to also change, leading to spin dephasing. This phenomenon occurs and directly after applying the RF pulse and randomizing the magnetization of excited spins with the same phase coherence. The spin phase starts to vanish in the xy-plane as a result of the various magnetic fields that the protons experience. The magnetic field difference is produced by the system's overall performance in shimming and the magnetic properties of the imaging materials. However, the inhomogeneity of the static magnetic field caused by the system's imperfections is typically decreased by a variety of applications. These include shimming coils and shimming algorithms, or the usage of the spin echo sequence which opposes this effect and impacts on the decay of transverse magnetization. Since they are of another source of field inhomogeneity, the magnetic properties of imaging physical objects can cause phase incoherence. The spin-spin interaction between the hydrogen nuclei and electrons results in a loss of transverse coherence, which creates the T_2-weighted images of body tissues (Hayat, 2007).

The first category of a particular T_1 contrast agent is based on its nano-structured frames that consist of many anchoring sites for paramagnetic ions. Those particles can carry a large number of paramagnetic payloads and produce a strong T_1 contest. T_1

relaxation is a method of equilibration of the net magnetization after applying a radio frequency pulse (Zarihah et al., 2003). This change of Mz is caused by the energy transfer between the proton spin system and the neighboring molecules. Almost all biological systems consist of various molecules and organisms in which their water protons have different relaxation characteristics and different T_1 relaxation times. The presence of the paramagnetic iron, an ion near the tissue, increases its own relaxation and shortens the T_1 relaxation time. Transition and lanthanide and metallic ions in particular, with a large number of unpaired electrons, such as Gd^{3+}, Mn^{2+} and Fe^{3+} (Shanehsazzadeh et al., 2014), have been confirmed to induce effective relaxation enhancement. Gadolinium (III) complexes are the most typical MRI contrast agents. They have seven unpaired electrons that result in a large magnetic moment. It has a substantially prolonged relaxation time if the toxicity of the Gd^{3+} ions is not considered. These metal ions are known as coordinated complexes or chelating ligands (Shellock & Spinazzi, 2008).Various surface modification, such as silicas, dendrimers, perfluorocarbons, and nanotubes, have been used to increase the application of the mentioned ions.

T_1 contrast agents are light compounds in terms of molecular weight and contain a single Lanthanide chelate, due to their long lifetime, to produce contrast. The concentration of these contrast agents for imaging is greater than iron oxide when molecular quantity is measutred in mmolar. It has been estimated that the T_1 relaxivity value is in the range of 5-80 $(mMs)^{-1}$. As such, a T_1 contrast agent would seem to have the potential to be active by target-mediated methods to increase relaxivity.

T_1 contrast agents enhance T_1 relaxation, which causes signal-increasing imaging effects. The most significant advantage of T_1 contrast agents compared to T_2 contrast agents is that they improve imaging by signal enhancing, which can maximize the results of MR imaging and produces anatomic imaging with higher spatial resolution. Furthermore, their bright signals are typically recognized clearly from some other pathogenic or biological conditions. Whereas with T_2 contrast agents are essentially paramagnetic they will not effect the magnetic homogeneity over considerable dimensions, which usually have the capability to disturb other anatomic backgrounds. Despite the fact that contrast agents, formulated with gadolinium (III) increase longitudinal relaxations, there exist absolutely no natural Gd (III) ion-based biochemistry components in humans. Moreover, manganese, iron, iron (III), and copper have different magnetic moments and applications due to less unpaired electrons. Contrast agents have short life spins inside the body and work in a nonspecific manner. Approximately all of the T_1 contrast agents will be within the extracellular space and usually interact with the blood, which is a disadvantage of molecular probes due to their short tracing time. The representative of the T_2 MR contrast agent category is SPIO. The synthesized nanoparticles are in general coated with hydrophilic polymers and improved to be much more stable inside the body (Cheon & Lee, 2008; Xu & Sun, 2013).

2.5.2 Negative contrast agent

The magnetization of paramagnetic components, for example, gadolinium complexes, is specifically dependent on the variety of ions, and they do not have magnetization in the absence of an external magnetic field. However, ferromagnetic iron oxide possesses a very large magnetic susceptibility, which might persist even upon the