UNIVERSITI PUTRA MALAYSIA

EXPLORING EFFICACY OF GOLD NEAR INFRARED DYE CONJUGATED CALCIUM CARBONATE NANOPARTICLES DERIVED FROM COCKLE SHELL FOR POTENTIAL MOLECULAR IMAGING

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By

KIRANDA HANAN KARIMAH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillments of the Requirements for the Degree of Master of Science

October 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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October 2017

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Institute : Bioscience

The development of biocompatible and economical bio nanomaterial for molecular imaging modalities rapidly increases, with aim of enhancing and improving detection. Ultimately, providing information at a molecular and cellular level. This is a promising, long term non-toxic and biocompatible approach for decreasing mortalities, and advancement to molecular imaging. Presently, the molecular imaging modalities such as the Computed Tomography (CT) and optical modalities suffer limitations like poor specificity, low sensitivity and poor signal penetration through tissues. Additionally, the current imaging agents used for molecular imaging are known to be associated with non-biodegradability or slow excretion and high toxicity, challenging the production of a strong imaging signal However, the complexity of cellular and molecular processes of any biological system pose a challenge for the development of novel nanomaterial like the conjugated near infrared gold cockle shell-derived calcium carbonate nanoparticles (Au-$CsCaCO_3$NPs). Thus, biocompatibility assessment and proof of cellular uptake is essential to further biomedical applications. This research developed and characterized Au-$CsCaCO_3$NPs derived from cockle shell calcium carbonate nanoparticles ($CsCaCO_3$NPs) and gold nanoparticles (AuNPs).

The obtained spherical shaped nanoparticles diameter size 35 nm ± 11, were characterised using Transmission Electron Microscope (TEM), Field Emission Scanning Electron Microscope (FESEM) equipped with Energy Dispersive X-ray (EDX) for their physicochemical properties and elemental analysis. Fourier transform infrared spectroscopy (FTIR) revealed significant supporting interactions between the conjugated nanoparticles, Zetasizer highlighted the stability with the highly negative nanoparticles charges and Uv-Vis spectrophotometer displayed significant synthetic regions of the nanoparticles. For biocompatibility assessment and cellular uptake

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imaging; the studies were done on breast cancer cell line (MCF-7) against mouse fibroblast normal cell line (NIH3T3). This was done using 3-Dimethylthiazo-2,5-diphynyltetrazolium bromide (MTT), lactate Dehydrogenase (LDH), Reactive Oxygen Species (ROS) assays and fluorescent confocal imaging which confirmed nontoxic on normal cells and evidence of cellular interactions. Furthermore, IC_{50} was noted 23 – 25 µg/ml for the conjugated nanomaterial. The threshold of significance was $p < 0.05$. Based on the results, Au-$CsCaCO_3$NPs were most biocompatible and proved to be excellent potential candidate for enhancing molecular cancer imaging and other biomedical applications.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

MENEROKAI KEBERKESANAN EMAS INFRARED DYE BERHAMPIRAN TERKONJUNGSI NANOPARTIKEL KALSIUM KARBONAT DARI KULIT KERANG UNTUK PENGIMEJAN MOLEKUL YANG BERPOTENSI

Oleh

KIRANDA HANAN KARIMAH

October 2017

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Pembangunan bionanomaterial yang ekonomi dan biokompatibel untuk modaliti pengimejan molekul meningkat dengan cepat, dengan tujuan untuk meningkatkan dan memperbaiki pengesanan. Akhirnya, memberikan maklumat pada tahap molekul dan selular. Ini adalah pendekatan jangka panjang yang tidak beracun dan biokompatibel yang menjanjikan dapat mengurangkan mortaliti, dan kemajuan dalam pengimejen molekul. Pada masa ini, modaliti pengimejan molekul seperti Tomography Computed (CT) dan modaliti optik adalah terbatas seperti kekhususan yang rendah, kepekaan yang rendah dan penembusan isyarat yang lemah melalui tisu. Selain itu, ejen pengimejan semasa yang digunakan untuk pengimejan molekul adalah tidak biodegradabel atau mempunyai perkumuhan yang perlahan dan ketoksikan yang tinggi, menyebabkan pengeluaran isyarat pengimejan yang kuat menjadi sukar. Walau bagaimanapun, kerumitan proses molekul dan sel mana-mana sistem biologi menimbulkan cabaran untuk membangunkan nanomaterial yang novel seperti emas inframerah dye berhampiran terkonjungsi nanopartikel kalsium karbonat dari kulit kerang (Au-CsCaCO$_3$NPs). Oleh itu, penilaian biokompatibiliti dan bukti pengambilan selular adalah penting untuk aplikasi bioperubatan selanjutnya. Kajian ini membangunkan dan mencirikan Au-CsCaCO$_3$NPs dan nanopartikel emas (AuNPs).

Saiz nanopartikel berbentuk sfera yang diperolehi adalah 35 nm ± 11, dicirikan menggunakan Mikroskop Transmisi Elektron (TEM), Mikroskop Pengimbasan Pelepasan Medan (FESEM) yang dilengkapi dengan X-ray Dispersive Tenaga (EDX) untuk sifat fizikokimia mereka dan analisis unsur. Transformasi Fourier spektroskopi inframerah (FTIR) menunjukkan interaksi sokongan yang signifikan antara nanopartikel konjugasi, Zetasizer menyerlahkan kestabilan dengan caj nanopartikel yang sangat negatif dan Spektrofotometer Uv-Vis memaparkan kawasan sintetik nanopartikel yang penting. Untuk

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penilaian biokompatibiliti dan pengimejan pengambilan sel; kajian dilakukan terhadap sel kanser payudara (MCF-7) dan juga sel normal fibroblast tikus (NIH3T3). Ini dilakukan menggunakan 3-Dimethylthiazo-2, 5-diphynyltetrazolium bromide (MTT), lactate Dehydrogenase (LDH), ujian Reaksi Oksigen Reaktif (ROS) dan pengimejan pendarfluor yang mengesahkan tidak beracun pada sel normal dan membuktikan interaksi selular telah berlaku. Tambahan pula, IC_{50} untuk nanomaterial konjugated ialah 23 - 25 µg / ml. Had ambang signifikan adalah $p < 0.05$. Berdasarkan kepada hasil kajian, Au-$CsCaCO_3$NPs adalah paling biokompatibel dan terbukti menjadi calon berpotensi yang sangat baik untuk meningkatkan pengimejan kanser molekul dan lain-lain aplikasi biomedikal.

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LIST OF ABBREVIATIONS

GLOBOCAN	Comprehensive cancer surveillance database managed by the International Association of Cancer Registries [IARC]
˚C	Degree Celsius
µg	Microgram
AO	Acridine Orange Fluorescent Dye
Au-$CsCaCO_3$NPs	Gold Near Infrared Dyed Conjugated Cockle Shell-derived Calcium Carbonate Nanoparticles
AuNPs	Gold Nanoparticles
BS-12	Dodecyl Dimethyl Betaine
$CaCO_3$NPs	Calcium Carbonate Nanoparticles
C-C	Carbon-Carbon Bond
-cm	Per Centimetre
C-N	Carbon-Nitrogen bond
C-O	Carbon-Oxygen bond
CO_2	Carbondioxide
$CsCaCO_3$NPs	Cockle Shell-derived Calcium Carbonate Nanoparticles
CT	Computerized Tomography
DAPI	4', 6-Diamidino-2-Phenylindole
DCF	2′-7′-Dichlorofluorescein
DCF-DA	2′-7′-Dichlorofluorescin Diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EDX	Energy Dispersive X-ray

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FBS	Fetal Bovine Serum
FESEM	Field Emission Scanning Electron Microscope
FRGS	Fundamental Research Grant Scheme
FTIR	Fourier Transform Infrared Spectroscopy
H_2O_2	Hydrogen Peroxide
HeLa cells	Human cervical cancer cell line
IC50	50% inhibition concentration
ICG	Indocyanine Green Dye
JCRB	Japanese Collection Research Bioresource
LDH	Lactate Dehydrogenase
LSPR	Localized Surface Plasmon Resonance
MCF-7	Human Breast Adenocarcinoma Cell Line
MI	Molecular Imaging
MID	Molecular Imaging Devices
Mins	Minutes
ml	Millilitre
MRI	Magnetic Resonance Imaging
MRSI	Magnetic Resonance with Spectroscopy
MTT	3-Dimethylthiazo-2, 5-diphynyltetrazolium Bromide
NIH3T3	Mouse Embryonic Fibroblast Cell Line
NIR	Near Infrared
nm	Nano Metre
OD	Optical Density

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O−H	Oxygen-Hydrogen Bond
PBS	Phosphate-Buffered Saline
PET	Positron Emission Tomography
PI	Propodium Iodide Fluorescent Dye
ROS	Reactive Oxygen Species
Rpm	Revolutions Per Minute
SD	Standard Deviation
SPECT	Single Picture Emission Scanner
TEM	Transmission Electron Microscope

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CHAPTER 1

INTRODUCTION

1.1 Background

Molecular imaging is a type of medical imaging that provides a full anatomical view, biological and cellular functioning of the body processes at a molecular level (De La Zerda et al., 2008). Molecular imaging scans are done for various purposes including locating, detecting, visualizing and characterizing tumors in patients (van der Meel et al., 2010). In addition, molecular cancer imaging technologies have grown explosively over the years, and now play a great role in clinical oncology. This allows clinicians to image cell proliferation, gene expression, angiogenesis, and apoptosis (J. Chen et al., 2012; Ria et al., 2009; Xu et al., 2002; Zhu et al., 2010). Also, investigating the biochemical functioning of the tumors and monitoring progression using cancer therapy. However, the true transformative power of cancer imaging and clinical management lies ahead (Shah et al., 2004; Weissleder, 2006).

Today cancer is a global problem and leading cause of deaths worldwide (X. Ma & Yu, 2006). In 2012, GLOBOCAN reported 14.1 million new diagnosed cancer cases and 8.2 million cancer deaths worldwide. Additionally, GLOBOCAN further predicted an estimation of 21.7 million new cases, and 13 million deaths by 2030 arguing that the growing population and changing lifestyles in society pose a great risk in the increase of cancer threat. (Release, 2013). In Malaysia, cancer is the 4th leading threat to human life causing numerous medically certified deaths amongst Malaysians. An estimated 30,000 cancer incidences are reported annually, and are expected to increase. Furthermore, this horrifying disease has raised public health concerns in the Malaysian communities especially with breast cancer which generally affects a greater ratio of the female population (G. Lim, 2002; Lim et al., 2013).

Significantly optical imaging has played a crucial role in early cancer diagnosis particularly for fluorescence and bioluminescence imaging which focus on imaging screens. Disease management is achieved through electromagnetic interactions with living tissue and fluids of non-specific tumor biomarkers. This uses the principle of reflection, scattering, and frequency shift of acoustic waves except for ultrasound. This has led to the reduction in cancer mortalities and improved cancer survival rates as compared to the previously later stage diagnosis (Fass, 2008).

Today, molecular imaging devices (MID) used include Positron Emission Tomography (PET), Computerized Tomography (CT), Single Picture Emission Scanner (SPECT), Magnetic Resonance with Spectroscopy (MRSI) and Magnetic Resonance Imaging (MRI) which offer improved clinical cancer care (Buck et al., 2010; Kurhanewicz et al.,

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2002). The PET, SPECT have very high sensitivity but low spatial resolution, non-specific and use ionizing radiation imaging agents (Pysz et al., 2010). CT and MRI have high spatial resolution but lower sensitivity and also use expensive imaging agents (Weissleder, 2006b). For example, the PET can be used to detect radioactive cancer-related biomarkers in the targeted organs of interest (Wu et al,, 2013). The procedure is done by injecting the patient with radio-marker substance also known as radiopharmaceutical into the blood stream prior to the procedure (Phelps, 2002). In turn, these act by attaching to the target organ of interest and are detected using PET/SPECT, CT or MRI (Jacobson & Chen, 2013; Juergens et al., 2016; Khalil et al., 2011; Lu & Yuan, 2015).

Additionally, the devices are known to track the pattern of distribution, activity of the cell changes, effects, and progression of the disease using the radioactive biomarkers in the system to obtain the scans with a relatively small amount of ionizing radiation (Walker, 2011). Future developments using Raman spectroscopy and nanoparticle-tumor targeted biomarkers have been reported to show great promise coupled with the use of molecular cancer imaging techniques. Nanoparticle based imaging agents could alleviate some limitations like, the lack of specificity and low sensitivity associated with the current imaging modalities (Ma et al., 2017). This allows for information associated with the biological tissues, tumor anatomical structure, and tumor metabolism to be provided (Kurdzeil et al., 2008). However, cancer detection at earlier stages before failure functioning of a vital organ (s) or metastasis, still poses a challenge. Nonetheless, a promising noninvasive, real-time and high resolution for cancer detection with the use of less radiation as compared to the invasive surgical procedures and diagnosis using biopsy samples is possible (Sadeghi, 2016).

Molecular imaging strategies are known to include a direct or indirect approach. The direct approach involves imaging the target directly with target specific probe such as the use of monoclonal antibodies to target a particular cell membrane epitope or imaging activity of a particular enzyme with enzyme specific probe (Dobrucki and Sinusas, 2010; Kobayashi et al., 2010; Willmann et al., 2008). The indirect approach is said to involve the use of reporter genes or probes. Show casing the fact that molecular imaging techniques have better advantages over the invasive methods used in diagnosing cancer. Also, offering a more subtle non-invasive approach which is easier on patients, makes data collection easier, faster and possible for post therapy evaluation (Dobrucki and Sinusas, 2010; Kircher et al., 2012; Kobayashi et al., 2010; Willmann et al., 2008).

The current challenge for future cancer diagnosis is earlier detection of the disease before the cancer compromises the functioning of the major body organs and metastasis (Kamba et al., 2013). This research could prove vital in molecular imaging technology and also could improve detection of multiple biomarkers or cancer-related activities, elaborating tumor physiology and features. The gold near infrared dye conjugated calcium carbonate nanoparticles derived from cockle shell can be synthesized using a simple and cost efficient conjugation method. The gold nanoparticles are conjugated with the cockle shell derived calcium carbonate nanoparticles into a conjugated

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nanomaterial, retaining most of the useful parental traits associated with the individual nanoparticles respectively. The outcome of this research could pave way for future studies, such as detection of the smallest tumor sizes using nanotechnology with appropriate multi-functionalization of the conjugated nanomaterial.

1.2 Problem Statement

In this era, cancer is a global burden, a health challenge, and the leading cause of death worldwide (Youlden et al., 2012). In 2012, it has been documented that 14.1 million new cases were recorded resulting into 8.2 million number of death worldwide. 21.7 million cases and 13 million deaths are expected by the year 2030 (Release, 2013). With millions of people dying each year and among the leading causes of medically certified deaths in Malaysia, especially with breast cancer the principal female cause of cancer deaths (Hortobagyi et al., 2005; Youlden et al., 2014). The National Cancer Registry has recorded more than 21 thousand Malaysian cancer cases per 100,000 population. Also estimating almost 10,000 unregistered cases each year, a ratio of (1:4) people developing cancer by 75yrs. There are more affected female ratio to the males (1:1.2) and a < 10% of cancer happens in children compared to the > 50% men and 35% women by 50 years and above (Omar et al., 2006; Othman et al., 2008).

Evidently, cancer causes the most premature deaths with 30 - 40% that are medically certified, meaning no precise figure for the deaths. The National Cancer Society Malaysia (NCSM) also estimated about 90-100,000 cases of people in Malaysia living with cancer at any one time (Al-Dubai et al., 2011; Zainal and Nor Saleha, 2011). Hence improvement in cancer detection and treatment could significantly lead to better survival rates for the people with cancer. Hundreds of people are still dying each year due to challenges associated with early detection of cancer. More often the disease is detected at later stages usually when one or more vital organs are compromised (Hortobagyi et al., 2005; Kurdzeil et al., 2008; Youlden et al., 2014). However, the fundamental aspect in cancer detection is to explore and improve methods for the early detection of localised and disseminated tumours in patients. This is crucial in the success of cancer therapy, treatment and management (Kircher et al., 2012; Saadatmand et al., 2015; Sadeghi, 2016).

Currently, the imaging modalities for clinical detection and diagnosis suffer limitations like poor specificity, low sensitivity and poor signal penetration through tissues such as the CT and optical modalities. In addition, the current imaging agents are known to be associated with non-biodegradability or slow excretion and high toxicity, challenging the production of a strong imaging signal (Cheng et al., 2016; Galbn et al., 2010; Quon & Gambhir, 2005). The use of multi-functionalized nanoparticles for imaging agents such as gold near infrared dye conjugated calcium carbonate nanoparticles derived from cockle shell could resolve these limitations. However, there is need to evaluate the efficacy, safety and their cytotoxic effect. Hence improvement in cancer detection and treatment could significantly lead to better survival rates for the people with cancer.

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1.3 General Objective

The aim of this research was to explore the efficacy of gold near infrared dye conjugated calcium carbonate nanoparticles derived from cockle shell for potential molecular imaging.

1.4 Specific Objectives

- To synthesize and characterize gold nanoparticles, cockle shell derived calcium carbonate nanoparticles and the conjugated nanomaterial.
- To incorporate near infrared dye into the conjugated nanomaterial, evaluate *in vitro* cytotoxicity and biocompatibility on both normal and cancer cell lines.
- To test possible imaging of the conjugated nanomaterial *in vitro* using cancer cell line and study its cellular uptake using confocal microscopy.

1.5 Hypothesis of Study

1.5.1 Null Hypothesis (H0)

Gold near infrared conjugated cockle shell-derived calcium carbonate nanoparticles (Au-CsCaCO$_3$NPs) cannot be used for potential molecular imaging.

1.5.2 Alternative Hypothesis (Ha)

Gold near infrared conjugated cockle shell-derived calcium carbonate nanoparticles (Au-CsCaCO$_3$NPs) can be used for potential molecular imaging.

1.5.3 Research Question?

How do the Au-CsCaCO$_3$NPs impact molecular imaging for cancer diagnostics?

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