SYNTHESIS AND CHARACTERIZATION OF HYPERTHERMIA INDUCED SPIONS-PEG-HER IN TARGETED BREAST CANCER

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Dedicated to

My beloved parents and siblings, my main supportive supervisor Prof. Dr. Ani Idris and also my helpful co-supervisor Prof. Dr. Fadzilah Adibah Abdul Majid

Thank you very much for being supportive, helpful and understanding

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ABSTRACT

Hyperthermia has opened up key avenues in cancer therapy. Nevertheless, engineering a smart and efficient tumor targeting superparamagnetic fluid agent capable of elevating the temperature of targeted sites as well as exposing a safe level of biocompatibility remain remarkably demanding and challenging. In this study, a novel core-shell tumor-targeting superparamagnetic iron oxide nanoparticle-polyethylene glycol-Herceptin (SPIONs-PEG-HER) was developed and evaluated for an efficient hyperthermia treatment of HER2+ breast cancer using an alternating magnetic field (AMF). Both *in vitro* and *in vivo* treatment models using four different cell lines and 7, 12 dimethylbenz (α) anthracene (DMBA)-induced balb/c mice were developed, respectively. SPIONs modification was carried out by PEGylation to provide biocompatibility and conjugation of Herceptin to add the tumor-targeting features to the SPIONs. The morphological characterization and physico-chemical analyses of SPIONs-PEG-HER, were carried out using transmission electron microscopy (TEM), field emission scanning electron microscopy, dynamic light scattering (DLS), x-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), vibrating sample magnetometer (VSM) and specific absorption rate (SAR). The *in vitro* biocompatibility confirmation of SPIONs-PEG-HER was performed using tetrazolium dye (MTT) assay, Trypan blue staining method and blood compatibility tests. The ability of SPIONs-PEG-HER binding to HER2+ cell line (SK-BR-3) was measured comparatively to HER2- cell lines (HSF 1184, MDA-MB-231, MDA-MB-468) via *in vitro* binding studies. The qualitative and quantitative data were obtained using Prussian blue staining, TEM assay and atomic absorption spectroscopy (AAS) analysis. The comparison on the changes in growth inhibition rates in HSF 1184, MDA-MB-231, MDA-MB-468 and SK-BR-3 cell lines at different temperatures (40 °C, 42 °C, and 45 °C) were studied using a post-hyperthermia MTT assay. Then, post-hyperthermia morphological investigations were performed via inverted microscope and acridine orange/ethidium bromide (AO/EB) staining method. The *in vivo* model using DMBA-induced balb/c mice were injected with SPIONs-PEG-HER via tail vein. The biodistribution of SPIONs-PEG-HER at tumor site as well as in vital organs were qualitatively and quantitatively measured using colorimetric methods (AAS and TEM). Finally, the survival rate of the DMBA-induced balb/c mice injected with SPIONs-PEG-HER was measured in the presence and absence of AMF and the daily monitoring of the weight of the treated DMBA-induced babl/c mice during the treatment period was carried out. The morphological characterization and physico-chemical analyses revealed that the synthesized SPIONs-PEG-HER had a size of almost 17 nm and possessed a nearly spherical appearance as well as greater hydrodynamic diameter (~84 nm) and a wider distribution compared to the bare SPIONs. Moreover, XRD and FT-IR analyses confirmed that the processes of PEGylation and conjugation were successfully accomplished while the VSM and SAR analyses showed that the SPIONs-PEG-HER possess efficient magnetic properties to be used as a hyperthermia fluid agent. SPIONs-PEG-HER showed relatively low levels of toxicity even at the extremely high concentration of 1000 μg/ml. The results of the binding studies indicated that the SPIONs-PEG-HER could selectively bind to the HER2+ cancer cells. The results obtained from the post-hyperthermia MTT assay indicated that exposing the HER2+ cells to the temperature of 45 °C for 20 minutes inhibited the growth of the cells by 90% where they did not regain their normal proliferation like the HER2- cell lines which corroborates the results obtained through post-hyperthermia morphological analyses where it was shown that the temperature of 45 °C induced significant apoptosis compared to the other temperatures. Through morphological alteration studies by inverted microscope and AO/EB staining method, it was disclosed that the SK-BR-3 cells had undergone apoptosis since apoptotic signs such as shrunk cells as well as apoptotic bodies were obviously seen. The results of biodistribution studies showed significantly higher accumulation of the SPIONs-PEG-HER in the tumor site compared to SPIONs-PEG. Based on the results obtained through hyperthermia treatment of DMBA-induced balb/c mice, it was revealed that the survival rate in the experimental group treated with SPIONs-PEG-HER in the presence of AMF was much higher than other experimental groups where 50% of the DMBA-induced balb/c mice survived and maintained their average body weight. Findings in this study illustrated that SPIONs-PEG-HER-mediated hyperthermia is a potent breast cancer treatment.

ABSTRAK

Hipertermia telah membuka ruang utama dalam terapi kanser. Walau bagaimanapun, pembentukan tumor yang pantas dan berkesan menyasarkan ejen cecair super paramagnet yang mampu meningkatkan suhu asas yang disasarkan serta mendedahkan tahap selamat kesesuaian-bio agar kekal sebagai sesuatu yang amat mencabar. Dalam kajian ini, rangkaian teras-kelompang tumor mensasarkan partikel nano ferum oksida super paramagnet-polietilena-*Herceptin* (SPIONs-PEG-HER) telah dibangunkan dan dinilai untuk rawatan *hipertermia* HER2+ kanser payudara dengan menggunakan medan magnet selang-seli (AMF). Kedua-dua *in vitro* dan model rawatan *in vivo* menggunakan 4 baris sel yang berbeza dan 7, 12 dimetilbenz (α) antrasena (DMBA) yang disebabkan tetikus *balb/c* telah dibangunkan. Pengubahsuaian SPIONs telah dijalankan oleh *PEGylation* untuk menyediakan kesesuaian-bio dan hubungan antara HER untuk menambah ciri-ciri tumor yang disasarkan ke atas SPIONs. Pencirian morfologi dan analisis fiziko-kimia SPIONs-PEG-HER telah dijalankan dengan menggunakan mikroskopi pancaran elektron (TEM), mikroskop elektron pengimbas pancaran medan, penyerakan cahaya dinamik (DLS), pembelauan sinar-x (XRD), spektroskopi inframerah transformasi Fourier (FT-IR), magnetometer sampel bergetar (VSM) dan kadar penyerapan tertentu (SAR). Pengesahan kesesuaian-bio *in vitro* daripada SPIONs-PEG-HER dilakukan dengan menggunakan asai pewarna tetrazolium (MTT), kaedah pewarnaan biru *Trypan* dan ujian keserasian darah. Keupayaan SPIONs-PEG-HER mengikat sel garisan HER2+ (SK-BR-3) telah diukur secara perbandingan dengan rangkaian sel HER2- (HSF 1184, MDA-MB-231, MDA-MB-468) melalui kajian ikatan *in vitro*. Data kualitatif dan kuantitatif telah diperoleh dengan menggunakan pewarnaan biru *Prussian*, asai TEM dan analisis spektroskopi penyerapan atom (AAS). Perbandingan terhadap perubahan dalam kadar pertumbuhan perencatan dalam HSF 1184, MDA-MB-231, MDA-MB-468 dan SK-BR-3 baris sel pada suhu yang berbeza (40 °C, 42 °C dan 45 °C) dikaji dengan menggunakan asai MTT pasca hipertermia. Setelah itu, kajian morfologi pasca hipertermia telah dijalankan melalui mikroskop songsang dan kaedah pewarnaan oren akridina/etidium bromida (AO/EB). Model *in vivo* menggunakan DMBA yang disebabkan oleh tetikus *balb/c* telah disuntik dengan SPIONs-PEG-HER melalui hujung salur darah. Taburan-bio daripada SPIONs-PEG-HER ke atas tumor asas dan juga di organ-organ penting telah diukur secara kualitatif dan kuantitatif menggunakan kaedah kolorimetri (AAS dan TEM). Akhir sekali, kadar hayat DMBA yang disebabkan oleh tetikus *balb/c* disuntik dengan SPIONs-PEG-HER telah diukur dengan kehadiran dan ketiadaan AMF dan pemantauan harian ke atas berat DMBA yang disebabkan oleh tetikus *babl/c* dirawat semasa tempoh rawatan telah dijalankan. Analisis pencirian morfologi dan fiziko-kimia mendedahkan bahawa SPIONs-PEG-HER yang telah disintesis mempunyai saiz hampir 17 nm dan mempunyai penampilan yang hampir setara serta diameter hidrodinamik yang lebih besar (~ 84 nm) dan pengedaran yang lebih luas berbanding dengan pendedahan SPIONs. Selain itu, analisis XRD dan FT-IR mengesahkan bahawa proses *PEGylation* dan hubungan antaranya telah berjaya dicapai manakala analisis VSM dan SAR menunjukkan bahawa SPIONs-PEG-HER memiliki sifat-sifat magnet berkesan untuk digunakan sebagai ejen cecair hipertermia. SPIONs-PEG-HER menunjukkan tahap ketoksikan yang rendah walaupun pada kepekatan yang sangat tinggi 1000 µg/ml. Keputusan kajian menunjukkan bahawa SPIONs-PEG-HER terpilih boleh mengikat kepada HER2+ sel-sel kanser. Keputusan yang diperoleh dari asai MTT pasca hipertermia menunjukkan bahawa pendedahan HER2+ sel-sel dengan suhu 45 °C selama 20 minit menghalang pertumbuhan sel-sel sebanyak 90% di mana mereka tidak mendapatkan semula percambahan normal mereka seperti bahagian-bahagian sel HER2- yang menguatkan keputusan yang diperoleh melalui analisis pasca hipertermia morfologi di mana ia telah menunjukkan bahawa suhu 45 °C secara signifikan amat ketara berbanding dengan suhu lain. Melalui kajian perubahan morfologi oleh mikroskop songsang dan kaedah pewarnaan AO/EB, telah dinyatakan bahawa sel-sel SK-BR-3 telah menjalani apoptosis sejak tanda-tanda apoptotik seperti kemerosotan sel-sel serta badan-badan apoptotik telah jelas dilihat. Dapatan kajian taburan-bio mendedahkan bahawa pengumpulan SPIONs-PEG-HER di dalam laman tumor jauh lebih tinggi berbanding SPIONs-PEG. Berdasarkan keputusan yang diperoleh melalui rawatan hipertermia daripada DMBA yang disebabkan oleh tetikus *balb/c*, telah mendedahkan bahawa kadar hayat dalam kumpulan eksperimen yang dirawat dengan SPIONs-PEG-HER di hadapan AMF adalah lebih tinggi daripada kumpulan eksperimen lain di mana 50% daripada DMBA yang disebabkan oleh tetikus *balb/c* terselamat dan mengekalkan berat badan purata mereka. Penemuan dalam kajian ini menggambarkan bahawa hipertermia SPIONs-PEG-HER-pengantara adalah rawatan kanser payudara yang mujarab..

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AAS	-	Atomic Absorption Spectroscopy
AC	-	Alternating Current
AMF	-	Alternating Magnetic Field
AO	-	Acridine Orange
APTT	-	Activated Partial Thromboplastin Time
AR	-	Androgen Receptor
ASR	-	Age-Sstandardised Rate
DLS	-	Dynamic Light Scattering Analysis
DMBA	-	7,12-Dimethylbenz[a]anthracene
DNA	-	deoxyribonucleic acid
EAC	-	Ehrlich Ascites Carcinoma
EB	-	Ethidium Bromide
EDC	-	(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)
EDTA	-	Ethylenediaminetetraacetic Acid
EGFR	-	Epidermal Growth Factor Receptor
EPR	-	Enhanced Permeability and Retention
FA	-	Folic Acid
FB	-	Fibrin
FESEM	-	Field Emission Scanning Electron Microscope
FRα	-	Folic Acid Receptor
FT-IR	-	Fourier Transform Infrared Spectroscopy
Hc	-	Coercive force
HER	-	Herceptin
HER2	-	Human Epidermal Growth Factor Receptor 2
HER3	-	Human Epidermal Growth Factor Receptor 3
HRTEM	-	High-Resolution Transmission Electron Microscopy

IGF-IR	-	Insulin-like Growth Factor Receptor
IR	-	Infrared Radiation
MFH	-	Magnetic Fluid Hyperthermia
MNP	-	Magnetic Nanoparticle
MOH	-	Ministry of Health
Mr	-	Remanent Magnetization
MRI	-	Magnetic Resonance Imaging
Ms	-	Saturation Magnetization
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NCR	-	National Cancer Registry
NHS	-	N-Hydroxysuccinimide
NPs	-	Nanoparticles
NSCLC	-	Non-Small Cell Lung Cancer
PARP	-	Poly(ADP-Ribose)Polymerase
PBS	-	Phosphate-buffered saline
PDI	-	Polydispersity Index
PEG	-	Polyethylene Glycol
PSMA	-	Prostate Specific Membrane Antigen
PT	-	Prothrombin Time
RBC	-	Red Blood Cell
S.E.M	-	Standard Error of the Mean
SAED	-	Selected Area Electron Diffraction Pattern
SAR	-	Specific Absorption Rate
SPIONs	-	Superparamagnetic Iron Oxide Nanoparticles
TEM	-	Transmission Electron Microscopy
TT	-	Thrombin Time
UKMAEC	-	Universiti Kebangsaan Malaysia Animal Ethics Committee
VEGF-A	-	Vascular Endothelial Growth Factor A
VEGFR	-	Vascular Endothelial Growth Factor Receptor
WHO	-	World Health Organization
XRD	-	X-Ray Diffraction

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Breast cancer is the most commonly diagnosed cancer among American women and it is the second leading cause of cancer death. In 2012, an estimated 226,870 new cases of invasive breast cancer and 39,510 breast cancer deaths were expected to occur among American females. Breast cancer rates vary largely by race/ethnicity and geographic region. Historically, breast cancer was known as a disease in western countries. However, over the past two decades, breast cancer incidence and mortality rates have been growing up rapidly in economically less developed regions too. According to 2012 GLOBOCAN estimates, 52.6% of the new worldwide breast cancer cases (882,900) and 62.1 % of the breast cancer deaths in women (324,300) occurred in developing countries [1].

Among Malaysians in Peninsular Malaysia, approximately, a total 18,219 new cancer cases were diagnosed in 2007 according to a report published in February 2011 by National Cancer Registry (NCR), Malaysia. Breast the first most common cancer among population regardless of sex in Malaysia. There were 3,242 female breast cancer cases diagnosed in 2007 and reported to NCR, accounted for 18.1% of all cancer cases reported and 32.1% of all female cases [2].

Nowadays, cancer rate is increasing and it is predicted that the number of cancer patients will reach to just over 15 million until the year 2020 [3]. Breast cancer is known as the most frequently diagnosed cancer among women all over the world. Different common treatment methods including partial or radical mastectomy, chemotherapy and radiotherapy are not completely successful and induce unwanted side effects on healthy tissues in the body. So, novel treatment methods are highly required to be invented and designed [4].

"To do no harm" has always been the physician's faith. Although, the harmful side effects of cancer treatments is known as a great paradox since chemicals or radioactive agents used in cancer treatments has the potential to adversely affect a patient's overall health. The cancer treatment complication is due to the alterations and deviations in the function of the genes and the pathways controlling the cell cycles. It is clear to the world that acute and chronic side effect are always associated with the current cancer treatments. In addition, over the last thirty years, the rate of successful therapies for the majority of human cancers has marginally improved. Therefore, it is so clear that advances in cancer therapies are required especially the advances in the treatments during which not only a patient's health is highly improved, but also the adverse side effects are minimized. Targeted cancer treatment has been exclusively developed to specifically affect the tumors and cancerous cells while the other surrounding tissues are spared throughout the chemotherapy procedure. Achieving this goal, the characteristics of the cancer cells must be distinguished amongst trillions of normal cells in the body so that the therapeutic agent can be specifically delivered to the cancer cells and destroy them while the surrounding tissues are remained unaffected. The idea of targeted cancer treatment has been actively followed up for many years aiming to improve the survival rate of the cancer patients. To implement such a complicated task, many advances are needed for the detection and the treatment of the cancer; and nanotechnology is considered as a key knowledge to resolve the challenging and problem of tracking and treating the cancer cell-by-cell [5].

Surgery, systemic therapy and radiation therapy are the most practiced breast cancer treatment methods. Since the natural history of breast cancer and the

understanding of the molecular biology have improved, an evolution in the breast cancer surgery has been seen over the past few decades. However, historically, aggressive nature of breast cancer surgery has always been a key issue since the breast and the surrounding tissues undergo removal [6].

Radiation therapy is practiced subsequent to the modern breast-conserving surgical treatments in order to control the cancer recurrences in the breast. Radiation is mostly practiced subsequent to the tumor removal (mastectomy) once the tumor size exceeds 5 cm or when the cancer is seen in lymph nodes. In under-developed regions, radiation therapy is mostly utilized to control the cancer symptoms and not used to cure the cancer since the majority of the patients present in the hospitals with a metastatic disease at its advanced stages. Radiation is exclusively effective to control the painful symptoms resulted from bone metastases [6].

Hormone therapy, chemotherapy and targeted biological therapies fall under category of systemic therapies. The advantages of chemotherapy depend on multiple factors: cancer size, the number of lymph nodes involved, the existence of hormone receptors, and the amount of human epidermal growth receptor 2 (HER2) protein overexpressed on the cancer cells. According to the availability of the resources, different agents are utilized. Hormone therapy like tamoxifen can be given to the patients with HER2+ breast cancer. However tamoxifen is known as an affordable treatment, sufficient pathology services are not accessible in order to monitor status of the hormone receptors in the lower-resource settings. Moreover, carrying out an appropriate tissue handling and processing is inevitable for valid hormone test results. The utilization of the HER2-targeted monoclonal antibody-based treatment herceptin together with chemotherapy has shown high effectiveness in curing HER2+ cancer, but is cost-prohibitive in most of the world. Herceptin has been considered for inclusion in the World Health Organization (WHO) Essential Medicine list, sparking a debate about how health care systems can and should balance high cost against proven curative benefits [6].

1.2 Problem Statement

Conventional cancer therapies including surgery, radiotherapy, and chemotherapy are not so effective in the treatment of certain cancers such as breast cancer since normal tissues surrounding the cancerous tissues are also adversely affected. Thus, a novel and effective method of treatment is required. Hyperthermia is a promising therapy for cancers such as breast cancer. Hyperthermia therapy is based on the fact that cancer cells are much more sensitive to heat than normal cells. However, the difficulty of delivering the necessary selective heating remains an important technical problem to be resolved [7].

1.3 Research Objectives

The aim of this study is to develop a stable and biocompatible magnetic tumor-targeting hyperthermia complex "SPIONs-PEG-HER" and assess its effectiveness via *in vitro* and *in vivo* evaluations. Thus, the objectives of the study were phrased as follow:

1. To develop SPIONs-PEG-HER targeting complex which include stabilization and vectorization of iron oxide nanoparticles (γ-Fe_2O_3).
2. To perform physico-chemical characterization of the synthesized SPIONs-PEG-HER.
3. To investigate the effectiveness of magnetic targeted hyperthermia *in vitro*.
4. To investigate the effectiveness of targeted hyperthermia *in vivo*.

1.4 Scope of Research

In order to achieve the goals of this study, the scope of the research is as follows.

i) Superparamagnetic iron oxide nanoparticles/maghemite (γ-Fe_2O_3) was stabilized by PEG (MW 2000) in order to increase the biocompatibility, solubility and stability in aqueous solution as well as in physiological saline.

ii) Then, HER (monoclonal anti-her2 antibody/herceptin) was conjugated to the previously coated SPIONs through EDC/NHS click chemistry method for detection of HER2/neu antigen on HER2+ breast cancer cells.

iii) Investigation of stability level of SPIONs-PEG-HER in the blood compartment was the next step during the experimental work to assure that the synthesized complex is stable and do not aggregate when exposed to the physiological conditions.

iv) SK-BR-3 cell line (human breast cancer cell line that over expresses HER2) was used as targeted cells while HSF-1184 cell line (human skin fibroblast cell line), MDA-MB-231 cell line (human breast cancer cell line that does not over express HER2) and MDA-MB-468 cell line (human breast cancer cell line that does not over express HER2) were used as control cells.

v) Attachment of SPIONs-PEG-HER to HER2+ cells were investigated prior to proceed to the *in vitro* evaluation. Then, magnetic hyperthermia using SPIONs-PEG-HER was performed to investigate the treatment effects of the proposed treatment method on the nominated cells.

vi) Finally, treatment effects of the *in vivo* magnetic tumor-targeting hyperthermia on the tumor bearing balb/c mice were investigated.

In brief, successful synthesis of SPIONs-PEG-HER and magnetic tumor-targeting hyperthermia treatment effects using SPIONs-PEG-HER as the targeting agent on HER2+ cells and tumor bearing balb/c mice were focused in this study.

1.5 Significance of Study

Advances in new technologies such as molecular biomarkers and nanoparticles are considered as highly qualified tools to diagnose and effectively treat the breast cancer. During the breast cancer improvement stages, genetic mutations occur and create certain molecular effects that can be used as biomarkers. Attaching the nanoparticles to the specific biomarkers of breast cancer has the potential to follow and limit the cancer cells with higher sensitivity and selectivity [8]. There are many advantages in using the targeted iron oxide nanoparticles combined with intracellular hyperthermia as targeted treatment in chemotherapy, radiotherapy, conventional hyperthermia, immunotherapy and mastectomy [9]. These advantages are explained as the matters of significance in this study.

Possibility of treatment of metastasis is the first advantage. Metastasis is the growth of secondary malignant cells at a distance from a primary site of cancer. One of the most important obstacles in treatment of cancerous tumors is the unsuccessful treatment of metastasis. Tumor itself can be treated by mastectomy or the other treatment methods, but metastasis does not appear at first or cannot be treated by the conventional methods. In the proposed targeted treatment with iron oxide nanoparticles, monoclonal antibody against a specific antigen is attached to the nanoparticles and detects the antigen wherever it is and attaches to it. Then, after the heat is induced by iron oxide nanoparticles using magnetic hyperthermia, cancer cells and metastasis are effectively treated [4],[9].

Minor systemic side effects compared to chemotherapy, radiotherapy and conventional hyperthermia is the second advantage. In the conventional methods, radiation dose absorbed by the skin, heat absorbed by the surrounding tissues and

organs on the way towards the tumor are the issues forcing the dosage of the treatment to be highly increased. But, in the targeted treatment of breast cancer via coated nanoparticles (biocompatible and far less toxic) attached to specific monoclonal antibodies, nonspecific heating of the surrounding tissues is eliminated and the dosage of the monoclonal antibody as a drug is significantly reduced which is so cost effective and less harmful. Moreover, iron oxide nanoparticles are only accumulated in the tumor area in its metastasis so that only these areas are under the influence of magnetic field. So, the surrounding tissues remain healthy and unaffected [4],[9].

Reduction of immune system response compared to unaccompanied immunotherapy is the third advantage of the proposed treatment. In some situations, immunotherapy is practiced to treat the cancer. In this case, not only super expensive cost of treatment is imposed to the patient, but also severe response of the immune system is faced due to the injection of large amounts of antibody. Normally immune system should be suppressed in this case but the amount of antibody used in the proposed treatment is highly reduced compared to the unaccompanied immunotherapy. So, this problem is solved by the proposed targeted treatment [4].

The proposed treatment method by SPIONs-PEG-HER by magnetic tumor targeting hyperthermia has never been studied anywhere else in the world. So, applying hyperthermia using magnetic coil where SPIONs-PEG-HER are injected intravenously to target the tumor cells and turn the magnetic energy to heat is introduced as the novelty of this study.

1.6 Thesis Organization

This thesis includes five chapters. The first chapter describes the key information of the study: research background, problem statement, significance of study, research objectives, scope of research.

The second chapter comprehensively reviews the literature regarding the topic of the study. The introduction to the breast cancer treatments, the nature of SPIONs, the stabilization of SPIONs, the vectorization of the SPIONs and magnetic tumor-targeting hyperthermia are critically discussed and reviewed.

The third chapter describes the methodology in this study. This chapter elaborates the step-by-step synthesis of SPIONs-PEG-HER, physico-chemical characterization of the engineered system, *in vitro* and *in vivo* evaluation of the SPIONs-PEG-HER.

The fourth chapter of this thesis illustrates and discusses the obtained results of the experiments. It discusses on the properties of the developed SPIONs-PEG-HER and its effectiveness.

The entire findings of the research are concluded in chapter five. This chapter recommends the future works regarding this research.

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