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SYNTHESIS AND CHARACTERIZATION OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES-GOLD CORE-SHELL PORPHYRIN CONJUGATE FOR PHOTODYNAMIC THERAPY

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Thesis in fulfilment of the requirement for the degree

PHILOSOPHIAE DOCTOR (PhD)
in
CHEMISTRY

in the

FACULTY OF SCIENCE

of the

UNIVERSITY OF JOHANNESBURG

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DEDICATION

To Almighty God, the owner and giver of life.

To my lovely wife, Chidinma and our Great Son, Demilade.

To all who had inspired me and that I had inspired during the course of my academic career.
ACKNOWLEDGEMENTS

I am grateful to my supervisor, Prof. Samuel Oluwatobi Oluwafemi and co-supervisor, Prof. Sandile Phindile Songca for standing by me throughout the process of achieving this goal.

Also, I am grateful to the National Research Foundation (NRF), South Africa, under the Nanotechnology Flagship Programme (Grant no: 97983) for financial support.

Furthermore, I thank the Laser Research unit, Faculty of Health Science, University of Johannesburg, especially Prof. Heidi Abrahamse and Dr. Cherie-Ann Kruger, for the training, technical guidance and provision of laser resources employed for the photodynamic therapy applications.

Finally, I thank Dr. Charles Sheppard (Department of Physics, University of Johannesburg) for the technical assistance on the vibrating sample magnetometry’s measurement.
ABSTRACT

A nuclear-targeting fluorescent SPIONs-gold core-shell meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate was synthesized, characterized and used for fluorescence imaging and magnetic-targeting in photodynamic therapy against breast cancer cells (MCF-7). In addition, series of gluconic acid capped SPIONs, SPIONs-gold and meso-tetrakis(4-hydroxyphenyl)porphyrin derivatives were synthesized via new greener approaches and characterized using ultraviolet-visible spectrophotometry (UV-Vis), Fourier Transform infrared spectroscopy (FT-IR), transmission electron microscopy (TEM), high resolution transmission electron microscopy (HRTEM), selected area electron diffraction (SAED), X-ray diffractiometry (XRD), vibrating sample magnetometry (VSM) and energy dispersive X-ray spectroscopy (EDS). After conjugation, the obtained polymeric nanomagnetic porphyrin conjugate was highly soluble in water and exhibited ultraviolet, indigo, blue and red emissions at specific excitation wavelengths varying from UV@254 nm to red@680 nm with high magnetic response to external magnetic field. Furthermore, the conjugate together with some other newly synthesized conjugates displayed high singlet oxygen generation potentials sufficient for the eradication of MCF-7 breast cancer cells in vitro. In addition, the conjugate showed no cytotoxicity against the cancer cells in the dark but became highly toxic to cells after irradiation with light of 673 nm for 14 min 51 s. Moreover, the cells that were exposed to external magnetic field displayed higher phototoxicity than those that were without exposure. Overall, these results indicate that the nano-porphyrin drug system exhibit excellent potential to function as a new promising magnetic-field targeting agent for theranostic photodynamic eradication of cancer diseases.
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LIST OF ABBREVIATIONS

$^1$H NMR – Proton nuclear magnetic resonance

ASCT – Alcoholic solvent conjugation technique

ATP – Adenosine triphosphate

CMF - Cells incubated with conjugate that were exposed to external magnetic field

CNMF - Cells incubated with conjugate that were not exposed to external magnetic field

DIC - Differential interference contrast

DLS - Dynamic light scattering

DMEM - Dulbecco’s Modified Eagle’s Medium

FBS - Fetal bovine serum

FT-IR - Fourier transform infrared spectroscopy

HBSS- Hank’s Balanced Salt Solution

LDH – Lactate dehydrogenase

MCF-7 – Epithelial breast cancer cells

mPEGOH – methoxypolyethyleneglycol

PBS - Phosphate buffer saline

PC – Porphyrin conjugate

PDT- Photodynamic therapy

PL – Photoluminescence spectroscopy

POP - Purified porphyrin

Porphyrin – meso-tetrakis(4-hydroxyphenyl)porphyrin

PS- Photosensitizer
PSD – Polar solvent deposition
SBP- Solid-based precursor
SOQY – Singlet oxygen quantum yield
SPIONs-Superparamagnetic iron oxide nanoparticles
SPIONs@Au – Gold coated SPIONs core-shell
TEM - Transmission electron microscopy
TOAB – Tetraoctylammonium bromide
UV-Vis - Ultraviolet-visible spectroscopy
VSM - Vibrating sample magnetometry
XRD - X-ray diffractiometry
CONFERENCE AND SYMPOSIUM PRESENTATIONS

• Facile Green Synthesis and Characterization of Water Soluble Superparamagnetic Iron Oxide-Gold Porphyrin Conjugate for Improved Photodynamic Therapy – The Materials and Energy International Conference, San Diego, California, USA (February 26 - March 2, 2017)-(Oral Presentation)

• The Photodynamic Therapy Efficacy of Nano-superparamagnetic Iron-Oxide-Gold meso-tetrakis (4-hydroxyphenyl) porphyrin conjugate- Frank Warren International conference (December 4-8, 2016), Grahams town, South Africa-Poster Presentation.


• Removal of neutral red dye from aqueous saline solution using gluconic acid capped superparamagnetic iron oxide nanoparticles – NanoAfrica International Conference (April 3-6, 2016), University of South Africa, Florida, South Africa-Oral Presentation.


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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. BACKGROUND

The high rate of death caused by non-communicable diseases (NCDs) such as cardiovascular disorders, cancer, diabetes and chronic respiratory diseases (Beaglehole et al., 2007) is a global concern and calls for improvement on the existing prevention and treatment modalities of these diseases (Hunter & Reddy, 2013). Among the leading life-threatening NCDs, cancer is rated second after the heart disease (Aziz et al., 2003). Cancer affects many parts of the human body including the vital organs such as the brain (Silva et al., 2011), lung (Vermaelen & Brusselle, 2013), cervix (Arbyn et al., 2011), breast (Eckstein, 2011) and prostate (Turkbey et al., 2009). About 40 % increase in annual deaths caused by cancer has been reported to occur over the last two decades and this trend has been predicted to increase in the coming years (Cavalli, 2013). About 8 million people die of cancers every year and this figure has been projected to increase to more than 13 million by 2030 (Cavalli, 2013). Typically, many factors that initiate cancer such as tobacco smoking (Chang et al., 2009), exposure to environmental toxins (Goubran et al., 2014), poor diet and alcohol drinking (Kanavos, 2006) can be prevented. However, the higher mortality rate reported over the last two decades shows that the prevention campaign has been unsuccessful in preventing people from developing cancer. The conventional methods for treating cancer such as chemotherapy,
radiotherapy (Chiaviello et al., 2011) and surgery (Sanjay et al., 2012), have also been inefficient (Aziz et al., 2003) for many years (Zhao et al., 2014). This in part may be due to the emergence of new cases of cancer (Kanavos, 2006), toxicity of therapeutic agents (Tang et al., 2010), surgical operation discomfort, resistance of cancer to therapeutic agents (Eckstein, 2011), recurrence of cancer after therapy and high cost of therapeutic methods especially for people living in low-income developing countries (Katz & Wright, 2006 and Cavalli, 2013). Every year, the world spends about 900 billion dollars from the world economy to control cancers without success (Cavalli, 2013). As a result, alternative means for controlling cancer have become highly imperative.

1.2. PHOTODYNAMIC THERAPY AS AN ALTERNATIVE TECHNIQUE FOR CANCER TREATMENT

1.2.1. Principle and Mechanism

Photodynamic therapy (PDT) was discovered in the early 1900s (Cengel et al., 2016). It is a non-invasive cancer treatment technique (Allison & Moghissi, 2013) that entails generation of in situ reactive oxygen species for the destruction of cancerous and non-cancerous disease cells. It involves activation of a photosensitizer (PS) with non-toxic light (Master et al., 2013) of a suitable wavelength, from visible to near infrared (Gao et al., 2012; Li et al., 2013). The PSs are usually fluorescent materials that have the capability to undergo transformation from their excited singlet state to corresponding triplet state population (TSP) via photochemical intersystem reactions. The TSP produces reactive oxygen species via Type I or Type II reactions (Fig. 1.1). This involves transfer of electrons or energy to molecular oxygen in the presence or absence of biological substrate respectively (Chiaviello et al., 2011). In most cases, Type II reaction involving generation of singlet oxygen predominates (Ethirajan et al.,
destroy cancer cells via apoptosis and necrosis (Gariboldi et al., 2009; Piette et al., 2003; Hirohara et al., 2015; Master et al., 2013; Chiaviello et al., 2011; Samaroo et al., 2007). Apoptosis is a form of programmed cell death that is controlled via a natural energy-dependent biochemical pathway. It could be triggered by both external and internal signals (Zamaraeva et al., 2005). Its effect has been associated with mitochondrion damage (Chen et al., 2013; Yan et al., 2010). On the other hand, necrosis is an uncontrollable process (Montagne et al., 2015) that could be triggered under systemic conditions that are beyond the control of the apoptosis process. It damages the cell membrane causing the release of inflammatory molecules (Oleinick et al., 2002) into the surrounding environment. As a result, the process could be predictably irreversible. The necrosis effect has been associated with calcium ion stress (Montagne et al., 2015; Trump et al., 1997) within the tissue environment. This generally leads to the ceasing of the normal biochemical activities such as angiogenesis (Acharya & Sahoo, 2011) of the tumour. Both apoptosis and necrosis can be detected using various biological staining assays coupled with microscopic techniques (Hirohara et al., 2015; Yan et al., 2010; Meyers et al., 2015). Importantly, for effective PDT, certain parameters are highly essential. These include the amount of energy delivered per unit area (fluence or dose), power (fluence rate or intensity) (Chiaviello et al., 2011) and wavelength of the irradiating light (Postiglione et al., 2011), tissue oxygen concentration (Ethirajan et al., 2011), singlet oxygen generation potential of the photosensitizer, optical absorptivity of the photosensitizer, photobleaching resistance of the photosensitizer, irradiation time, area of irradiation and the drug incubation-light irradiation time interval (Chiaviello et al., 2011). The relationship between the light dosimetry parameters and time of irradiation is shown in Fig. 1.2.
However, despite all these advantages, PDT has many challenges. These include post-therapeutic photosensitivity (Zheng Huang et al., 2008; Schuitmaker et al., 1996), optical absorption limitation (Taniguchi et al., 2008) and non-specific localization of photosensitizers (Connor et al., 2009; Josefsen & Boyle, 2008) leading to light-associated toxicity (Wang et al., 2014). The first clinically-acceptable photosensitizer, photofrin, a mixture of hematoporphyrins (Schuitmaker et al., 1996) lacks purity integrity. Porphyrin PSs have a long history of biosafety as many are found directly in nature (Aravindu et al., 2013). However, photofrin has limited optical attributes and thus has only been used to treat superficial tumours (Connor et al., 2009). Moreover, the need to treat deep-seated tumours becomes more and more imperative everyday leading to the development of the second generation porphyrin PSs (Josefsen & Boyle, 2008) such as Tookad, benzoporphyrin, phthalocyanines (Connor et al., 2009), chlorins and bacteriochlorins (Mccarthy et al., 2009). Unlike photofrin, which is a mixture of oligomers, second generation PSs are pure compounds that absorb light of higher wavelengths including those that fall within the biological therapeutic window (Schuitmaker et al., 1996; Mccarthy et al., 2009; Taniguchi et al., 2008). The second generation porphyrin PSs also have faster clearance indices compared to photofrin with reduced photosensitivity (Schuitmaker et al., 1996; Zheng Huang et al., 2008). Nevertheless, all porphyrin PSs show non-specific therapeutic effect leading to the demand for the development of third generation porphyrin prodrugs. Third generation PSs’ synthesis involve attachment of porphyrin prodrug to cancer-surface targeting (CST) agents such as antibody, protein (Hu et al., 2011), reducing carbohydrate (McCarthy et al., 2009), folate (Schneider et al., 2005) or nanomaterials functionalized with any of these biomolecular-targeting agents, or capable of being driven by an external magnetic
field (Li et al., 2013). The CST agents specifically bind to their receptors that are overexpressed on the surface of tumours. This avoids non-specific reactions within the physiological fluid. Similarly, the use of external magnetic field-driven nanomaterials helps to ensure the prodrug is actually delivered into tumour sites (Li et al., 2013) via magnetic response. The advantages of making porphyrin to be tumour-specific are enormous. These include safety of normal cells within the vicinity of the irradiated tumour, reduced photosensitivity time, very high therapeutic function and reduction in therapeutic agent’s dose (Wang et al., 2014).

1.2.4. Photosensitizers in PDT

The prodrugs used in PDT can be classified as porphyrin and non-porphyrin photosensitizers (Chiaviello et al., 2011). The porphyrin photosensitizers include the free base porphyrin molecules and derivatives such as benzoporphyrin, chlorins and bacteriochlorins. These can further be classified as first (e.g. photofrin) and second free base porphyrin (e.g. chlorins and bacteriochlorins) generation porphyrin PSs. On the other hand, the non-porphyrin photosensitizers include cyanine (Jing et al., 2016; Dongsheng Wang, Fei, L. V. Halig, et al., 2014), methylene blue (Zhao et al., 2014), rose bengal (Tang et al., 2015) and boronated dyes (Adarsh et al., 2010).

Nevertheless, the porphyrin PSs have higher clinical acceptance than the non-porphyrin counterparts due to their relative biocompatibility, non-toxicity, and high clinically-proven safety (Hampton et al., 1994; Connor et al., 2009). Different porphyrin photosensitizers have evolved over many years due to an attempt to improve the properties of original porphyrins. For example, photofrin, the first generation clinically-acceptable porphyrin PS exhibits purity disintegrity and optical limitation (Schuitmaker et al., 1996). Also, the problem of photosensitivity due to prolonged delay in clearance from circulation has long been experienced with this
photosensitizer (Schuitmaker et al., 1996). In order to resolve these drawbacks, second generation porphyrin PSs such as chlorins (Chiaviello et al., 2011) and bacteriochlorins (Dąbrowski et al., 2012) have been developed. These porphyrin PSs have been reported to possess relatively high purity, longer wavelength of absorption and faster clearance from the body after PDT (Schuitmaker et al., 1996). However, one problem yet unresolved by all types of photosensitizers is non-specific therapeutic function due to accumulation in both normal and tumour cells. This has caused destruction of normal cells within the vicinity of the tumour environment (Wang et al., 2014). As a result, the development of new generation photosensitizer systems has evolved and is currently undergoing unrelenting exploitation. In terms of porphyrin PSs, these are known as the third generation PSs. Among these third generation photosensitizer systems are, nanomaterial-based photosensitizer bioconjugates (Huang, 2005; Mccarthy et al., 2009) which consist of a core-shell structure in which the core is made up of a nanomaterial (nanocarrier or vehicle) and the shell, pure or functionalized photosensitizer entity. The incorporation of nanomaterials into PS prodrug systems has enabled PS to be efficiently delivered directly into the tumour leading to an improvement of PDT efficacy. Strategies for the development of the nanomaterials-based PS conjugate platform may include conjugation with gene structures (Sargsyan et al., 2012), carbohydrates (Mccarthy et al., 2009), peptides (Wang et al., 2014), quantum dots (Fowley et al., 2012) or superparamagnetic iron oxide nanomaterials (SPIONs) (Li et al., 2013). The carbohydrates and peptides target respective receptors overexpressed on tumour cells while SPIONs target tumour sites by both enhanced permeability and retention (EPR) effect and attractive response toward the direction of an external magnetic field (Li et al., 2013). Quantum dots can also target cancer location by EPR effect
(Drbohlavova et al., 2009). The EPR is a significant phenomenon shown by most nanomaterials of certain polymer (Jing et al., 2016; Acharya & Sahoo, 2011), noble metal (Dreaden et al., 2012) or metal oxide (Gobbo et al., 2015) origin. The principle explores the fact that, tumour vasculature exhibits bigger opening than the normal cells and thus the smaller nanoscale materials relatively accumulate more in them than in normal cells. Also, due to poor lymphatic system in tumour vasculature, these nanomaterials are retained for longer period within the tumour environment (Danhier et al., 2012).

1.3. NANOMATERIALS IN MEDICINE

Nanotechnology has played vital roles in many fields of human endeavor. In medicine, for example, nanomaterials have been used to improve both the diagnosis of disease and therapeutic functions of existing chemotherapeutics drug (Bae et al., 2011). Nanomaterials exist at the nanoscale level and thus have some unique properties lacking in their bulk counterparts. These properties such as size, shape, surface chemistry, superparamagnetism, therapeutic heat generation and specific light absorptions have been used in wide array of biomedical applications such as magnetic resonance imaging (Vu-Quang et al., 2012), specific-site drug delivery (Huang et al., 2013) and disease-treatment modalities such as immunotherapy (L. Xu et al., 2016), gene therapy (Ramamoorth and Narvekar, 2015), photothermal therapy (Zhou et al., 2009), photodynamic therapy (Li et al., 2013; Banfi et al., 2004; Hu et al., 2014) and magnetic hyperthermia (Silva et al., 2011). For example, well-dispersed hydrophilic functionalized gold nanorods exhibit unique plasmonic effect which can be sensitized by near infrared light to generate thermal energy capable of lysing cancer cells (Huang et al., 2008; Huang & El-sayed, 2011). Also, water-soluble surface-capped superparamagnetic iron oxide nanoparticles have unique
magnetic properties capable of generating a high contrast image for detection of cancer cells in magnetic resonance imaging (Shevtsov et al., 2014) and therapeutic heat efficient for the destruction of tumour cells under the influence of alternating current magnetic field (Mohammad et al., 2010; Muñoz de Escalona et al., 2016). Quantum dots possess unique optical properties (Drbohlavova et al., 2009; Bera et al., 2010) that are capable of improving the optical properties of new and existing therapeutic agents. Nonetheless, for nanomaterials to be useful for medical use, certain criteria need to be fulfilled. For example, they need to be water soluble. Hydrophilicity (water-solubility) is important since the body fluid is an aqueous system which helps to convey the materials round about the body and in and out of cells. In addition, the size and surface chemistry including functional groups and charge of the nanomaterials are highly important. These two factors are essential to avoid being recognized and thus eliminated from the circulation by the body’s defense system (Weinstein et al., 2010; Kim, et al., 2012). Biocompatible polymeric materials such as polyethylene glycol (Ichikawa et al., 2005) and dextran capable of by-passing the defense system without being recognized may therefore be highly desired for capping the surface of nanoparticles. Also, the cell membrane is negatively charged at the physiological pH. Thus, positively charged nanomaterials may have relatively better access to the cell interior via electrostatic interaction with the cell membrane’s surface.

1.4. FATE OF NANOMATERIALS FOR BIOMEDICAL APPLICATIONS

The concern that nanomaterials may infer toxicity when employed for biomedical application has been a long time debate. However, reports have proven non-toxicity of some nanomaterials both in vitro and in vivo. Thus, for a nanomaterial to be acceptable for biomedical applications, its biocompatibility as well as cytotoxicity
properties must fulfill biomedical safety requirements. Many pristine nanoparticles especially noble metals, metal oxides and quantum dots aggregate in aqueous solution due to their hydrophobic character. Aggregation within biological fluid raises a concern since aggregated nanomaterials can cause embolism of blood vessels (Kim, et al., 2012). Naked nanoparticles can also react readily with cellular composition inducing toxicity. As a result, these nanomaterials are usually covered with biocompatible hydrophilic materials such as dextran (Wang et al., 2010), liposomes (Vincent et al., 2013; You et al., 2014), dendrimers (Tajabadi et al., 2013; Daou et al., 2009), lipoic acid (Tournebize et al., 2012) and polyethylene glycol (Ichikawa et al., 2005; Penon et al., 2015) which infer on them stability against aggregation and help to prevent toxicity. Nanomaterials may be administered into the human body via oral, injection and intravenous routes (Cortajarena et al., 2014). Nonetheless, nanomaterials need to overcome certain barriers such as protein interaction, phagocytic invasion and cellular resistance before they can become efficient for biomedical use. Again the surface charge, shape and nature of coating materials (Cortajarena et al., 2014), in addition to size (Kim, et al., 2012; Weinstein et al., 2010) play essential roles. Neutral nanomaterials may be required to avoid protein interaction while an intermediate optimum size profile (< 200 nm) may be needed to avoid phagocytic elimination (Weinstein et al., 2010). Furthermore, cellular resistance against internalization may be prevented using positively charged nanomaterials (Cortajarena et al., 2014). As a result, the evaluation of the pharmacokinetic parameters such as absorption and internalization into cells, circulation half-life as well as elimination mechanism from the body is crucial for the determination of the fate of nanomaterials within biomedical environments.
1.5. SURFACE MODIFICATION OF NANOMATERIALS

Nanomaterials for biomedical applications are core-shell configurative functional materials consisting of structured nanoparticles covered by an inorganic material or a polymeric structure capable of inferring or improving at least one unique property lacking in the underlying material. Most unique properties inferred by the shell on the core materials include water-solubility (Kim et al., 2012; Kikuchi et al., 2011), optical property improvement, disease-site location (Vincent et al., 2013), prolonged biological circulation half-life (Kim et al., 2012) and therapeutic function (Wang et al., 2014; Meyers et al., 2015; Li et al., 2013).

1.6. CHARACTERIZATION OF NANOMATERIALS

Characterization of nanomaterials is essential to determine their safety and efficacy and to prove their structure. Nanomaterials are characterized for the elucidation of their inherent physicochemical properties such as composition, size, shape, stability, solubility, surface properties, molecular weight, identity, purity and optical properties such as light absorption and emission which are of paramount importance for physiological interactions. There are series of characterization techniques that are available for the measurement of the physical and chemical properties of nanomaterials. These include ultraviolet-visible spectrophotometry (Dong et al., 2016), photoluminescence spectroscopy (Zhang et al., 2016), scanning electron microscopy (SEM) (Goswami et al., 2016; Iram et al., 2010), transmission electron microscopy (TEM) (Jing et al., 2016; Saville et al., 2014), energy dispersive X-ray (EDX) (Shervani & Yamamoto, 2011), X-ray photoelectron spectroscopy (XPS) (Azhdarzadeh et al., 2016; Dong et al., 2016), dynamic light scattering (DLS) (Zamora-Mora et al., 2014), zeta potential (Iram et al., 2010; Zhang et al., 2016), inductively coupled plasma optical emission spectroscopy (ICP/OES) (Saville et al., 2014).
2014), vibrating sample magnetometry (Shen et al., 2014) and powder X-ray
diffraction (XRD) (Ngenefeme et al., 2013). However, determination of the core
nanocrystallite particle size distributions, hydrodynamic size distribution, shape
morphology and crystallinity are usually done using collaborative data from SEM,
TEM, DLS, HRTEM, SAED and XRD (Lu et al., 2010; Shete et al., 2014; Behdadfar
et al., 2012). Surface chemistry such as surface chemical functional group
composition, specific surface area and surface reactions are often characterized
using Fourier transform infrared spectroscopy (FTIR) (Shen et al., 2014; Behdadfar
et al., 2012), Brunauer-Emmett-Teller (BET) surface area analysis (Aphesteguy et
al., 2015) and Surface Enhanced Raman Spectroscopy (Bera et al., 2010; Han et al.,
2012) respectively. In terms of surface charges and elucidation of the oxidation
states of metallic components, zeta potential (Ngenefeme et al., 2013) and X-ray
photoelectron spectroscopy (XPS) (Dong et al., 2016; Azhdarzadeh et al., 2016) are
especially useful. This is because zeta potential measures the overall net charge on
the surface of a material while the XPS detects the different oxidation states of a
metal within a material. For the optical absorption and fluorescence probing,
ultraviolet-visible spectrophotometry (Shervani & Yamamoto, 2011) and
fluorospectrophotometry (Mandal et al., 2013) are the most commonly employed.
Moreover, elemental composition determination is usually done using energy
dispersive spectroscopy (EDS) (Shervani & Yamamoto, 2011) and ICP/OES (Saville
et al., 2014) to prove the presence of core and shell materials in the nanostructures.
1.7. LITERATURE AND REVIEW

1.7.1. PORPHYRIN MACROMOLECULES

1.7.1.1. Structure and Electronic Transitions

Porphyrins are heterocyclic tetrapyrrole macromolecular organic compounds consisting of 22-conjugated \( \pi \) electrons, 18 of which are delocalized within a ring supported by porphine background system (Giovannetti, 2012). The four pyrrole ring systems are joined together by four methine groups representing the four meso-positions (5, 10, 15, and 20) in the entire macromolecular ring system (Fig. 2.1a). Positions 2, 3, 7, 8, 12, 13, 17 and 18 represent \( \beta \)-positions on the pyrolic system (Fig. 2.1a).

The absorption spectrum of a typical free base porphyrin (Fig. 2.1b) consists of two separate electronic transitions, namely the strongly intense soret or B band (Bx, By) transitions consisting of the transition from the ground states \( S_{HOMO\,1}, S_{HOMO\,2} \) to the second excited state \( S_{LUMO\,2} \) and weakly intense Q-bands consisting of the transition from the ground state \( S_{HOMO\,1}, S_{HOMO\,2} \) to the first excited state \( S_{LUMO\,1} \) (Hashimoto et al., 1999; Giovannetti, 2012) (Fig. 2.1c). According to Fig. 2.1c, the soret band transition requires higher energy than the Q band and thus the absorption signal is usually seen at a relatively lower wavelength range, typically between 380 and 500 than the Q bands which usually exist within the visible region, typically between 500 and 750 nm (Giovannetti, 2012). In the theoretical context, both soret and Q bands evolve as two distinct energy-differentiation excited states emanating from the mixing of the two occupied \( \pi \)-orbitals and two unoccupied \( \pi^* \)-orbitals, viz: \( a_{2u}, a_{1u}, e_g(x) \) and \( e_g(y) \) corresponding to the \( HOMO_1, HOMO_2, LUMO_1 \) and \( LUMO_2 \) respectively (Fig. 2.1c).
1.7.1.2. Classification of Porphyrins

Many porphyrins are found widely distributed in nature (Aravindu et al., 2013; Hyland et al., 2016). They are especially utilized to carry out certain biochemical functions such as molecular oxygen binding, charge separation, light harvesting, solar energy conversion and metal ion binding, storage and transport (Hashimoto et al., 1999; Giovannetti, 2012; Lindsey et al., 1987; Shy et al., 2014). Such porphyrins are referred to as naturally-occurring porphyrins. Examples are heme, chlorophyll (Hashimoto et al., 1999) and bacteriochlorophylls (Taniguchi et al., 2008; Krayer, Yang, Diers, et al., 2011). Over the years, due to the high demand of porphyrins in medicine, synthetic porphyrins have also emerged. The latter enable the availability of various forms of porphyrins with different chemical structures (McCarthy et al., 2009; Krayer, Yang, Diers, et al., 2011; Aravindu et al., 2013) and physiological functions. The synthetic protocol has also enabled the production of high purity porphyrins (Taniguchi et al., 2008; Lindsey et al., 1987) for various biomedical applications.

In the context of their use as photodynamic therapy photosensitizers, the evolution of different forms of porphyrins with time can be classified as first, second or third generation. The first generation porphyrins include hematoporphyrin and its derivatives (Connor et al., 2009). The first commercially available form of this porphyrin was Photofrin, which was successfully used as the first clinically accepted porphyrin photosensitizer to cure topical tumour diseases (Connor et al., 2009). However, due to issues of purity, prolonged photosensitivity, non-directional biodistribution and limited optical characteristics, the need to develop second generation porphyrins arose (McCarthy et al., 2009; Schuitmaker et al., 1996). Accordingly, the second generation porphyrins were made in order to resolve the...
challenges encountered with the first generation porphyrins. These porphyrins were
derived by either modification of the native porphyrin structure or synthesis of an
entirely new porphyrin moiety (Krayer, Yang, Diers, et al., 2011; McCarthy et al.,
2009). Although, the second generation porphyrins were able to circumvent some of
the problems associated with the first generation porphyrins, the problem of non-
specific biodistribution still remains unresolved to this day. As a result, many normal
cells have been vulnerable to the toxicity effects of the irradiating light under the
photodynamic therapy procedure (Wang et al., 2014). To resolve this, unrelenting
efforts are currently ongoing for the development of third generation porphyrins with
tumour-specific targeting characteristics. Two major approaches are currently being
exploited, viz: the receptor-mediated strategy (McCarthy et al., 2009; Schneider et
al., 2005) and magnetic targeting (Li et al., 2013). The receptor mediated approach
involves attachment of certain biomolecules such as proteins (Hu et al., 2011),
antibodies, peptides (Wang et al., 2014) or carbohydrates (McCarthy et al., 2009) to
porphyrins which target their specific receptors overexpressed on the surface of
cancer cells. Through this method, porphyrins are safely delivered into tumour sites.

On the other hand, magnetic targeting involves coupling of functional
superparamagnetic nanomaterials with porphyrins, which aid the delivery of the
porphyrin into tumour via their response to an external magnetic field (Li et al.,
2013). Making photosensitizers generally tumour specific via magnetic field targeting
has several advantages. These include relatively higher accumulation of PS within
tumour cells (Li et al., 2013) leading to lower PS dose requirement, faster treatment
time and higher PDT efficacy (Zhao et al., 2014). The low dose requirement factor
induces cost effectiveness on the whole PDT approach.
1.7.1.3. Synthesis of Porphyrins

Typically, symmetrical meso-substituted porphyrins are synthesized via acid-catalyzed condensation reaction between pyrrole and a suitable aldehyde under appropriate acidic pH and temperature (Fig. 2.2a). The first synthetic attempt for tetraphenyl porphyrin was performed by Rothemund and coworkers under reflux at 150 °C for 24 h but with relatively low yield (about 5% yield) (Lindsey et al., 1987) due to a large proportion of chlorin forming along side with the targeted porphyrin (Giovannetti, 2012). In order to resolve this yield challenge, many efforts have been made to reduce the temperature of the synthesis while introducing alternative conditions of the reaction. For example in the 1960s, Adler and coworkers obtained yields of up to 30-40% (Giovannetti, 2012) of symmetrically meso-substituted porphyrins by carrying out the Rothemund synthesis in open air at 141 °C (Lindsey et al., 1987) under reflux for 30 min. Moreover, in 1986-1987, Lindsey and coworkers designed a two-step sequential condensation and oxidation synthetic procedure (Fig. 2.2b) involving the synthesis of meso-substituted porphyrins at room temperature using boron trifluoride (BF₃) or trifluoroacetic acid as trace acid-catalyst and 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) or p-chloranil as the oxidizing agent (Lindsey et al., 1987). Their strategy involved the synthesis of the stable cyclic porphyrinogen and subsequent oxidation to the target porphyrin. A yield of 45-55% was reported by the Lindsey group (Lindsey et al., 1987). In recent years, a two-step mechanochemical synthesis of porphyrins (Fig. 2.2c) mimicking the Lindsey approach has been reported by Shy and coworkers (Shy et al., 2014).
1.7.2. NANOMATERIALS IN PDT

1.7.2.1. Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

SPIONs are magnetic iron oxide nanoparticles with a core diameter usually less than 16 nm (Mürbe et al., 2008) and hydrodynamic diameter less than or equal to 300 nm (Corot et al., 2006). For biomedical use, they may be classified based on their routes of administration and hydrodynamic sizes as oral SPIONs (300 nm) (Corot et al., 2006), intravenous standard SPIONs (50-180 nm), ultrasmall (10-50 nm) and very small (< 10 nm) (Weinstein et al., 2010). The SPIONs with sizes between 10 and 180 nm may also be injectable (Cortajarena et al., 2014). They are generally employed as excellent magnetic resonance imaging contrast agent for cancer diagnosis, as therapeutic agents in magnetic hyperthermia and as drug delivery agents in other disease treatment strategies such as chemotherapy (Wagstaff et al., 2012) and photodynamic therapy (Wang et al., 2014).

The main strategy for the synthesis of SPIONs revolves around breaking down the size of their bulk material below a critical size necessary to infer the unique super paramagnetic properties on the nanoparticles. Thus, many factors that promote production of smaller size nanoparticles such as increase in stirring rate, pH and concentration of the precursor and coating materials are taken into consideration. Typically, SPIONs have been made using various techniques such as co-precipitation (Lu et al., 2010; Wagstaff et al., 2012), hydrothermal (Ge et al., 2009a), micro-emulsion (Nassar & Husein, 2006), solvo-thermal (Hou et al., 2003b) and electrochemical methods (A. Rodríguez-López et al., 2012). However, for biomedical purposes, SPIONs synthesized via co-precipitation are commonly employed. This may be as a result of its clearer chemistry, cost advantage, and greener procedures compared to other techniques. SPIONs utilized for biomedical applications are of two
categories, viz: magnetite (Fe$_3$O$_4$) and maghemite (Fe$_2$O$_3$). Both exhibit cubic inverse spinel crystal structure. However, iron (II) ion vacancy exists in maghemite. The presence of this vacancy in maghemite makes it to be less toxic and thus more desirable for biomedical applications. Moreover, maghemite can also exhibit tetragonal configuration under certain thermal conditions (Kazeminezhad & Mosivand, 2014). Under co-precipitation, magnetite nanoparticles are first produced, while maghemite can be subsequently obtained if desired under suitable oxidizing conditions. Stereotypically, magnetite nanoparticles are produced by precipitating stoichiometric molar solutions of iron (II) and iron (III) ions (ratio 1:2) in an alkaline medium in the presence of an inert gas such as nitrogen or argon. The inert gas ensures prevention of oxidation of magnetite to either hematite or maghemite. However, nowadays, the use of inert gases may be less embraced as new methods such as green techniques that generate in situ reducing capping agents capable of preventing oxidation are emerging (Lu et al., 2010). Pristine SPIONs aggregate in aqueous solution. Thus, to make SPIONs suitable for biomedical application, they need to be stabilized in water. This is achieved by coating their surfaces with hydrophilic polymers such as dextran and polyethylene glycol. The coating process may involve encapsulation, covalent or electrostatic linkage.

Synthesis of super paramagnetic magnetite nanoparticles can be described under two categories, viz: Low and high temperature syntheses. The low temperature synthesis (LTS) involves synthesis with temperature ranging between room temperature and 100 °C under normal atmospheric pressure whereas the high temperature synthesis involves synthesis which requires temperature above boiling point of water and pressure greater than normal atmospheric pressure. Co-precipitation, microemulsion and electrochemical syntheses fall under the low
temperature category whereas hydrothermal and solvothermal fall under high temperature synthesis.

The co-precipitation method involves precipitation of magnetite nanoparticles at some temperatures below the boiling point of water: 40 °C (Mürbe et al., 2008), 60 °C (Lu et al., 2010), 70 °C (Daou et al., 2006), 80 °C and 90 °C (Thapa et al., 2004) usually in an alkaline medium in the presence or absence of an inert gas such as nitrogen or argon. The magnetite nanoparticles are produced from 1:2 molar ratio of iron (II) and iron (III) ions which can be obtained from direct addition of iron (II) and iron (III) salts (Tajabadi et al., 2013; Wagstaff et al., 2012), partial reduction of ferric salts by suitable reductants (Lu et al., 2010; Qu et al., 1999) or partial oxidation of ferrous salts by suitable oxidizing agents (Mürbe et al., 2008; Shete et al., 2014).

On the other hand, micro-emulsion involves co-precipitation of ferrous and ferric ions within the matrix of two immiscible liquids such as oil and water. This is one of the easier ways in which smaller magnetite nanoparticles can be obtained since reactants are confined within a limited space so that co-precipitation occurs within the microdroplets formed as a result of the immiscibility of the organic and aqueous phases (Okoli et al., 2012; Nassar & Husein, 2006; Chin & Yaacob, 2007; Okoli et al., 2011).

Electrochemical synthesis involves synthesis of magnetite from an iron salt solution using electrochemical setup. Typically, iron is the anode while the electrolyte consists of a mixture of salt and stabilizer. Many authors such as Cabrera et al., 2008, Rodríguez-López et al., 2012, Yu et al., 2014 and Kazeminezhad & Mosivand, 2014 used this method to synthesize magnetite nanoparticles of well-defined characteristics. Moreover, it has been reported that the distance between the anode
and cathode and optimized low potential favour formation of magnetite nanoparticles without metallic iron as impurity (Cabrera et al., 2008, Rodríguez-López et al., 2012).

Hydrothermal synthesis is a form of aqueous-based co-precipitation but occurs at higher temperature and pressure than the boiling point of water and normal atmospheric pressure (Wu et al., 2008; Ge et al., 2009; Haw et al., 2010). Typically, it involves heating aqueous mixture of ferrous and ferric ion at molar ratio of 1:2 in a sealed container (usually a Teflon stainless steel autoclave) at temperature above 100 °C. Highly crystalline monodispersed magnetite nanoparticles may be obtained under this method. The higher temperature and pressure seem to promote higher crystallinity and monodispersity.

Solvothermal synthesis is similar to hydrothermal synthesis but employs high boiling point polar or non-polar organic solvents instead of water. For example, Hou et al., 2003 synthesized magnetite nanoparticles by heating mixture of Iron (III) acetyl acetonate, hydrazine (b.p. 114 °C) and ethylene glycol (b.p. 197.3 °C) in a Teflon stainless steel autoclave at 180 °C.

Sun and coworkers (Sun et al., 2004) synthesized crystalline monodispersed magnetite nanoparticles by refluxing a mixture of Iron (III) acetyl acetonate, 1,2-hexadecanediol, phenyl ether, oleic acid and oleylamine at 265 and 300 °C after initial heating of reactants to 200 °C. This is similar to solvothermal technique but the experimental set up is done in a reflux system rather than in an autoclave. High temperature reflux synthesis seems to produce highly monodispersed uniformly shaped narrow size distribution nanomaterials (Saville et al., 2014).

Aerosol assisted chemical vapour deposition involves co-precipitation of magnetite under high temperature thermal decomposition of ferrous salt solution in the
presence of regulated constant flow of a given volume of air/argon mixture usually at
temperature above 420 °C. The air is responsible for the partial oxidation of ferrous
ion while the argon keeps the synthesis environment inert. The setup is such that the
magnetite can be collected in methanol and be separated from other components by
a permanent magnet (Monárrez-Cordero et al., 2014).

Maghemite nanoparticles are typically synthesized by oxidizing magnetite
nanoparticles (Mürbe et al., 2008, Bee et al., 1995; Sun et al., 2004) either in the
presence of air at 100 °C (Sun et al., 2004) or 300 °C (Mürbe et al., 2008) or by
using other suitable oxidizing agents such as nitric acid (Wagstaff et al., 2012) or
ferric nitrate (a. Bee et al., 1995) at 90 °C. This is usually accompanied by a
change of colour from black magnetite nanoparticle solution to a light brown
(Kazeminezhad & Mosivand, 2014) or reddish brown colour of maghemite
nanoparticle (Sun et al., 2004; Kazeminezhad & Mosivand, 2014) solution depending
on synthesis conditions. Moreover, sintering of magnetite nanoparticles at 200 °C
and 650 °C has also been reported for the synthesis of pure maghemite
nanoparticles of different structures (Kazeminezhad & Mosivand, 2014).

Many biocompatible polymers such as dextran (Saraswathy et al., 2014),
polyethylene glycol (Zhang et al., 2002; Silva et al., 2016), polyvinyl pyrrolidone
(Arsalani et al., 2010), liposomes (Vincent et al., 2013), dendrimers (Tajabadi et al.,
2013), copolymers such as poly(aspartate)-graft-poly(ethylene glycol)-dodecylamine-
hydrazone-(adriamycin-levulinic acid) (Huang et al., 2013), polyethylene glycol-
polylactic acid (Zhang & Xie, 2011) or poly (3-(trimethoxysilyl) propyl methacrylate-r-
PEG methyl ether methacrylate –r-N- acryloxysuccinimide) (Lee et al., 2007), protein
(Lee et al., 2006) as well as functionalized gold (Azhdarzadeh et al., 2016; Wagstaff
et al., 2012; Liu et al., 2008) and metalloid such as silica (Tadić et al., 2012) have
been reported to stabilize SPIONs in aqueous solution. Besides coatings with organic polymers, organic acids such as gluconic acid (Lu et al., 2010), citric acid (Klein et al., 2012) or mercapto propionic acid (Lee & Woo, 2006) have also been reported to be suitable for capping SPIONs’ surface. Modification of SPIONs’ surface is usually achieved by direct addition of these coating agents during or after synthesis or by ligand exchange. The latter involves interchange of hydrophobic ligand on the surface of SPIONs with a hydrophilic ligand in order to make SPIONs water soluble (Zhou et al., 2014; Silva et al., 2016).

In photodynamic therapy, SPIONs have been successfully used to deliver both porphyrin (Li et al., 2013) and non-porphyrin photosensitizers (PSs) into tumour-target sites (Zeng et al., 2013). The success of this strategy has led to the development of multifunctional SPIONs-PS conjugate system capable of performing a wide array of specific functions as one entity. For example, Zeng and coworkers developed a multicomponent prodrug system consisting of SPIONs, up-conversion nanoparticles and phthalocyanine PS (Zeng et al., 2013). However, despite this success, SPIONs-prodrug conjugation has many challenges. These include inefficient loading of targeting agent and prodrug on SPIONs, aggregation (water-solubility issue) and hydrodynamic size expansion. In order to resolve these drawbacks, a new synthesis and conjugation approach may have to be developed. Also, optimizing encapsulation of SPIONs-prodrug conjugate into polymers such as polyethylene glycol, liposomes or dendrimers may have to be fully exploited.
1.7.2.2 Gold Nanoparticles

Gold nanoparticles have a long history in biomedical applications basically as passivating, drug delivery, imaging and photothermal agents. They have typically different characteristic shapes such as spherical, rod and cages with tunable sizes (Huang & El-sayed, 2011). However, for the purpose of photodynamic therapy, spherical gold nanoparticles are used solely or as part of multifunctional nanomaterial hybrid systems for drug delivery purposes. The spherical gold can be obtained using simple established laboratory procedures such as sodium borohydride (Shervani & Yamamoto, 2011), citrate (Huang & El-sayed, 2011; Verissimo et al., 2016; L. Zhao et al., 2016), amino acid (Katti et al., 2009; C. Wang et al., 2016; Vijayakumar & Ganesan, 2012), UV-irradiation (Cai & Yao, 2013), gamma-ray (Hien et al., 2012) and carbohydrate (Shervani & Yamamoto, 2011;Engelbrekt et al., 2009; Pienpinijtham et al., 2011) reduction methods. Stereotypically, the reaction undergoes many colour change phases depicting different sizes and electronic transition transformation with time (Shervani & Yamamoto, 2011). Classically, the colour changes from yellow to blue to purple to wine red (Zhao et al., 2016; Hussain et al., 2009)(Fig.1.6). This mechanistically suggests that at first the reaction was very fast and many small nanoscale gold nanoparticles are produced. However, these nanoparticles are very unstable due to their high surface energy. Thus, they aggregate in order to minimize this surface energy causing the surface plasmon resonance (SPR) wavelength to shift to higher wavelength causing a change in the colour of the medium from yellow to blue (Fig. 1.6). Moreover, as the reaction proceeds, the aggregate redissolves in the solution forming small particles causing lowering of the SPR wavelength. This causes a change in the colour of the solution from blue to purple and finally to red (Fig. 1.6) or
even yellow-brown (Pal, 2004). This phenomenon is particularly attractive because specific size distribution can easily be controlled by evolution of each colour.

Two commonly employed methods for gold synthesis for biomedical applications include the citrate and carbohydrate reduction strategies. The citrate method involves reduction of gold precursor salt into its nanoscale dimension by citrate ion at boiling temperature of water. Many authors have used this method to obtain gold nanoparticles of variable sizes (Zhang et al., 2012; Wang et al., 2012; Verissimo et al., 2016; Zhao et al., 2016). Also, the citrate ion can serve as both reducing and capping agent, making this approach more cost-effective than some techniques (e.g. sodium borohydride) which require stabilizing agents in the synthesis.

The mechanism of reduction of gold salt to its nanoparticle dimension by citrate ion could follow the following pathway: oxidation of citrate ion by gold (III) ion leading to reduction of the latter to its zero oxidation state. After reduction, excess citrate ions surround the gold (0) nanoparticles preventing it from aggregation. However, it has been reported that citrate-capped gold nanoparticles are prone to aggregation in the presence of high ionic strength (40-100 mM NaCl) (Wang et al., 2010).

On the other hand, the carbohydrate reduction technique involves utilization of sugars such as glucose (Shervani & Yamamoto, 2011; Engelbrekt et al., 2009), starch (Pienpinijtham et al., 2011), dextran (Wang et al., 2010) and some natural occurring gum (Tagad et al., 2014) as reductants for gold nanoparticle production. Effectively, this typically takes place in the presence of an alkaline promoter or high temperature or both (Pienpinijtham et al., 2011) as many of these sugars inherently could not reduce at ordinary room temperature due to their intrinsic structural orientation. For example nearly all glucose molecules exist as ring structures in
aqueous solution preventing their reducing aldehydic functional groups from functioning. However, under hot environment, the ring structures are transformed to open chain, exposing the aldehydic groups, and thus activating the reducing property.

The use of carbohydrate reductants for the synthesis of gold nanoparticles has several advantages over other methods. These include cost effectiveness, availability of reductant materials, non-toxicity and biocompatibility. Other advantages include ability of some of these carbohydrates to serve as both reducing as well as stabilizing agents. Furthermore, reducing monosaccharides such as glucose can generate in situ acidic stabilizing agents making the approach more cost-effective.
Not so much has been reported about gold nanoparticles in PDT research other than using them as passivating agents for SPIONs (Wagstaff et al., 2012) and carriers of anticancer drugs and PSs (Li et al., 2015; Jang et al., 2011; Cheng et al., 2014). However, a challenge that may be encountered might be aggregation of gold nanoparticles in physiological medium leading to clearance by the body’s immune system before reaching the target site. Nonetheless, aggregation may be prevented by using suitable biocompatible materials that are capable of preventing protein adsorption and escaping the evasion of the phagocytic system. Another challenge is absorption of light by gold nanoparticles. Gold nanoparticles, being plasmonic in nature (Huang & El-Sayed, 2011) are capable of absorbing visible and near infrared lights and thus may reduce the amount of light absorbed by the photosensitizers to achieve a complete therapeutic efficacy. As a result, it is necessary to tune the absorption wavelength’s peak of gold nanoparticles far away from those of the photosensitizers in order to avoid interference which may cause reduction in therapeutic function. Also, it is of paramount importance to use gold nanoparticles of uniform morphology (size and shape) as deviation from this may lead to different light effects causing unpredictable and unmodellable therapeutic functions. Nevertheless, gold nanoshells are specialized gold nanostructures with unique uniform coating of nano-metallic gold on core nanomaterials (Tintoré et al., 2015; Sabale et al., 2017). They differ from the typical spherical gold nanoparticles by forming lining-like structures (shells) round about the core materials to form core-shell nanostructures (Tintoré et al., 2015; Sabale et al., 2017). Gold nanoshells help to prevent the leaching of the core materials into the surrounding environment by passivating or covering the surface of the core materials. This helps to reduce the toxicity of the core materials.
1.7.2.3 Quantum Dots

Quantum dots are specialized types of zero dimensional nanomaterials with unique quantum confinement effect characteristics (Drbohlavova et al., 2009). Thus, they exhibit unique optical properties (Lu et al., 2013; L. Zhang et al., 2014; Sun et al., 2014). Quantum dots (QDs) have excellent broad light absorptions with unique narrow emissions (L. Zhang et al., 2014). They have excellent photobleaching resistance (Dong et al., 2016), conjugable surfaces (Liang et al., 2009) and tunable size and composition-dependent fluorescent properties (Oluwafemi et al., 2016; Liang et al., 2009). Their tunable size can produce emission from the visible to near infrared regions (Lu et al., 2013; Yang et al., 2013; Liang et al., 2009), thus making deep-seated optical imaging easier to visualize in vivo. The large surface area, high brightness and flexible surface of QDs permit their use in the development of drug delivery and fluorescence bio-imaging systems. As a result of these properties, QDs have been used extensively in many biological applications such as biosensors (Wegner & Hildebrandt, 2015), cell labelling (Chang et al., 2008) cellular imaging (Biju et al., 2010) and cancer diagnostics (Malik et al., 2013). Like other nanoparticles, the focus on the synthesis of QDs is directed toward reduction of the bulk precursors to a small size with zero dimensional property. Classically, this can be achieved via hot injection high temperature decomposition of organometallic precursors (Mohan et al., 2014; Beri et al., 2010; Jana et al., 2008). However, the QDs produced under this method are highly hydrophobic making them unsuitable for biological applications. To resolve this challenge, these QDs are subjected to ligand exchange (Gao et al., 2014) via interaction with hydrophilic materials which help to solubilize them in water. Alternatively, QDs are now directly synthesized in aqueous
environment (Rong et al., 2012; Wei et al., 2014; Zhou et al., 2011) using suitable reducing agent and water-solubilizing capping materials.

In respect to photodynamic therapy, Quantum dots can simultaneously function as diagnostic as well as therapeutic tools. Due to their unique narrow light emission properties (between visible and near infrared), they can be used with cancer-surface targeting moieties such as antibody or peptides to track down the site of tumour occurrence in the body. The moieties determine the location of the tumour and bind to their respective receptors that are overexpressed on the tumour surface while the QDs give the signal by light emission after light irradiation within the tumour vasculature (Lu et al., 2013; Dong et al., 2016). In terms of the therapeutic function, QDs can be used as Förster resonance energy transfer (FRET) agents, transferring energy to a neighbouring conjugated photosensitizer (PS) under a two-photon irradiation mechanism (Fowley et al., 2012) or up and down conversion mechanisms (Dong et al., 2016) at a wavelength where normally the PS does not absorb light. This enables a native visible light absorbing photosensitizer to function excellently well under near-infrared light irradiation without having to modify its structure for it to absorb near-infrared light.

However, despite these advantages, the use of QDs in PDT has faced some few challenges. For example, the concern of using toxic heavy metals such as cadmium as part of the core or shell structure of QDs has been a long term debate. Thus, there is a need to develop cadmium free QDs (Mandal et al., 2013), probably focusing on less toxic or more biocompatible metal with comparable or better optical properties. Also, poor loading efficiency of both cancer-targeting agents and photosensitizers on the QDs is another difficulty. To resolve this, new strategies must be develop to optimize the loading capability of these materials on QDs.
Another challenge is the solubilization of hydrophobic QDs in aqueous solution. Although, many greener approaches are emerging, the techniques may not work for the generality of the QD precursors. Thus, there may be a need for improving the existing solubilization techniques involving conversion of hydrophobic QDs to water-soluble QDs.

1.7.2.4 Lanthanide Series Nanoparticles

Lanthanide series are rare-earth elements having atomic number ranging from 57 – 71. Their ions are exceptional UV, visible or near infrared light emitters when irradiated with near infrared (Up-conversion) (Hemmer et al., 2013; Zeng et al., 2013; Yang et al., 2015) or X-ray (Down-conversion) (Chen et al., 2015) light of appropriate wavelength. Thus, they have found major use in up and down-conversion optical applications such as visible or ultraviolet up-conversion luminescence PDT (Chen et al., 2015) and X-ray down-conversion visible luminescence PDT (Chen et al., 2015)

Typically, they are synthesized by doping in a suitable host such as yttrium oxide (Hemmer et al., 2013) or yttrium chloride (Guo et al., 2007) or sodium gadolinium fluoride (Yang et al., 2015). This can be achieved by microemulsion (Guo et al., 2007) or high temperature reflux (Zeng et al., 2013) or thermal decomposition of the precursor salts (C. Wang et al., 2011) usually in the presence of a suitable organic stabilizing agent such as oleic acid (C. Wang et al., 2011) or cetyl trimethylammonium bromide (CTAB) (Guo et al., 2007). However, for biomedical purposes, ligand exchange may be employed using carbohydrates such as α-cyclodextrin (Tian et al., 2013) or polymers such as polyethylene glycol (Yang et al., 2015; Zeng et al., 2013) to prevent toxicity and ensure aqueous stability and prolong circulation half-life. The core-shell architecture of up and down-conversion lanthanide series nanoparticles embedded in their host matrix used for the purpose of
leading to an emission of light that is capable of exciting an inherent visible light absorbing photosensitizer (PS). By this approach, the PS does not need to absorb within the therapeutic window before it can be used to treat deep-seated tumours. Another advantage of this approach is its cost- effectiveness. There may not be any need for manipulation of the structure of the PS in order to make it absorb in the near infrared region. This is very important as manipulating PS’s structure may involve expensive chemicals which in addition may be toxic to both humans and the environment. For example, efficient conversion of a free base porphyrin to its corresponding chlorin or bacteriochlorin may require the use of osmium tetroxide and hydrogen sulphide (Mccarthy et al., 2009), both of which are toxic to humans and the environment. On the other hand, the down-conversion PDT involves excitation of the lanthanide series nanoparticles by a light of appropriate shorter wavelength (e.g. X-ray) leading to an emission capable of exciting an inherent visible light absorbing PS (Chen et al., 2015). The use of X-ray light has been argued to be suitable for the excitation of PS for the purpose of treating deep-seated tumour in the presence of a scintillation nanoparticles (Tang et al., 2015) since currently, X-ray is being used clinically for imaging body parts located deep inside the body. The advantage of this approach, like up-conversion, includes avoidance of structural modification of the primary PSs, which are easier and less toxic to make and can be produced in large scale proportion than their structurally modified derivatives. However, despite this advantage, a concern about the safety of lanthanide series nanoparticles and toxicity emanating from prolonged exposure to X-ray light may limit the future credibility of this interesting PDT approach.
1.8. Magnetic Targeting Using Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

Tumour-targeting strategy using biocompatible functionalized SPIONs has been identified as one of the vital ways of delivering therapeutic agents specifically into tumours (Zhang et al., 2014; Wang et al., 2014; Yeol et al., 2012). Due to their high surface to volume ratio, inherent enhanced permeability and retention (EPR) characteristics (Zhao, Chen, Zhao, Zhang, et al., 2014; Mulens et al., 2013; H.-L. Xu et al., 2016) and magnetic properties (Cheraghipour et al., 2012; Huang et al., 2013), these SPIONs have been used effectively as unique nanocarriers for the delivery of therapeutic agents into the tumour arena including the brain (H.-L. Xu et al., 2016; Shevtsov et al., 2015), cervical (Zhao, Chen, Zhao, Zhang, et al., 2014) and breast cancer cell (Kumar et al., 2014) environments.

Typically, SPIONs deliver their payloads into the tumour environment via two different routes, viz: the enhanced permeability and retention (EPR) and external magnetic field pathways. The EPR route occurs as a result of the passage of SPIONs through the leaky tumour vasculature due to their permeable small sizes, followed by subsequent retention due to the poor lymphatic drainage of the vasculature (Mulens et al., 2013). On the other hand, the magnetic field channel involves dragging SPIONs-payload system into tumour via their response to the direction of an external magnetic field (Zhao et al., 2014; Zhao et al., 2016). It has been reported that the magnetic field strength is inversely proportional to the distance from its source (Beinart & Nazarian, 2013). Thus, the closer the magnet, the higher the magnetic field effect (Li et al., 2013). The interaction of aqueous SPIONs in the absence and presence of an external magnetic field is shown in Fig. 1.9.
According to Fig. 1.9, the magnetic field re-orientates the SPIONs towards its direction based on a critical distance between the external magnet and the vessel containing the SPIONs. However, in the biological environment, it has been reported that static magnetic field may show some therapeutic effects on the imposed cancer cells (Ghodbane et al., 2013). Thus, in order to evaluate the photodynamic therapy efficacy of a particular magnetic targeting therapeutic system accurately, the effect of
the magnetic field on the morphology and proliferation of the cancer cells in the dark must be put into consideration.

In this project, towards the drive to make porphyrin tumour-specific via magnetic field targeting, a multifunctional nanostructure consisting of meso - 5, 10, 15, 20-tetrakis (4-hydroxyphenyl) porphyrin conjugated to gluconic acid capped superparamagnetic iron oxide nanoparticles-gold core-shell nanostructure encapsulated within a methoxy-polyethylene glycol-thiol matrix was successfully fabricated. The gold shell was used to passivate the surface of the SPIONs core to prevent undesired leaching of the iron species while the SPIONs played the role of magnetic field-driven nano drug delivery vehicle via their response to the direction of an external magnetic field. The porphyrin served the dual role of fluorescence imaging and photodynamic therapeutic agents. After successful conjugation, the singlet oxygen generation potential, fluorescence bio-imaging property and photodynamic therapy efficacy of the as-synthesized PEGylated magnetic porphyrin conjugate were evaluated.

1.9. RESEARCH PROBLEM
Photodynamic therapy suffers from light-associated toxicity (Wang et al., 2014) on normal cells due to lack of tumour-specificity and inadequate water solubility of most of the viable clinically-recognized photosensitizers (Master et al., 2013). To circumvent these challenges, many photosensitizers are currently being exploited for their feasible conjugation to hydrophillic biocompatible polymeric platforms (Li et al., 2013; Jing et al., 2016), while the tumour-specificity targeting issue is being addressed by employing various non-magnetic strategies such as tumour-targeting using biological molecules (e.g. carbohydrate, peptides and folic acid) which target specific receptors over-expressed on the surface of cancer cells (Master et al., 2013; Wang et al., 2014; Meyers et al., 2015) and magnetic targeting using functionalized
superparamagnetic iron oxide nanoparticles (SPIONs) which deliver photosensitizer payloads directly into the tumour site via response to an external magnetic field (Li et al., 2013). However, among these modalities, magnetic targeting using SPIONs is gaining more attention due to its several advantages. These include rapid concentration of photosensitizers in tumours, reduction in PS’s dose, and shorter therapeutic time. In this thesis, meso-tetrakis(4-hydroxyphenyl)porphyrin and its derivatives were conjugated to superparamagnetic iron oxide nanoparticles-gold core-shell for the purpose of making the porphyrin tumour-specific against MCF-7 metastatic breast cancer cells via magnetic targeting. While SPIONs have been conjugated to some photosensitizers in some reports (Li et al., 2013; Wang et al., 2014), no such reports exist on the conjugation of SPIONs-gold core-shell to meso-tetrakis (4-hydroxyphenyl) porphyrin for the improvement of PDT via magnetic targeting.

1.10. AIMS, OBJECTIVES AND HYPOTHESES

1.10.1. Aims
The aim of this project was to synthesize and characterize an efficient and tumour-specific superparamagnetic iron oxide nanoparticles-gold core-shell porphyrin conjugate for enhancement of photodynamic therapy. Moreover, this aim was achieved by carrying out the following objectives.

1.10.2. Objectives

- Synthesis and characterization of superparamagnetic iron oxide nanoparticles-gold core-shell nanomaterial (SPIONs@Au)
- Synthesis and characterization of a water-soluble therapeutic light-absorbing, magnetic targeting porphyrin conjugate (SPIONs@Au-TLA(P)
• Evaluation of SPIONs and porphyrin conjugates in exogenous aqueous solution
• *In vitro* evaluation of the singlet oxygen generation potential of the as–synthesized porphyrin conjugate (SPIONs@Au-TLA(P))
• *In vitro* evaluation of the dark toxicity and photodynamic therapy efficacy of the as-synthesized SPIONs@Au@TLA-P on MCF-7 metastatic breast cancer cells

Furthermore, these objectives were exploited to answer the following hypothesis statements.

1.10.3. Hypothesis

• Hypothesis 1: SPIONs@Au@TLA-P can be made water soluble
• Hypothesis 2: SPIONs@Au@TLA-P (and some other synthesized porphyrin conjugates) can be used to generate singlet oxygen.
• Hypothesis 3: SPIONs@Au@TLA-P can absorb the therapeutic light
• Hypothesis 4: SPIONs@Au@TLA-P shows no dark toxicity on MCF-7 metastatic breast cancer cells
• Hypothesis 5: SPIONs@Au@TLA-P can be used to destroy MCF-7 metastatic breast cancer cells via photodynamic therapy
CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

D-(+)-glucose monohydrate, ferric chloride hexahydrate, silver nitrate, sodium chloride, neutral red hydrochloride, ethyl acetate, propionic acid, ethanol, methanol, dichloromethane, sodium bicarbonate, gold (III) chloride hydrate, ammonium hydroxide (30-33%) methoxypolyethylene glycol-thiol (mPEGSH), silica gel, 4-hydroxybenzaldehyde, pyrrole, 1,3-diphenylisobenzofuran, dimethyl sulfoxide (DMSO), deuterated dimethylsulfoxide (DMSO-d6), methoxylporphyrin, petroleum ether, Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin-streptomycin, amphotericin-B solution, Hank’s Balanced Salt Solution (HBSS), TritonX-100 and Fluoromount™ Aqueous Mounting Medium were purchased from Sigma Aldrich. Ammonium hydroxide (5.02 N) and methylene blue were obtained from Fluka (USA) and Merck (USA) respectively. Fetal bovine serum (FBS) and Tryple™ Select were purchased from Life Technologies (USA). All reagents were analytical grades and used without further purifications except pyrrole which was distilled prior to use. Deionized water was used for all aqueous solution preparations.
2.2 METHODOLOGY

2.2.1 Synthesis of superparamagnetic iron oxide nanoparticles (SPIONs)

SPIONs are specialized nanomaterials with unique attraction towards the direction of applied external magnetic fields. Because of their essentiality, many approaches were introduced for the synthesis of SPIONs in this thesis via modification of existing methodology and a new synthetic route.

2.2.1.1 Synthesis of gluconic acid capped SPIONs at different temperatures (40-60 °C) (SPIONs 1-3)

The previously reported method by Lu and coworkers (Lu et al., 2010) was modified to achieve this current approach. Briefly, preheated mixture of glucose monohydrate solution (4 mL, 0.05 M) and ferric chloride hexahydrate solution (4 mL, 0.35 M) in a 100 mL Pyrex beaker was added dropwisely to a preheated ammonium hydroxide solution (10 mL, 1M) at 40 °C, under hierarchical heating and cooling pattern, for 6 min, under vigorous continuous stirring, to form a black colloidal solution. The latter was heated for another 4 min and afterward cooled to room temperature. At this time, SPIONs separated out from the solution and were dried in an oven at 68 °C (SPIONs 1). A repeat of the above procedure was performed at 50 °C and 60 °C to obtain SPIONs 2 and SPIONs 3 respectively.

2.2.1.2 Synthesis of ultrasmall-gluconic acid capped superparamagnetic iron oxide nanoclusters coprecipitated with goethite and hematite (USPIONCs-Goethem) (SPIONs 4A & 4B)

A glucose solution (anhydrous; 20 mL, 0.05817 M) was added to a ferric chloride solution (anhydrous; 20 mL, 0.3473 M). The mixture was heated to 73 °C and
subsequently maintained at this temperature under vigorous magnetic stirring for 30 min followed by cooling to room temperature. Afterwards, the solution was added drop-wisely to a preheated ammonium hydroxide solution (50 mL; 1M) between 75 and 55 °C at the rate of 2 mL/min for 16 min under hierarchical heating and cooling pattern and subsequently heated to 60 °C under vigorous continuous stirring for 4 min to obtain a black colloidal solution (SPIONs 4A). A similar procedure was used to synthesized SPIONs 4B employed for the simultaneous removal of neutral red, Ag+ and chloride from aqueous saline solution but with different concentration of the precursors (Glucose solution: 4 mL of 0.05M; ferric chloride solution (4 mL of 0.35M); (yield =0.1764 g).

2.2.1.3 Large scale one pot synthesis of gluconic acid capped superparamagnetic iron oxide nanoparticles (SPIONs 5)

Glucose monohydrate (100 mL, 2.5268 g/250 mL) was added to ferric chloride solution (100 mL, 23.6920 g/250 mL) and the resulting solution was heated until the volume changed from 200 mL to 40 mL (25 min) to obtain the yellow complex intermediate. A volume of the latter (20 mL) was added at once to a preheated ammonium hydroxide solution at 50 °C, after which the resulting solution was allowed to react for 10 min. The reaction was stopped by adding 500 mL of water. The resulting solution was subsequently aged for 22 min, followed by decantation. The final wet residue was dried at 60 °C. The whole process was repeated twice and the resulting nanomaterials combined together and stored under ambient condition (yield = 0.5987 g).
2.2.1.4 Synthesis of gluconic acid capped SPIONs by Lu et al (SP80S60) with modifications based on purification and drying conditions - (SPIONs 6)

A preheated solution of α-D-glucose monohydrate (5 mL of 0.05 M) and anhydrous ferric chloride solution (5 mL of 0.3 M) at 80 °C was added in drops to a preheated ammonium hydroxide solution (10 mL, 1M) at 60 °C on a pre-conditioned heating mantle, under vigorous mechanical stirring. The resulting solution was allowed to proceed for about 30 minutes while aliquot (1 mL each) was taken as the reaction proceeded to monitor the formation of the nanoparticles. Afterward, the solution was cooled to room temperature and washed several times with water and left in the laboratory environment to age for two days. This was followed by washing with water and subsequently removal of the supernatant to obtain black magnetic iron oxide colloid, which was dried under ambient conditions for two days. Addition of deionized water to the resulting matrix revealed the hidden tiny black needle-like magnetic nanocrystals.

2.2.1.5 Effect of switching between the precursor’s and synthesis’ temperatures (SPIONs 6 vs SPIONs 7)

The approach in section 2.2.1.4 was repeated to obtain SPIONs 6. Afterwards, a new experiment was conducted but with a change in the precursor’s temperature from 80 °C to 60 °C and synthesis’s temperature from 60°C to 80 °C to obtain SPIONs 7. However, all the solvent was allowed to evaporate for 15 min to obtain a red-brown precipitate. The latter was left to age for 7 days, followed by washing with deionized water and the obtained red SPIONs was allowed to dry for about two days under ambient condition.
2.2.1.6 Effect of changing the precursor's temperature from 82 °C (SPIONs 8) to 72 °C (SPIONs 9)

The method in section 2.2.1.5 was modified to achieve current procedure. Briefly, anhydrous solution of α-D-glucose (20 mL, 0.2094 g) was added to anhydrous ferric chloride solution (20 mL, 1.1262 g) to make a 40 mL dark brown glucose-ferric solution. This solution was heated under vigorous stirring from room temperature 25 °C to 82 °C until the colour changed from brown to yellow-orange. The latter was added to ammonium hydroxide solution (50 mL, 1 M) at 60 °C for 16 min under vigorous mechanical stirring. At this time, the resulting solution was allowed to react further for 4 min after which the reaction was stopped. Moreover, the solution was washed with water (200 mL) and subsequently aged for 12 h. This was followed by decantation and magnetization to obtain a dark chocolate brown colloid. The latter was dried under ambient condition to obtained black magnetic nanomaterials (SPION 8, yield = 0.0981 g). The above procedure was repeated but with a change in the precursor’s temperature from 82 °C to 72 °C under heat ON and Heat OFF conditions to obtain SPION 9 (yield = 2.4219 g).

2.2.1.7 Synthesis of magnetic solid-based precursor using glucose as reductant (SBP 1)

Glucose monohydrate solution (0.2523 g/25 mL) was added to a ferric chloride hexahydrate solution (2.3689 g/25 mL) and the mixture heated continuously nearly to dryness at 280 °C to obtain first a red-wine syrupy-oily mixture and finally a dark chocolate brown solid. At this time the heating was stopped. The latter was dried at 60 °C overnight to obtain a dark magnetic hygroscopic chocolate-brown solid. The latter was grind into powder and stored in a glass vial under ambient condition.
2.2.1.8 Synthesis of magnetic solid-based precursor using maltose as reductant (SBP 2)

Maltose monohydrate solution (0.6795 g/50 mL) was added to a ferric chloride hexahydrate solution (4.7401 g/50 mL) and the resulting mixture heated continuously to boiling for some time to obtain a yellow-orange solution. The latter was then dried in an oven at 68 °C to obtain hygroscopic chocolate brown solid. The latter was further heated in an oven at 125 °C to drive out water molecules and air and subsequently stored in a glass vial for further analysis. Furthermore, some amount of the stored solid was reduced to powder via grinding. The latter was highly soluble in water and its aqueous solution exhibited strong attraction towards external magnetic field.

2.2.1.9 Synthesis of magnetic iron oxide nanoparticles (maghemite- \( \gamma \)-Fe\(_2\)O\(_3\)) using solid-based precursor SBP 2

Precursor powder (SBP 2)(0.2241 g)(Section 2.2.1.10) was added to an ammonium hydroxide solution (3 mL, 1 M; prepared by diluting 5.4 mL of 30-33% NH\(_4\)OH to 100 mL standard flask mark) in a closed vial at room temperature (25 °C) for 12 min to obtain a green-black magnetic residue with green supernatant. Afterwards, the mixture was subjected to multiple series of washing with water, followed by magnetization and decantation of the supernatant until the supernatant pH became 6.25. At this time, the resulting magnetic residue was heated in air at 60 °C to obtain a red-brown magnetic powder (yield = 0.0399 g).
2.2.1.10 Synthesis of magnetic solid-based precursor using maltose, ferric chloride and sodium bicarbonate mixture (SBP 3)

A mixture of maltose monohydrate (2.3497 g) and sodium bicarbonate (3.1376 g) were heated on a flat ovenware ceramic plate at 150 °C for 12 min to obtain a yellow flake-like solid (YFS). A given amount of the YFS (1.1512 g) was added to a ferric chloride hexahydrate solution (30 mL of 0.35 mol/L diluted to 45 mL) to obtain a red violet/red grape solution with effervescence. Afterwards, this solution was heated to dry in an oven at 150 °C to obtain a dark chocolate brown magnetic solid (SBP 3).

2.2.1.11 Synthesis of non-magnetic solid-based precursor using maltose and sodium bicarbonate mixture (SBP 4)

A mixture of maltose monohydrate (20.0016 g) and sodium bicarbonate (46.7329 g) was heated on a rectangular flat ovenware plate at 153±2 °C for 5 h to obtain a dark chocolate brown dough-like solid (SBP 4). Afterwards, it was cooled to room temperature and stored under ambient condition.

2.2.1.12 Synthesis of magnetic iron oxide nanoparticles (maghemite- $\gamma$-Fe$_2$O$_3$) using solid-based precursor prepared from preheated mixture of sodium bicarbonate and maltose (SBP 4)

Ferric chloride solution (25 mL, 0.35 M) was heated with 25 mL solution (0.1214 g/100 mL) of SBP-IV in a 250 mL Boro Lassco beaker for 60 min and the resulting greenish-brown precipitate added slowly to sodium bicarbonate solution (50 mL, 2.66%) at room temperature. Afterward, the solution was heated to dry in an oven at 60 °C to obtain a red-brown magnetic nanomaterial.
2.2.1.13 Synthesis of gluconic acid capped SPIONs for photodynamic therapy (SPIOns 10)

The approach employed under section 2.2.1.1 was followed with some modifications. Briefly, a mixture of ferric chloride hexahydrate solution (25 mL, 0.3506 M) and D- (+)-glucose monohydrate solution (0.051 M) in a 100 mL Pyrex beaker was heated from room temperature to 66 °C and maintained at this temperature until the colour of the solution changed from brown to dull yellow-orange covering a total heating period of 60 min. The resulting solution was then cooled for 42 min after which it was added dropwise to 50 mL of ammonium hydroxide solution (1 M) in a 100 mL Pyrex beaker at 50 ± 2 °C for 21 min. This was followed by further heating at this temperature for another 24 min. Afterwards, the as-synthesized SPIONs (SPIOn 9) were washed five times with deionized water (4 x 250 mL; 1 x 75 mL) and magnetically harvested, followed by decantation and drying in an oven at 75 ± 1 °C.

2.2.2. Synthesis of gold nanomaterials

2.2.2.1. Synthesis of maltose-reduced gelatin capped gold nanoparticles (Au 1)

Gelatin (1 g) was dissolved in 100 mL standard flask with deionized water at 100 °C to obtain gelatin solution. A given volume of this solution (10 mL) was added to a gold solution (4 mL, 0.00053 M), followed by addition of maltose solution (4 mL, 0.02933 M) and the resulting mixture heated to 80 °C for a period of 15 min. The colour of the solution changed from yellow to light pink. Afterwards the as-synthesized gold solution was stored under ambient condition.
2.2.2.2. Synthesis of citrate-reduced gelatin capped gold nanoparticles (Au 2) in the presence of SPIONs

Gelatin (1 g) was dissolved in 100 mL standard flask with deionized water at 100 °C to obtain gelatin solution. A given volume of this solution (10 mL) was added to a solution containing gold (4 mL, 0.00053 M), SPIONs 8 (2 mL, 0.0012 g/100 mL) and maltose (4 mL , 0.02933 M) and the resulting mixture was heated to 80 °C for a period of 15 min. The colour of the solution changed from yellow to light pink. Afterwards, the as-synthesized gold solution was stored under ambient condition.

2.2.2.3. Synthesis of maltose-reduced gold nanoparticles (Au 3) – Method (I)

A gold solution (2 mL, 0.003155 M) was placed in a beaker and diluted to 12 mL with deionized water to obtain a concentration of 0.0005258 M. This was followed by the addition of 0.1011 g maltose. The resulting mixture was then subjected to continuous heating without stirring for 3 min to reach 80 °C at which point, the colour change from yellow to light pink. Afterward, the heating continued for further 2 min to reach 83 °C at which point the colour changed from the light pink to light blue and the reaction was stopped.

2.2.2.4. Synthesis of maltose –reduced gold nanoparticles (Au 3) – Method (II)

A gold solution (2 mL, 0.003155 M) was placed in a beaker and diluted to 12 mL with deionized water to obtain a concentration of 0.005258 M. This was followed by the addition of maltose solution (0.1011 g/10 mL). The resulting mixture was then subjected to continuous heating under vigorous stirring for 15 min. At the 8th min the
colour of the solution turned from yellow to almost colourless while at 9\textsuperscript{th} min a blue colour evolved at 80 °C. When the reaction was allowed to proceed further for 3 min the colour changed from light blue to dark blue and this colour was maintained till the end of the reaction at 83 °C.

2.2.3. Synthesis of superparamagnetic iron oxide nanoparticles (SPIONs)-gold core-shell using greener approaches

2.2.3.1. Synthesis of citrate-reduced gelatin capped gold-coated gluconic acid capped SPIONs – Red violet SPIONs@gold 1

Gelatin (1 g) was heated in a 50 mL deionized water in a 100 mL Pyrex beaker until it dissolved and the resulting solution was transferred into a 100 mL standard flask and diluted to mark with deionized water to obtain gelatin solution. A part of this solution (15 mL) was added to a solution containing mixture of 10 mL of gluconic acid capped SPIONs (section 2.2.1.6 (SPIONs 8)) and 11 mL of gold solution obtained by diluting 2 mL of 0.003 M HAuCl\textsubscript{4} \cdot xH\textsubscript{2}O to 12 mL using deionized water. This latter solution was heated for 10 min without stirring. At this time, sodium citrate (0.1716 g) was added and the mixture stirred vigorously for another 10 min. The colour of the solution changed from yellow to a red violet / pink colour at 12 min heating period and this colour was maintained till the end of the reaction at 85 °C.

2.2.3.2. Synthesis of gold-coated gluconic acid capped SPIONs at 50 °C (SPIONs@Au 2)

Gold salt (HAuCl\textsubscript{4}) (0.0341 g) was added to the colloidal solution of gluconic acid capped SPIONs (Section 2.2.1.1(SPI\textsubscript{ON} 2)) at 50 °C. The reaction mixture was allowed to proceed for some time (10 mins). A colour change was observed from black to antique gold colour. Afterward, the obtained gold-coated SPIONs
nanocomposite was cooled to room temperature, poured into a petri dish and dried in a fume hood overnight. The gold-coated SPIONs nanomaterial was slowly attracted to external magnetic field.

2.2.3.3. **Synthesis of gold-coated gluconic acid capped SPIONs at 74 °C (SPIONs@Au 3)**

A given amount of SPIONs 10 (0.0286 g) was sonicated in 100 mL of deionized water (Pyrex beaker) for 1 h 30 min to obtain a dark loamy-soil-like or dirty chocolate colour (DDC). Gold solution (25 ml; prepared from 0.0132 g dissolved in 25 mL of deionized water) was added to 40 mL of DDC solution in a 100 mL Pyrex beaker and the resulting solution was subsequently heated in an oven at 74 °C without stirring for 7 h to obtain an antique gold-orange-red colour. The solution was then cooled and magnetized for 5 h to ensure all the core-shell nanomaterials have been completely separated from the supernatant. The resulting core-shell nanoparticles (SPIONs@Au 3) were dried in an oven at 60 °C.

2.2.3.4. **Synthesis of gold coated SPIONs (SPIONs@Au 4) for POP-PEG-Brown**

Gold (III) chloride hydrate solution (0.2058 g/ 250 mL) and SPIONs solution (SPIONs 5 previously grinded to powder) (0.2658 g/ 250 mL) were premixed in a 500 mL Pyrex beaker, transferred to a rectangular glass ovenware and heated at 60 °C for 7 h without stirring to obtain SPIONs-gold nanostructure (SPIONs@Au 4). The supernatant was decanted and the residue washed with water, followed by magnetization and decantation. Finally, the nanomaterial was dried in an oven at 60 ± 2 °C to obtain a dark red brown nano-powder. The latter was stored under ambient condition until further analysis (yield = 0.2271 g).
2.2.4. Synthesis of meso-tetrakis(4-hydroxyphenyl)porphyrin (THPP (POP 1))

2.2.4.1. Synthesis, purification and characterization

A mixture of freshly distilled pyrrole (16 mL; colourless) and propionic acid (330 mL; colourless) in a 500 mL flask was refluxed at 138 °C for 40 min. Afterward, 4-hydroxybenzaldehyde (7.1727 g) was added slowly and the resulting mixture was refluxed further for 5 h to obtain a black purple solution (Caution: as soon as the aldehyde was added, a violent reaction took place; aggressiveness of boiling increased and the solution turned red purple violet). The resulting product was cooled to room temperature (2h, 20 min) and the supernatant decanted. The remaining black residue in the flask was labeled (BBRcrude). The BBRcrude (1.6617 g), dissolved in 2 mL ethyl acetate was mixed with 2.1315 g of silica gel and the resulting mixture subsequently heated to dry in an oven for a few minutes. The resulting dried crude-silica gel mixture was loaded on a silica gel column (20.1 x 180.7 mm), preloaded with a silica gel slurry, prepared by dissolving 36.8176 g silica gel in a 90 mL ethyl acetate-dichloromethane solvent mixture (1:2 v/v). The silica gel column had a flow rate of 7.5 mL/min and was run for 30 min. All pure porphyrin (POP) fractions were mixed together and the resulting solution subsequently dried in air under ambient condition to obtain a brilliant green dye which turned red brown in methanol. The pure porphyrin was identified using ultraviolet-visible spectrophotometry (UV-Vis), Fourier transform infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance spectroscopy (1H NMR). UV-Vis: soret: 426, QIV-I: 530, 566, 609, 656; FT-IR: O-H and N-H at 3381 (89.83 %), range 3682-3126, broad); C-H(benzene): 3100 (91.06 %); C-H(vinyl): 2932 (70.29 %); C=N(1730) (62.72 %); C=C(vinyl): (1610) (76.53 %); C=C(benzene): (1514) (71.76
% & (1467) (72.08 %); C-N(1274) (48.24 %); C-Ostr (1172) (61.47 %), 1129 (58.88 %) & 1075 (61.19 %) and bending peak for C-H(out- of plane ) (Benzene) : 746 (62.19 %) groups; $^1$H NMR : $\delta$(ppm) -2.90 (s, 2H, NH (within ring), 7.21 (d, 8H, m- C$_6$H$_4$OH), 7.95 (d, 8H, o-C$_6$H$_4$OH), 8.85 (s, 8H, HC=CH, Pyrrole), 9.97 (s, 4H, OH)(yield = 3.76 %).

2.2.4.2. Estimation of molar extinction coefficient of the purified porphyrin using methanol solution

A given amount of the purified porphyrin (0.0002 g) (section 2.2.4.1) was dissolved in 8 mL of Methanol (MeOH) to obtain a porphyrin stock solution (PPA)(Soret Absorbance = 2.7961 @421 nm). A given volume of this solution (1 mL ) was diluted to 3 mL with MeOH to obtain porphyrin solution PPB (Soret Absorbance 1.1644 @421 nm). In addition, 1 mL of PPB was diluted to 3mL with MeOH to obtain porphyrin solution PPC(Soret Absorbance = 0.45683 @421 nm ). Furthermore, 1 mL of PPC was diluted to 3 mL with MeOH to obtain porphyrin solution PPD(Soret Absorbance 0.20043 @421 nm ). Similarly, 1 mL of PPD was diluted to 3 mL to obtain porphyrin solution PPE (Soret Absorbance 0.11598 @421 nm). Lastly, 1 mL of PPE was diluted to 3 mL with MeOH to obtain porphyrin solution PPF (Soret Absorbance = 0.090546 @421 nm). The details of the estimation of the concentrations in mol/L is given below (Molecular weight of porphyrin = 678.73 g/mol).

Amount of porphyrin in 8 mL MeOH = 0.0004 g / 8 mL MeOH

= (0.0004 x 1000)/(8 x 678.73) mol/L

PPA = 0.0000736669 mol/L (stock)

1 mL of PPA diluted to 3 mL using MeOH gave PPB.
Using $C_1V_1 = C_2V_2$ where

$C_1 = $ initial concentration in mol / L; $C_2 = $ final concentration in mol / L; $V_1 = $ initial volume (mL) and $V_2 = $ final volume (mL)

implies that

$PPB = C_2 = \frac{(C_1 \times V_1)}{V_2} = \frac{(0.0000736669 \times 1)}{3} = 0.0000245556 \text{ mol/L}$

Similarly, 1 mL of PPB diluted to 3 mL gave PPC.

Thus,

$PPC = \frac{(0.0000245556 \times 1)}{3} = 0.0000081852 \text{ mol/L}$

Also, 1 mL of PPC diluted to 3 mL gave PPD.

Thus,

$PPD = \frac{(0.0000081852 \times 1)}{3} = 0.0000027284 \text{ mol/L}$

Furthermore, 1 mL of PPD gave PPE.

Thus,

$PPE = \frac{(0.0000027284 \times 1)}{3} = 0.00000090946666 \text{ mol/L}$

Finally, 1 mL of PPE gave PPF.

Thus,

$PPF = \frac{(0.00000090946666 \times 1)}{3} = 0.00000030315555 \text{ mol/L}$

### 2.2.4.3. Estimation of molar extinction coefficient of the purified porphyrin using methanol-water solution

A given amount of the purified porphyrin (0.0002 g) (section 2.2.4.1) was dissolved in a 10 mL methanol-water solution (ratio 1:1 v/v) to make the stock solution (mol/L) with absorbance of 0.64. This was followed by serial dilution of the stock solution
using the same methanol-water solution (1:1 v/v) to obtain porphyrin solutions with various concentrations. The absorbance values of the different concentrations (mol/L) of the purified porphyrin were measured using ultraviolet-visible spectrophotometer. The molar extinction coefficient of the porphyrin was estimated from the slope of the plots of absorbance at 422 nm vs concentration (mol/L) following Beer-Lambert’s principle.

2.2.5. Synthesis of meso-tetrakis(4-hydroxyphenyl)chlorin (THPC (POP 2))

Variant volumes (0.1 - 4 mL) of 30 % H₂O₂ were added consecutively to 3 mL methanolic solution of the purified 5, 10, 15, 20-tetrakis (4-hydroxyphenyl) porphyrin (section 2.2.4.1) to obtain a spontaneous green chlorin solution. The chlorin formation was confirmed using ultraviolet-visible spectrophotometry (UV-Vis).

2.2.6. Synthesis of water-soluble porphyrin conjugates (PC)

Many porphyrin derivatives are hydrophobic in nature and thus are required to be water-soluble for biomedical applications. In this thesis, many water-soluble porphyrin conjugates were synthesized using new approaches.

2.2.6.1. Synthesis of tetraoctylammonium bromide SPIONs-porphyrin (Hay yellow-PC 1) and PEGylated-thiol gold nanocomposites (Red violet-PC 2) conjugates.

Modified Brust-Schiffrin approach reported by Penon and coworkers was employed with some modifications. Briefly, some amount of methoxypolyethylene glycol thiol (0.02 g) was added to an aqueous solution (5 mL) of citrate reduced gelatin capped SPIONs-gold nanostructure (Section 2.2.2.2) and the resulting mixture shaking for 5 min. This solution was then added to a stirred solution (12 mL) of
tetraoctylammonium bromide in toluene containing 0.0002 g meso-tetrakis (4-
hydroxyphenyl) porphyrin. The resulting solution was left to interact under ambient
condition until all the organic and aqueous phases had evaporated. Two distinct
products were obtained, one magnetic water-soluble hay-like yellow material (PC 1)
that stucked to the top side of the beaker and the other non-magnetic red violet
material (PC 2) that lied low at the bottom of the beaker.

2.2.6.2. **Synthesis of white magnetic SPIONs-methoxyporphyrin conjugate
(PC 3)**

Glucose monohydrate (0.1269 g) was added to SPIONs 2 solution (0.0031g/ 20 mL)
to obtain a glucose-SPIONs solution mixture. A given amount of the latter (5 mL)
was added to methoxyporphyrin solution (0.0004 g / 5 mL absolute ethanol) and the
resulting solution sonicated for 4 mins to obtain a white suspension solution. This
solution was dried under ambient condition in the fume hood to obtain brown flake-
like mass. The latter was extracted three times with absolute ethanol and the
resulting combined greenish-yellow solution was transfered into a plastic cuvette and
left in a fume cupboard to dry overnight to obtain a white magnetic SPIONs-
porphyrin crystals. Addition of the latter to water releases porphyrin as a water-
soluble red violet/pink entity. On shaking, the red violet disappeared to obtain a
brown-yellow solution which showed attraction to external magnetic field.

2.2.6.3. **Synthesis of methoxypolyethyleneglycol-thiol SPIONs-gold core-
shell meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate (PC 4)**

Aqueous SPIONs@Au 3 core-shell solution (25 mL, 0.001394 g/mL) in a 100 mL
Pyrex beaker was sonicated for 10 min. This was followed by addition of 0.0802 g
methoxy-polyethylene glycol-thiol (mPEG-SH) and subsequent sonication of the
resulting solution for 5 min. Then, 20 mL of the resulting mixture was added to a 60 mL porphyrin solution in methanol (0.0005533 g/mL) in a 250 mL Pyrex beaker, followed by shaking for 10 min. The final solution was then evaporated to dryness under ambient condition to obtain the SPIONs-gold porphyrin conjugate.

2.2.6.4. Synthesis of methoxypolyethyleneglycol SPIONs-gold core-shell meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate (PC 5)

A given volume of PEGylated SPIONs@Au 4 solution (20 mL) taken from previously sonicated 100 mL solution containing 0.0045 g SPIONs 5 and 0.0425 g methoxypolyethyleneglycol (mPEGOH) was added to a methanolic solution of meso-tetrakis(4-hydroxyphenyl)porphyrin (60 mL, 0.0044 g/100 mL) in a 500 mL Erlenmeyer’s flask and the resulting solution subsequently shaken on a laboratory shaker (Merck Millipore) at 150 rpm for 10 min. Afterwards, the resulting solution was dried in an oven at 60 °C for 4 h to obtain a brown dye. To compare the effect of drying of the conjugate precursor solution at room temperature and at 60 °C, 10 mL each of the mixed PEGylated SPIONs-gold-porphyrin solution was dried at these temperatures (room temperature: 19.5 °C and 60 °C) and the respective brown aqueous solutions were evaluated using ultraviolet-visible spectrophotometry.

2.2.6.5. Synthesis of methoxypolyethyleneglycol SPIONs-gold core-shell carbonated meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate powder (PC 6)

The procedure in section 2.2.6.4 was repeated and conjugates obtained at room temperature and 60 °C were dissolved separately with 1.88% sodium bicarbonate (NaHCO₃) and the resulting solution evaporated at room temperature and 60 °C respectively to obtain two white crystalline materials. The latter were turned into
powder form after grinding in a mortal with pestle. Subsequently, the two powders were evaluated using ultraviolet-visible spectrophotometry and Fourier Transform infrared spectroscopy.

2.2.6.6. Synthesis of methylene blue meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate (PC 7).

20 mL of ethanolic methylene blue solution (0.0082 g/250 mL), 2 mL aqueous solution of methoxy-polyethyleneglycol (0.0237 g) and 20 mL of methanolic meso-tetrakis(4-hydroxyphenyl)porphyrin solution (0.0239 g/100 mL) were mixed and the resulting solution evaporated under ambient condition to obtain a greyish green fibrous material.

2.2.6.7. Synthesis of Iron(III) meso-tetrakis(4-hydroxyphenyl)porphyrin-methylene blue conjugate (PC 8)

Solutions of iron (III) chloride (0.0202 g/5 mL), porphyrin (5 mL, 0.0237 g/100 mL) and methylene blue (5 mL, 0.0082 g/250 mL ethanol) were added together in a 250 mL Pyrex beaker to obtain a green solution. Afterward, the latter was evaporated under ambient condition to obtain a water-soluble green powder dye.

2.2.6.8. Preparation of water-soluble carbonated porphyrin powder (PC 9)

Sodium bicarbonate solution (2 mL, 7.5 %) was added to 1 mL of the meso-tetrakis(4-hydroxyphenyl)porphyrin (Section 2.2.4.1, 0.0000736669 mol/L) in MeOH to obtain a spontaneous green-yellow precipitate. This was followed by filtration and the residue was dried under ambient condition. The dried porphyrin evolved in two forms, viz: a white powder and green powder of different solubilities. These porphyrin
powders were then characterized using FT-IR spectroscopy and stored under ambient condition.

2.2.6.9. Synthesis of water-soluble PEGylated thiol porphyrin conjugate (PC 10)

Methoxypolyethylene glycol-thiol (mPEGSH) (0.0587 g) was dissolved in 4 mL water and the resulting solution added to a 8 mL of POP 1 solution (prepared from 0.0011 g in 10 mL ethanol). The mixture was shaken at 135 rpm (Merck Millipore) for 8 min. This solution was then evaporated in a fume hood for 3 days to obtain a greenish brown solid (0.0499 g).

2.2.6.10. Synthesis of water-soluble methoxypolyethylene glycol-thiol SPIONs-porphyrin conjugate (PC 11)

Porphyrin (0.0004 g), mPEGSH (0.0227 g) and SPIONs 4 (0.0026 g) were added to a mixture of ethanol and water (10 mL: 5 mL) and the resulting solution shaken at 142 rpm for 10 min. This was followed by sonication of the resulting mixture for 10 min. This mixture was then dried in a fume hood for 2 days to obtain a magnetic greenish-yellow solid.

2.2.6.11. Synthesis of water-soluble methoxypolyethylene glycol-thiol SPIONs-gold porphyrin conjugate (PC 12)

Gold-coated SPIONs (SPIONs 10) (0.0084 g/30 ml) was sonicated for 5 min. Afterward, a given amount of mPEGSH (0.0081 g) was added to the solution, followed by sonication for another 5 min. At this time, a solution of porphyrin (0.0020 g/60 mL EtOH) was added to the mPEGSH-SPIONs-gold solution and the resulting
solution was shaken at 135 rpm for 12 min. This was followed by evaporation of the resulting solution to obtain a magnetic brown solid.

2.2.7. Estimation of % wt of porphyrin in porphyrin conjugate PC 5

Methanol-water mixture solution (1:1 v/v) of the PC 5 (oven-dried at 60°C) was subjected to ultraviolet-visible spectrophotometric analysis under the same conditions reported for the estimation of molar extinction coefficient of pure porphyrin (POP 1, section 2.2.4.1 and 2.2.4.3). The amount of porphyrin in this conjugate solution was then estimated from the calibration graph by converting the concentration in mol/L to mass in grams and subsequently divide the obtained mass by the initial mass of the conjugate multiplied by 100. In other words,

\[
\text{Mass of porphyrin (g)} = \frac{(\text{concentration of porphyrin (mol/L)} \times \text{volume (mL)} \times \text{MW})}{1000}
\]

\[
\% \text{ wt. of porphyrin} = \frac{\text{mass of porphyrin in the conjugate/Initial mass of conjugate}}{100}
\]

2.2.8. Applications of SPIONs and porphyrin conjugates in exogenous aqueous solutions

2.2.8.1. Separation of neutral red from aqueous saline/salt solution using ultrasmall-SPIONs (USPIONCs-Goethem)(SPIONs 4)

Sodium chloride (0.1051 g) was added to a 5 mL solution of neutral red hydrochloride (7.6 mg/L, pH 6.80) to form a neutral red saline solution (NRS) (pH 6.39). NRS was sonicated with SPIONs 4 (USPIONCs-Goethem) (1.3 mg) (section 2.2.1.2) for 15 min and the resulting mixture was allowed to settle under gravity (solution pH = 3.80). This solution was then made alkaline by adding some few drops of sodium hydroxide solution (0.06M). This changed the colour of the solution from
pink (pH 3.80) to yellow with a final pH of 12.27. The mixture was further subjected to sonication for another 15, 30, 45, and 60 min and the resulting solution subjected to separation via external magnetic field. A yellow-brown sun-flowery-like magnetic neutral red - iron oxide nanoparticle residue (NR-USPIONCs-Goethem) assembled at the magnet location after magnetization. The supernatant was decanted into a small vial while the residue washed with some deionized water to remove some adsorbed chloride ions on the surface of the magnetic residue. The new supernatant, which tested positive to the presence of chloride ion using silver nitrate solution (0.1 M) was removed and the neutral red sun-flowery-like magnetic nanostructure was again washed with water, reassembled using external magnetic field, dried under ambient condition after discarding the supernatant and finally stored under ambient condition for further analysis.

2.2.8.2. SPIONs as proton pump and electrostatic contributor for the simultaneous precipitation of protonated neutral red, \( \text{Ag}^+ \) and chloride ion from aqueous solution

\( \text{AgNO}_3 \) solution (1 mL, 0.1M) was added to a solution of neutral red hydrochloride (5 mL, 7.6 mg/L; pH 6.8) to obtain a yellow-orange complex solution (pH 5.44). This was followed by the addition of SPIONs (Section 2.2.1.2, SPIONs 4) which brought the solution pH to 3.78. The resulting mixture was sonicated for 15 min and subsequently left overnight. At this time, a greyish black-brown solid separated from a clear colourless supernatant. The supernatant was analyzed using ultraviolet-visible spectrophotometry to check for any possible change in neutral red peak. In addition, excess silver nitrate and sodium chloride crystals were added to two aliquots of the supernatant separately in order to test for the presence of Cl\(^-\) and Ag\(^+\) ions respectively.
2.2.8.3. Advanced colorimetric stripping technique for the detection and quantification of $H_2O_2$ in aqueous and pharmaceutical fluids using iron (III) meso-tetrakis (4-hydroxyphenyl) porphyrin-methylene blue (PC 13) strips

Solutions of iron (III) chloride (0.0202 g/5 mL water), porphyrin (POP 1: 5 mL of 0.0237 g/100 mL, section 2.2.4.3) and methylene blue (5 mL of 0.0082 g/250 mL ethanol) were added together in a 250 mL Pyrex beaker to obtain a green dye hybrid solution. This was followed by immersion of a Whatman No. 1 filter paper (90 mm) for 1h 30 min. Afterwards, the filter paper was removed and dried under ambient condition. Finally, the dried paper was cut into rectangular strips. To make calibration graph, each test strip was dipped into a 5 mL test solution containing a given amount of reference $H_2O_2$ concentration (between 0-30 %) and allowed to react for 10 min, followed by drying under ambient condition (room temperature 19.5 °C) for 15 min and capturing using a camera. Furthermore, a test strip was dipped into a 5 mL pharmaceutical 30%-labelled $H_2O_2$ disinfectant solution and allowed to react under the same conditions above. The colour of each paper strip was evaluated using the histogram of the RGB mode of image editor GIMP 2.8.20 for better colour discrimination.

2.2.9. Solubility and aqueous stability

A given amount of PC 4 (1.2 mg) was dissolved in 100 mL sterilized phosphate buffered saline solution (0.01M) and kept in the dark at 4 °C. Stability of the conjugate was monitored using ultraviolet-visible spectrophotometry.
2.2.10. Evaluation of photodynamic therapy potentials of the conjugates using singlet oxygen generation/quantum yield

2.2.10.1. Singlet oxygen generation of PC 11 (Absorption method)

A certain amount of 1,3-diphenylisobenzofuran (DPBF) solution (1 mL, 0.0180 g/100 mL DMSO) was added to a solution of PC 11 (section 2.2.6.10) (2 mL, 0.0001 g /4 mL DMSO) and the resulting mixture irradiated at 650 nm using the Xenon light (RF-6000 Shimadzu) at a scan rate of 6000 nm /min for 36 min. The decrease in absorbance of the DPBF was monitored at 417.64 nm on the Perkin Elmer spectrophotometry under the scan rate of 200 nm / min. Methylene blue (2 mL, 0.0001 g /4 mL DMSO) as a reference was made to undergo the same afore-mentioned conditions while the singlet oxygen generated was estimated using a modified relation reported by Adarsh and coworkers (Adarsh et al., 2010), viz:

$$[1^O_2]^x = \left(\frac{s^x}{s^y}\right) \times [1^O_2]^y \times \frac{A^y}{A^x}$$  \hspace{1cm} (1)

where $[1^O_2]^x$ = singlet oxygen yield of the conjugate; $[1^O_2]^y$ = singlet oxygen yield of the reference; $s^x$ = slope of the conjugate; $s^y$ = slope of the reference; $A^x$ = absorbance of the conjugate at irradiation wavelength and $A^y$ = absorbance of the reference at irradiation wavelength.

A similar approach was used for PC 12 (section 2.2.6.11).

2.2.10.2. Singlet oxygen generation of water-soluble PEGylated SPIONs-gold core-shell meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate (PC 4) (Fluorescence method)

A given amount of 1,3-diphenylisobenzofuran (DPBF) solution (0.1 mL of 0.2 mg dissolved in 10 mL DMSO) was diluted to 5.1 mL using DMSO (working solution). A
small volume (2 mL) of this solution was then added to 2 mL of conjugate solution prepared by dissolving 12 mg of conjugate in 3 mL of DMSO. The resulting solution was irradiated at 680 nm using a Xenon light (Shimadzu, RF-6000) for 200 s. The decrease in the fluorescence intensity of DPBF was monitored at 461 nm. Similarly, 0.1 mL of methylene blue solution (0.2 mg dissolved in 10 mL DMSO) was diluted to 10 mL using DMSO. A given volume of this solution (2 mL) was then added to the 2 mL of working solution of DPBF, followed by irradiation under the same light conditions described above.

The singlet oxygen quantum yield was estimated using the relation adapted from Adarsh and coworkers (Adarsh et al., 2010) as described in section 2.2.10.1.

2.2.10.3. Singlet oxygen generation of hay yellow conjugate (PC 1):

\[ \text{SPIONs-meso-tetrakis(4-hydroxyphenyl)porphyrin tetraoctylammonium bromide (Flourescence method)} \]

The method in section 2.2.10.2 was adapted except that the conjugate was changed to SPIONs-meso-tetrakis(4-hydroxyphenyl)porphyrin tetraoctylammonium bromide (Hay yellow conjugate (PC 1)(Section 2.2.6.1)).

2.2.10.4. Singlet oxygen generation of red violet PEGylated citrate-reduced gelatin-capped gold porphyrin conjugate (Red violet conjugate) (PC 2)

The method in section 2.2.10.2 was adapted except that the conjugate was changed to red violet conjugate (PC 2) (Section 2.2.6.1).
2.2.10.5. **A new approach for the estimation of singlet oxygen quantum yield**

A new approach is proposed in this thesis for the estimation of singlet oxygen quantum yield without reference or direct measurement of singlet oxygen phosphorescence at 1270 nm. This proposal was prompted based on the fact that many suitable references are not available and those that are available may not absorb light at desired excitation wavelength. The proposed formula for the estimation of singlet oxygen quantum yield is derived as follows:

The singlet oxygen ($^1\text{O}_2$) quantum yield has been reported to relate to the first order degradation rate constant (Kawasaki et al., 2014) and time for the first order exponential decay (Dai et al., 2014) and thus was estimated using the following proposed relation:

$$\varphi_{\text{yield}} = 1 - e^{k_{\text{phox}} \times t}$$ .................................................................(1)

Where $\varphi_{\text{yield}} = \text{singlet oxygen quantum yield}$, $k_{\text{phox}} =$First order reaction rate constant (slope) and $t = \text{First order reaction time}$, assuming constant volume, negligible or no $^1\text{O}_2\text{physical quenching}$ (Young et al., 1973).

The first order degradation rate constant $K_{\text{phox}}$ was estimated from the plot of $\ln \left[ \frac{I_t}{I_0} \right]$ vs. time following the relation:

$$\ln \left[ \frac{I_t}{I_0} \right] = - K_{\text{phox}} \times t$$ .................................................................(2)

Where $I_0 = \text{emission intensity of DPBF at time } t = 0$, $I_t = \text{emission intensity of DPBF at time } t$, $k_{\text{phox}} =$kinetic rate constant (slope) and $t = \text{First order reaction time}$.

The derivatisation of Eq. (1) was as follows:
The singlet oxygen quantum yield equals the sum of mole fraction of singlet oxygen reacted (e.g. with sensor and other chemical species, which represents true characteristics of therapeutic singlet oxygen) and mole fraction of singlet oxygen which undergoes physical quenching to triplet oxygen state (Young et al., 1973), i.e.

\[ \phi_{\text{yield}} = R + Q \] .................................................(3)

Where \( \phi_{\text{yield}} \) = singlet oxygen quantum yield, \( R \) = sum of the mole fraction of singlet oxygen reacted; \( Q \) = mole fraction of singlet oxygen physically quenched.

Assuming no physical quenching, then Eq.(3) becomes

\[ \phi_{\text{yield}} = R = f_{\text{dpbf}} + f_c \] .................................................. …………(4)

Where \( f_{\text{dpbf}} \) = mole fraction of singlet oxygen reacted with DPBF and \( f_c \) = mole fraction of singlet oxygen reacted with other chemical species.

Assuming no other reactions other than reaction with DPBF, then Eq (4) becomes

\[ \phi_{\text{yield}} = R = f_{\text{dpbf}} \] ..................................................... …………(5)

However, \( f_{\text{dpbf}} \) = mole fraction of DPBF degraded which is related to the first order reaction rate constant (Kawasaki et al., 2014) and time of first order reaction decay (Dai et al., 2014). That is,

\[ f_{\text{dpbf}} = 1 - e^{-k_{\text{phox}} \cdot t} \] .................................................(6)

Thus combining Eq. (5-6) gives

\[ \phi_{\text{yield}} = 1 - e^{-k_{\text{phox}} \cdot t} \] .................................................(7)

Where \( k_{\text{phox}} \) = first order kinetic rate constant and \( t \) = maximum time before DPBF’s intensity \( I_t \) deviates from first order exponential decay, assuming constant volume (V)
and negligible or no $^{1}O_2$ physical quenching or other side reactions (Young et al., 1973). The right side of Eq. (7) was derived from the following relationships:

Mole fraction of DPBF degraded =

$$V(C_0 - C_t) / VC_0 = (C_0 - C_t) / C_0 = 1 - (C_t / C_0)$$  \(\text{(8)}\)

Where \(V\) = volume of the solution and \(C\) = concentration in mol/ L.

However,

$$I = 2.303 \phi_F I_{ex} \epsilon b$$  \(\text{(9)}\)

Where \(I\) = Intensity of fluorescence emission, \(\phi_F\) = fluorescence quantum yield, \(I_{ex}\) = intensity of incident or excitation wavelength, \(\epsilon\) = molar extinction coefficient, \(C\) = concentration in mol /L and \(b\) = pathlength of the sample cell(Skoog et al. 1996, Fifield & Kealey, 1995). Since \(\phi_F, I_{ex}, \epsilon\), and \(b\) are all constants, then Eq. (9) becomes

$$I = K'C$$  \(\text{(10)}\)

where \(K' = 2.303 \phi_F I_{ex} \epsilon b = \) slope of the straight line calibration graph of the plots of intensity vs concentration in mol/L of DPBF.

Thus, rewriting Eq. (8) in terms of fluorescence intensity \(I\) gives

$$1 - (C_t / C_0) = 1 - [ (I_t / I_0) / (I_0 / K') ] = 1 - [I_t / I_0]$$  \(\text{(11)}\)

However, from first order relation,

$$\ln [I_t / I_0] = -k_{phox} t$$

which leads to

$$[I_t / I_0] = \exp^{-k_{phox} t}$$  \(\text{(12)}\)

Combining Eq.(11) and Eq.(12) gives
2.2.10.6. Singlet oxygen generation of methylene blue-porphyrin conjugate (PC 7) using the proposed method

A given amount of 1,3-diphenylisobenzofuran (DPBF) solution (2 mL, \(1.9729 \times 10^{-6}\) M in DMSO) and conjugate solution (2 mL, 0.0004 g/4 mL in DMSO) were pre-mixed and subsequently irradiated with light at 808 nm for 460 s. The decrease in emission intensity of DPBF (Kawasaki et al., 2014) was monitored at 459 nm with time. The first order degradation rate constant \(k_{phox}\) was estimated from the plot of \(\ln \left[ \frac{I_t}{I_0} \right] \) vs. time following the relation:

\[
\ln \left[ \frac{I_t}{I_0} \right] = -k_{phox} \times t \tag{2}
\]

Where \(I_0\) and \(I_t\) are emission intensities of DPBF at time \(t = 0\) and time \(t\) respectively, \(k_{phox}\) = first order rate constant and \(t\) = optimum time of irradiation for first order exponential decay.

The singlet oxygen quantum yield was reported to relate to the first order degradation rate constant (Kawasaki et al., 2014) and maximum time for first order exponential decay (Dai et al., 2014). Thus, the singlet oxygen quantum yield was estimated using the proposed relation:

\[
\phi_{yield} = 1 - e^{-k_{phox} \times t} \tag{1}
\]

Where \(\phi_{yield}\) = singlet oxygen quantum yield, \(k_{phox}\) = first order reaction rate constant (slope) and \(t\) = First order reaction time, assuming constant volume, negligible or no \(^1\text{O}_2\) physical quenching (Young et al., 1973).
2.2.11. BIOLOGICAL EVALUATION

2.2.11.1. Cell culture

Epithelial cells of breast cancer cells (MCF-7 cell line, ATCC HTB-22) derived from the tissue of a metastatic site of the mammary gland were employed and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1% (v/v) amphotericin-B solution and 0.1% (v/v) penicillin-streptomycin in a 5% CO₂, 85% humidified atmosphere at 37 °C. When the cells reached about 70-80% confluence, they were washed twice with Hank’s Balanced Salt Solution (HBSS) and detached using 1 mL / 25 cm² of Tryple™Select solution. This was followed by seeding the cells at a final concentration of 4 x 10⁵ cells/mL in a 35 mm diameter cell culture dishes (Corning-ware CR/430165) and subsequent incubation for 4 h for cellular attachment.

2.2.11.2. Dark cytotoxicity

The magnetic porphyrin conjugate (PC 4) (Section 2.2.6.3) was added at different concentrations of 0.5, 1.0 and 2 μg/Ml to the breast cancer cells (MCF-7; 4x 10⁵/ml) previously incubated in the dark in a 5% CO₂ incubator (85% humidity) at 37 °C for 4 h. This was followed by further incubation for 20 h and subsequent morphological imaging using inverted microscopy and biological assay’s evaluations, viz: Trypan blue, lactate dehydrogenase (LDH) and adenosine triphosphate (ATP) assays.

2.2.11.3. Uptake studies

Two separate groups of MCF-7 cells were grown on glass coverslips in 35 mm culture dishes together with the magnetic porphyrin conjugate (PC 4, Section 2.2.6.3) (1 μg/ml) in the absence and in the presence of an external magnetic field.
respectively. An hour before observation, cells in culture dishes were washed thrice with HBSS, fixed with 200 µl of 3.5% (v/v) Paraformaldehyde in DMEM and permeabilized with 200 µl of 0.5% (v/v) TritonX-100 in distilled water. This was followed by washing of the cells thrice again with HBSS, after which the coverslips were inverted onto glass microscope slides onto which a 30 µl of 20% Fluoromount™ Aqueous Mounting Medium in distilled water had been previously added. The coverslip borders were sealed and the slides were subsequently examined using the Carl Zeiss Axio Z1 Observer at 40x magnification. The 358 nm excitation/461 nm emission filter was used to detect fluorescent signal produced from the conjugate within the cell environment. Furthermore, these images were overlaid into simultaneously captured differential interference contrast (DIC) images in order to enhance the contrast of the cellular morphological features to ensure clearer visualization of the subcellular localization.

2.2.11.4. Photodynamic efficacy against MCF-7 breast cancer cells

The cell media containing the conjugate (PC 4, Section 2.2.6.3) were replaced with phosphate buffered saline (PBS) after 24 h dark incubation. This was followed by exposure to light dose of 10 J/ cm² at 673 nm wavelength for 14 min, 51 s. Afterward, the PBS was replaced with the cell media and the cells subsequently incubated for another 24 h before they were subjected to evaluation by biological assays, viz: Trypan blue, lactate dehydrogenase (LDH) and adenosine triphosphate (ATP).

2.2.11.5. Magnetic targeting effect on PDT’s efficacy

Cells incubated with the conjugate (PC 4, Section 2.2.6.3) were subdivided into two groups, namely, cells containing conjugate that were subjected to external magnetic
field exposure (CMF) and cells containing conjugate that were not exposed to external magnetic field (CNMF). The CMF group was exposed to a low magnetic field (68 G) for 1 h within the 20 h of dark incubation before light irradiation. The photodynamic therapeutic efficacies of these two cell groups were then evaluated using biological assays, viz: Trypan blue, lactate dehydrogenase (LDH) and adenosine triphosphate (ATP).

2.2.11.6. Changes in cell morphology

An inverted light microscope (Wirsam, Olympus CKX41) was used to probe morphological changes that occurred in different groups (experimental and control) of the metastatic breast cancer cells 24 h after addition of the conjugate and laser irradiation. Morphological images were taken using the SC30 Olympus camera.

2.2.11.7. Trypan blue assay

Trypan blue staining reagent (0.4 %, ThermoFisher Sci: T10282) was mixed with the cell suspensions in a 1:1 ratio (v/v) and the resulting solution loaded onto a cell counting chamber slide (Invitrogen, C10283) designed for use with Countess™ Automated Cell Counter (Invitrogen, C10227) which displayed the % live and % dead cells values.

2.2.11.8. Lactate dehydrogenase (LDH) cytotoxicity assay

The Cyto-Tox96® assay (Anatech, Promega AnaTech: PRG1780) was used to measure the released “Lactate Dehydrogenase” (LDH) within various cell groups. The LDH assay is typically used to evaluate the membrane integrity of cells after interaction with therapeutic agents which if cytotoxic will cause a release of the cytosolic enzyme lactate dehydrogenase (LDH) via damaging the cell membrane.
Briefly, 50 µl of reconstituted LDH assay reagent was added to an equal volume of cell culture medium, followed by incubation of the resulting solution in the dark at room temperature for 30 min. At this time, the resulting solution was subjected to spectrophotometric analysis at 490 nm using Perkin–Elmer, VICTOR3™.

2.2.11.9. Adenosine triphosphate (ATP) assay

The adenosine triphosphate (ATP) assay was used to evaluate mitochondrial activity which indirectly expresses the energy state of the cells. The lower the ATP value of a cell, the lower the cell viability and thus the higher the probability of cell death. For this purpose, the Cell Titer-Glo® luminescent proliferation assay (Anatech, Promega PRG7571) was employed. Briefly, equal volumes of cell suspensions of all groups (experimental and control) and reconstituted reagent were mixed to promote cell lysis. The resulting mixture was allowed to stabilize at room temperature for 10 min prior to ATP quantification using Perkin–Elmer, VICTOR3™ under luminescence mode.

2.2.11.10. Statistics

Dark cytotoxicity or PDT experiment was repeated four times (n = 4). Each assay was done in duplicate while all results were reported as average values. In all assays, untreated cells (control) were included throughout the course of the experiment and the results of all treated cells were compared to those of untreated cells (control) by means of one-way ANOVA analysis of variance to evaluate the statistical difference. Statistical analysis was conducted using Sigma plot 11.0 and the mean, standard deviation and standard error were obtained. Significant differences were considered at the 95th percentile.
2.2.12. Characterizations

2.2.12.1. Ultraviolet-visible (UV-Vis) spectrophotometry

The UV-Vis spectrophotometry was used to evaluate the ultraviolet and visible light absorptions characteristics of the as-synthesized nanomaterials and conjugate. The principle of UV-Vis spectrophotometry is based on the measurement of the electronic transitions of atoms or molecules from the ground state to higher energy excited state via absorption of ultraviolet or visible light from an electromagnetic radiation source, in a polar, non-polar or solid sample medium. For quantification purpose, the principle of Beer-Lambert law which relates the absorbance of light to the concentration of a given chemical species (analyte) is usually employed. In this study, the UV-Vis spectrophotometry analyses were conducted using Perkin Elmer UV-Vis Lambda 25 spectrophotometer (UK). The dilute aqueous solutions of SPIONs, SPIONs-gold and SPIONs-gold porphyrin conjugate and methanolic solution of pristine porphyrin were respectively put in a 4 mL pyrex glass cuvette and run with a scan range between 200-900 nm at a scan speed of 480 nm/min and resolution of 1 nm slit width. Deionized water was used as blank for aqueous solutions while methanol was used as blank for pristine porphyrin solution for baseline correction.

2.2.12.2. Fourier transform infrared spectroscopy (FT-IR)

The surface functional groups of the as-synthesized SPIONs, porphyrin and SPIONs-gold porphyrin conjugate were assessed using the Fourier transform infrared spectroscopy (FT-IR). FTIR measures the vibrational stretching and bending characteristics of bonds between two different atoms in an organic or inorganic molecule as a result of the absorption (or transmittance) of mid-infrared light from an
electromagnetic light source. Typically, the spectrum relates absorbance or % transmittance to the wavelength or wavenumber of the absorbed light for a given dried solid, liquid or gaseous sample. A Perkin Elmer Spectrum two UATR spectrometer (UK) was used for the FTIR analysis. The instrument was equipped with a LiTa03 detector and analysis was run on transmittance mode. The diamond sample holder was precleaned with 50 % ethanol and dried before sample analysis. The samples were analyzed in the dried form to avoid water molecule interference. The samples were placed on the diamond sample holder and pressed with stainless steel gauge cylindrical tool supplied with the instrument before analysis at a scan speed of 0.2 cm/s and a resolution of 4 cm\(^{-1}\). A scan wavenumber range between 400-4000 cm\(^{-1}\) was employed for the analysis.

### 2.2.12.3. Spectrofluorophotometry

The photoluminescence properties of the pristine porphyrin, SPIONs@Au, SPIONs@Au porphyrin conjugate and singlet oxygen generation potential of the conjugate were evaluated using the Shimadzu RF-6000 spectrofluorophotometer. The spectrofluorophotometry is an analytical technique based on the measurement of the fluorescence light emitted by organic and inorganic fluorophores via excitation by an ultraviolet, visible or near infrared light from an electromagnetic radiation source. The spectrofluorophotometry analysis was performed by running aqueous solutions of the respective materials in a 4 mL all-round transparent Pyrex glass cuvette on the above instrument with a scan range between 300-800 nm and a scan rate of 6000 nm/min.

### 2.2.12.4. Proton nuclear magnetic resonance (\(^1\)H NMR)

The chemical structure of the pure porphyrin was confirmed by probing the different hydrogen environments present in the structure of the molecule using proton nuclear
magnetic resonance spectroscopy (\(^1\)H NMR). \(^1\)H NMR measures the response of magnetic spinnable charged hydrogen nuclei in different chemical environments within a molecular structure to an applied magnetic field. The shielding or deshielding effects (measured by chemical shifts) of electrons surrounding the different hydrogen nuclei within a molecular structure indicates the different chemical environments of the hydrogen atom and thus are useful for structural elucidation and quantification of a given organic molecule.

For \(^1\)H NMR analysis, the porphyrin sample was dissolved in deuterated dimethylsulfoxide (DMSO-d6) and run on a proton NMR instrument, Bruker Advance III HD, (Germany) at a frequency of 500 MHz and temperature of 302 K. Tetramethylsilane was used as internal standard.

2.2.12.5. Transmission electron microscopy (TEM)

The morphological characteristics of the SPIONs, SPIONs-gold and SPIONs-gold-porphyrin conjugate materials were evaluated using transmission electron microscopy technique. TEM captures images of nanoparticles by allowing high energy accelerating electrons to pass through a very thin layer of dried sample usually placed on a carbon-coated copper grid. The principle employs the wave property of electron rather than its particle characteristics. TEM analyses were performed on TEM instrument, JEOL 2100 (Japan), operated at 200 KeV. Each sample was dissolved in water and dropped on a carbon-coated copper-grid, followed by drying in the air at room temperature prior to analysis. The crystallinity properties of SPIONs and SPIONs-gold nanostructures were probed using the high resolution mode of the JEOL 2100 (HRTEM). The HRTEM images were captured by increasing the resolution of the normal TEM images so that a single nanoparticle can be enlarge and visualized clearly. The samples analyzed using lower TEM resolution
were subsequently used for this purpose. The samples were analyzed by increasing the resolution of TEM images up to 2 nm.

2.2.12.6. Selected area electron diffractiometry (SAED)

The nanocrystallinity of the SPIONs was further probed using the selected area electron diffractiometry (SAED) mode of the JEOL 2100 TEM microscope. SAED image was captured based on the diffraction of the high energy accelerating electron beam incident on a selected part of a dried solid sample. The sample analyzed using TEM technique was also used for this purpose.

2.2.12.7. X-ray Diffractiometry (XRD)

The iron oxide phase and nanocrystallinity property of the as-synthesized SPIONs were further evaluated using the powder X-ray diffractiometry (XRD) technique. XRD measures the diffraction of X-ray electromagnetic radiation by electrons present in a regularly arranged and spaced atoms within a crystalline structure of a given powder sample. As a result, the extent of crystallinity and atomic or molecular structure of a given material can be elucidated. X-ray analysis was performed using the SmartLab X-ray diffractometer, Rigaku (Japan), equipped with 1.54056Å Cukα radiation at 2θ scan range between 10 and 80 degrees at 9 KW, 45 KV and 200 mA. The sample was pre-ground in an ethanolic precleaned and dried ceramic mortal with pestle to obtain a fine powder and subsequently placed on the sample holder prior to analysis.

2.2.12.8. Vibrating sample magnetometry (VSM)

The superparamagnetic properties of SPIONs were evaluated using the vibrating sample magnetometry (VSM). VSM employs the principle of Faraday's law of induction which relates electric field generation with a changing magnetic field. In
VSM study, a magnetic sample placed in a constant magnetic field and subjected to an alternating magnetic field motion will produced an electric current which is proportional to the magnetization of the sample. In other words, the higher the electric current produced, the higher the magnetization characteristic of the magnetic sample. VSM analysis was performed using a vibrating sample magnetometer, cryogenic device (UK), at a temperature range between 1.8 – 320 K and magnetic field range of -14 to +14 Tesla. The sample was pre-ground in an ethanolic precleaned and dried ceramic mortal with pestle to obtain a fine powder and subsequently loaded on the sample holder prior to analysis.

2.2.12.9. Dynamic-light scattering (DLS)

The hydrodynamic sizes of SPIONs, SPIONs-gold and SPIONs-gold porphyrin conjugate were evaluated using the dynamic light scattering technique (DLS). DLS measures the differential intensity of a scattered light (Rayleigh scattering) of a particular wavelength, incident at a particular angle, by the different sizes of nanoparticles, caused by their different Brownian motions in aqueous solution. The fluctuation of the intensity could be used to determine the extent of Brownian velocity and thus the particle size estimation using the Stokes-Einstein relationship. The output information is typically displayed as size by intensity but can also be obtained as size by volume or by number by converting the intensity data to volume or number data using mathematical relationships. For DLS study, the aqueous solution of each sample was run using the DLS operational mode of the Anton Paar Litesizer 500 (Austria) and in some cases by the Marven Zetatizer (UK) in a 4 mL DLS disposable plastic cuvette at room temperature.
2.2.12.10. Zeta Potential

The overall surface charges of materials were evaluated using the zeta potential operational mode of the Anton Paar Litesizer 500 (Austria). Zeta potential employs the electrophoretic light scattering principle which involves measurement of the electrophoretic mobility of particles or molecules with net charge, migrating towards a particular stationary charged electrode placed within a solution. The output of the process is then converted to zeta potential value using mathematical relationships. To perform zeta potential analysis, aqueous solution of the sample was injected into a standard zeta potential electrode sample holder and run on the above instrument at room temperature.

2.2.12.11. Energy dispersive X-ray spectroscopy (EDS)

The elemental compositions of the SPIONs were evaluated using the Oxford energy dispersive X-ray spectroscopy device associated with the transmission electron microscope, JEOL 2100 (Japan). The EDS measures the emission of different X-ray lights, characteristic of different elements present in a sample excited by a suitable light source. For EDS analysis, the sample analyzed using TEM technique was used due to the interconnectivity of the two instrumentations.
CHAPTER 3

RESULTS AND DISCUSSION

3.1. SYNTHESSES, CHARACTERIZATIONS AND APPLICATIONS OF SPIONs, GOLD, SPIONS-GOLD, PORPHYRIN AND PORPHYRIN CONJUGATES)

3.1.1. General synthesis scheme – Gluconic acid capped SPIONs

Different forms of water soluble gluconic acid capped superparamagnetic iron oxide nanoparticles (SPIONs) were synthesized using new green approaches. The general procedure employed for the synthesis of these nanomagnetic materials is shown in Scheme 3.1. According to scheme 3.1, brown solution of glucose and ferric chloride mixture transformed to a yellow-orange intermediate solution which served as a precursor for the generation of the gluconic acid capped superparamagnetic iron oxide nanoparticles (Fe$_3$O$_4$@gluconic acid (magnetite) or Fe$_2$O$_3$@gluconic acid (maghemite)). Mechanistically, a redox reaction induced the conversion of glucose (reducing agent) and ferric chloride (oxidizing agent) to gluconic acid and ferrous ion respectively, the mixture of which appeared as a yellow-orange complex in the presence of excess ferric ion. This mixture spontaneously reacted with aqueous ammonia solution to form the desired gluconic acid capped SPIONs. Technically, co-precipitation and neutralization reactions concurrently occurred leading to the formation of the magnetic nanomaterials with associative reduction in the pH of the medium. Interestingly, two different behaviours were observed.
Another advantage of the new approach over the Lu et al. approach was that there was no need for precipitation with toxic organic solvents such as ethanol or centrifugation as employed by Lu and co-workers to separate the SPIONs from the mother liquor, thus making the new approach relatively safer and greener in nature (Chart 3.3). Table 3.1 summarizes some major advantages of using the new approach and modifying Lu et al approach for better cost, safety and energy efficiency.

**Table 3.1** Comparison between the present synthesis approach and Lu et al. approach

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lu et al. Approach</th>
<th>Present Approach</th>
<th>Advantage/Contribution to knowledge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor synthesis’ temperature</td>
<td>80 °C</td>
<td>Different temperatures between 60-80 °C</td>
<td>Energy efficient, synthesis at lower temperature</td>
</tr>
<tr>
<td>SPIONs synthesis’ temperature</td>
<td>60 °C</td>
<td>Different temperatures between Room temperature – boiling temperature of water (100 °C)</td>
<td>Energy efficient, synthesis at lower temperature</td>
</tr>
<tr>
<td>Precipitating / harvesting mode</td>
<td>Used ethanol as precipitating agent</td>
<td>No requirement for precipitating solvent; the material settled under gravity; water was used as washing agent</td>
<td>Energy efficient, no use of toxic organic solvent; water is used as washing agent</td>
</tr>
</tbody>
</table>
3.1.2. Synthesis using solid-based precursors

In addition to modifying Lu et al.’s approach, SPIONs were synthesized using solid-based precursors (SBP) made from preheated mixture of glucose and ferric chloride (SBP 1), maltose and ferric chloride (SBP 2), and maltose, ferric chloride and sodium bicarbonate (SBP 3 and SBP 4) (Chapter 2, section 2.2.1.7-8,10-11). SBPs were synthesized as a result of the need to develop solid-based precursors for facile synthesis of magnetic iron oxide nanomaterials in large scale as well as improve commercial qualities and attributes. Also, SBP exhibits good storage properties compared to the liquid-based precursor. Furthermore, some SBPs evolved as magnetic nanomaterials (e.g. SBP 1, SBP 2 and SBP 3) and thus can be used directly as magnetic entities (Chapter 2, section 2.2.1.7-8,10; Fig 3.1a-d). overall, gluconic acid capped maghemite SPIONs (γ-Fe$_2$O$_3$) can easily and directly be synthesized using both the magnetic and non-magnetic SBP powders (Chapter 2, section 2.2.1.9,12; Inset(Fig. 3.1d);Fig. 3.2a-d). In its uniqueness, SBP 4 powder produced maghemite-SPIONs with some attributes resembling the orientation of iron fillings within a magnetic field even without placing the nanomaterials in an external magnetic field (Fig. 3.2c). The versatility of maghemite-SPIONs in various fields of human endeavour such as data/information storage (e.g. applications in bank ATM cards, identity electronic screenings, music/data compact disks, etc) and biomedicals (such as their use as magnetic resonance image contrast agents and drug delivery nanovehicular systems, owing to their biocompatibility and stability), makes the current SBP approach very attractive, industrially. In addition, the current approach shows greener characteristics than the conventional approach since it does not involve the use of toxic oxidizing chemicals such as nitric acid (Wagstaff et al., 2012)
to the mother liquor (Lu et al. approach). This was possible, since large amount of SPIONs separated from the solution under precipitation. On the other hand, the materials that stuck to the mother liquor required several washing cycles during which many of the as-synthesized SPIONs could have been lost. This explained why lower yield were observed for materials that stuck to mother liquor than those that precipitated out of solutions.

**Table 3.2** Effects of synthesis’ conditions on the yields of SPIONs

<table>
<thead>
<tr>
<th>Synthesis conditions</th>
<th>SPIONs 8</th>
<th>Precursor’s temperature</th>
<th>Synthesis’ temperature</th>
<th>Glucose concentration (g/mL)</th>
<th>Ferric chloride concentration (g/mL)</th>
<th>Cooling time before synthesis (min)</th>
<th>State of SPIONs after synthesis</th>
<th>Yield (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stuck to mother liquor</td>
<td>0.0981</td>
</tr>
<tr>
<td>SPIONs 9</td>
<td>72</td>
<td>60</td>
<td></td>
<td>0.01047</td>
<td>0.05635</td>
<td>8</td>
<td>Precipitate out of solution</td>
<td>2.4219</td>
</tr>
<tr>
<td>SPIONs 4</td>
<td>73</td>
<td>60</td>
<td></td>
<td>0.01047</td>
<td>0.05635</td>
<td>31</td>
<td>Stuck to mother liquor</td>
<td>0.1764</td>
</tr>
<tr>
<td>SPIONs 2</td>
<td>66.5</td>
<td>50</td>
<td></td>
<td>0.01047</td>
<td>0.05635</td>
<td>5</td>
<td>Precipitate out of solution</td>
<td>0.1491</td>
</tr>
<tr>
<td>SPIONs 1</td>
<td>66.5</td>
<td>40</td>
<td></td>
<td>0.01047</td>
<td>0.05635</td>
<td>9</td>
<td>Precipitate out of solution</td>
<td>0.2334</td>
</tr>
</tbody>
</table>

### 3.1.4. Characterization of gluconic acid capped SPIONs

#### 3.1.4.1. Ultraviolet-visible spectrophotometry (UV-Vis) and Fourier Transform infrared spectroscopy (FT-IR)

The results of the evaluation of the UV-Vis properties of some of the as-synthesized gluconic acid capped SPIONs are shown in Fig. 3.3. According to Fig. 3.3, all tested SPIONs exhibited absorption band peaks between 300 and 400 nm, typical of iron oxide nanoparticles absorption (Fazio et al., 2016; Ghandoor et al., 2012).
synthesized from solid-based precursor (SBP 2), several absorption band differences were observed relative to the SBP 2 and black SPIONs (Fig. 3.4b-d). The SBP 2 spectrum showed that free or less intermolecular hydrogen bonded O-H\textsubscript{str} groups were present on the surface of the materials. This was evident by the presence of a very narrow band peak at 3399 (3700-3000 cm\textsuperscript{-1}) (Fig.3.4c). The presence of Fe-O\textsubscript{str} band peak at 592 cm\textsuperscript{-1} was also visible. The latter coupled with black colour of the SBP 2 confirmed the presence of magnetite (Fe\textsubscript{3}O\textsubscript{4}) nanomaterials. In contrast, the spectrum of the maghemite obtained by heating the SBP 2 at 60 °C showed the presence of carboxylic acid on the surface of the nanomaterials (Fig. 3.4d). In addition, the presence of small amount of goethite was detected at 715,755 and 821 which was absent in the precursor (Andrade et al., 2009). The narrower magnetite peak at 592 red shifted to a broader 560 cm\textsuperscript{-1} peak while the band peak at 485 cm\textsuperscript{-1} red shifted to 467 cm\textsuperscript{-1} after synthesis. Altogether, all these facts confirmed the formation of gluconic acid capped maghemite nanomaterials. With respect to the black SPIONs, the broader C-O\textsubscript{str} band peak at 1000 cm\textsuperscript{-1} became narrower with sharp pointing end after blue shifting to 1033 cm\textsuperscript{-1}. In addition, the peak at 434 cm\textsuperscript{-1} blue shifted to 467 cm\textsuperscript{-1} on the surface of the maghemite nanomaterials.
3.1.4.1.1. Monitoring the course of synthesis for black SPIONs (SPIONs 6)

A closer look at the absorption bands of the yellow-orange preheated ferric chloride-glucose mixture with respect to Fe$^{3+}$ absorption showed that two bands were present, viz: the ultraviolet band peak at 301 nm and the visible band peak at 491 nm (responsible for the brown colour of the ferric chloride solution)(Fig. 3.3a, Fig. 3.5a). Thus, the disappearance of these two bands coupled with appearance of new bands within the ultraviolet-visible regions can serve as a lead towards elucidating the mechanism of formation of the nanomaterials. To achieve this objective, the
Furthermore, as the reaction proceeded, a decrease in the absorbance to initial position at 1 min was observed at 16 min reaction. Afterwards, the absorbance increased again at 21 min but relatively with lower values compared with the initial absorbance at 11 min and subsequently decreased again with time below the initial starting point of the second cycle (16 min) at 30 min. At 1 min interaction, the rate of breakdown of the bulk species was very rapid in such a way that both narrow peaks at 301 nm and 491 nm disappeared. This means that the formation of nanoparticles was very fast at this time, howbeit with a given wide particle size distribution (flattened line at 1 min) of a particular concentration. However, as the reaction proceeded, Ostwald ripening occurred whereby smaller size nanoparticles dissolved into the solution matrix and diffused to the surface of the larger particles where they were absorbed leading to the formation of a new well-defined size distribution system (peak at 463 nm) (Baldan, 2002; Tadros, 2013; Fairhurst & Lee, 2008) (Fairhurst & Lee, 2008; Tadros, 2013; Baldan, 2002) at 6 min (Fig. 3.5b). This new blue shift of the nanoparticles with respect to the initial bulk peak at 491 nm further confirmed the transformation of the bulk to the nanoparticle size distribution dimension range. According to the Ostwald ripening principle, smaller particles are less energetically or thermodynamically-stable than the larger particles due to the possession of higher energy on their surfaces and thus possess higher concentration of less stable molecules on their surfaces (Fairhurst & Lee, 2008; Tadros, 2013; Baldan, 2002). These unstable molecules are more reactive and thus move into the solution matrix and diffuse to the surface of the larger molecules where they are absorbed. Thus, the sizes of smaller particles decrease while those of the larger particles increase. This explained why a new size distribution curve was obtained at 6 min relative to 1 min (Fig. 3.5b). Moreover, since heating and mechanical stirring
Table 3.3 Various particle size distribution band peak evolved during synthesis

<table>
<thead>
<tr>
<th>Time</th>
<th>Red shift relative to 301 nm</th>
<th>Absorbance</th>
<th>Blue shift relative to 491 nm</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>301</td>
<td>1.220261</td>
<td>491</td>
<td>0.074829</td>
</tr>
<tr>
<td>1</td>
<td>387</td>
<td>0.057109</td>
<td>387</td>
<td>0.057109</td>
</tr>
<tr>
<td>6</td>
<td>460</td>
<td>0.078644</td>
<td>463</td>
<td>0.078644</td>
</tr>
<tr>
<td>11</td>
<td>392</td>
<td>0.13077</td>
<td>392</td>
<td>0.13077</td>
</tr>
<tr>
<td>16</td>
<td>399</td>
<td>0.055932</td>
<td>399</td>
<td>0.055932</td>
</tr>
<tr>
<td>21</td>
<td>444</td>
<td>0.080477</td>
<td>444</td>
<td>0.080477</td>
</tr>
<tr>
<td>26</td>
<td>529</td>
<td>0.066083</td>
<td>529</td>
<td>0.066083</td>
</tr>
<tr>
<td>30</td>
<td>379</td>
<td>0.047263</td>
<td>379</td>
<td>0.047263</td>
</tr>
</tbody>
</table>

Similarly, FT-IR was used to evaluate how functional groups interacted during the course of synthesis. The results of the FT-IR evaluations are given in Fig. 3.6b-d. According to Fig. 3.6 b-c, the intensity of absorption of O-H\textsubscript{str} (3475 cm\(^{-1}\), broad (3700-2500)), OH\textsubscript{bend}(1419 cm\(^{-1}\)), C-H\textsubscript{str} (3000 cm\(^{-1}\)), (C=O\textsubscript{str}(1636 cm\(^{-1}\)), C-O\textsubscript{str} (1078 cm\(^{-1}\)) and Fe-O\textsubscript{str} (669 cm\(^{-1}\)) decreased with time from 0 min (yellow complex precursor) till 6 min and afterwards increased with a burst at 11 min correlating with the results obtained from the UV-Vis analysis (Fig. 3.5a-b,d, 3.6a). Also, at 11 min, the Fe-O\textsubscript{str} band was clearly defined compared to that of the precursor at 0 min (Fig. 3.6b). In addition, all functional groups showed enhanced intensities at 11 min (Fig. 3.6b-c). Moreover, C=O\textsubscript{str} group band split into two and red shifted at 11 min while the O-H\textsubscript{str} band (carboxylic acid) became broader relative to that at 0 min(Fig. 3.6b). This splitting of the C=O\textsubscript{str} and wider bands of the O-H\textsubscript{str} groups showed that the feasible binding of gluconic acid group (-COOH) to the surface of the SPIONs via complexation (Sarkar et al., 2012) occurred at 11 min of reaction. Moreover, O-H\textsubscript{str} group showed a deformed appearance relative to the precursor at 0 min as well as the usual well defined carboxylic acid OH\textsubscript{str} curve (Fig. 3.4b,d). This may be as a result of the interaction of the O-H group of the gluconic acid with the hydroxide ion.
in the alkaline medium. This interaction could be responsible for the reduction in the intensity/concentration of the O-H$_{\text{str}}$ groups in the first 6 min (Fig. 3.6b). For example, neutralization reaction occurred in the base medium leading to the formation of gluconate ion, thus reducing the number of hydroxy (O-H) group within the matrix of the acid. However, since the precursor is very acidic in nature (pH 1.8), a possible neutralization of the base solution occurred spontaneously and the remaining excess hydrogen ion concentration returned the solution to acidic pH. This obviously would increase the number of O-H$_{\text{str}}$ group again within the matrix of the gluconic acid, thus explaining why the intensity of the O-H$_{\text{str}}$ group increased at 11 min relative to 6 min (Fig. 3.6b). The extent of shift in wavenumbers (cm$^{-1}$) with respect to these functional groups was also evaluated. The result is shown in Fig 3.6d. According to Fig. 3.6d, both O-H$_{\text{str}}$ and Fe-O$_{\text{str}}$ group bands red shifted up to 1 min, remained stable till 6 min and then red shifted again till 11 min. C-H$_{\text{str}}$ and O-H$_{\text{bend}}$ bands, first blue shifted till 1 min and then red shifted till 6 min and remained stable till 11 min. The C=O$_{\text{str}}$ and C-O$_{\text{str}}$ showed a mirror image pattern, i.e. taking C=O$_{\text{str}}$ first, the band initially blue shifted up to 6 min and then red shifted till 11 min.

Altogether, combining information from the UV-Vis and FT-IR, 11 min could be regarded as the minimum time required for the SPIONs to be fully formed from 1 mL of the precursor solution. This information is essential for reproducibility and projection for industrial large scale synthesis.

3.1.4.1.2. Effect of aging

The as-synthesized SPIONs (SPIONs 6) were left to age under ambient condition for three days before further drying was employed. The result of the aging effect is shown in Fig 3.7a. According to Fig.3.7a, the nanoparticles increased in population with broader band width associated with a formation of a new larger particle size.
distribution, evident by a bathochromic shift in the band peak from 379 nm (30 min synthesis time, first day) to 402 nm (third day aging time) as their solution aged with time (Inset, Fig. 3.7). The red shift and the formation of a new PSD with wider band showed that Ostwald ripening occurred. Afterward, further drying ensued to obtain the black needle-like magnetic nanomaterials with a final band peak at 386 nm. The FTIR analysis of the dried SPIONs showed characteristic bands between 3600-2500, 2800-3000, 1500-1700, 1400-1600, 1300-1500, 1000-1200 and 500-600 corresponding to O-H

str (carboxylic acid), C-H

str (saturated), C=O

str (carboxylic acid), C-H

bend (saturated), C-O

str (alcohol and carboxylic acid) and Fe-O

str (iron oxide system), typical of magnetic iron oxide magnetite nanoparticles (Lu et al., 2010; Andrade et al., 2009), in addition to a very narrow needle-like monomodal peak between 600 and 700. This unusual peak may be due to the presence of Fe-O

str of akaganeite (β-Fe

III

−O(OH)) generated during the course of synthesis (Parameshwari et al., 2011; Andrade et al., 2009). Moreover, this peak gradually disappeared together with its associated peak between 700-800 (Andrade et al., 2009) as the material was left to dry in the air overnight. The disappearance of this peak may be due to the possible reduction of akaganeite to magnetite (Fig. 3.7a-b) by Fe

II present on the surface of the SPIONs (Blesa et al., 1986).
3.1.6. Synthesis, Characterization and application of USPIONCs-Goethem (SPIONs 4A)

3.1.6.1. Synthesis

The summary of the synthesis procedure for USPIONCs-Goethem is given in scheme 3.3. According to scheme 3.3, the as-synthesized nanomaterial separated from the mother liquor as a black colloid. The systematic heating and cooling effect employed during addition of the preheated precursor mixture to the alkaline solution probably enforced this precipitation. Mechanistically, the preheating treatment of the precursor's mixture in the air ensured feasible redox reaction between the gluconic aldehyde group and ferric ion. This led to the simultaneous generation of ferrous ion and gluconic acid. The feasibility of this reaction was evident by the change in the colour of the solution from brown to yellow-orange. The yellow-orange solution contained the stoichiometric ratio of ferrous and ferric ions (0.5) and gluconic acid and thus served as the transitional material for the evolution of the as-synthesized USPIONCs-Goethem (Eq. 13-17). The ferrous and ferric ions reacted with the hydroxide ion in the presence of oxygen in the air to form iron (II, III) oxide while gluconic acid surrounded the iron oxide nanoparticles, stabilizing and protecting them from air oxidation (Scheme 3.3). Furthermore, since the synthesis took place in the presence of air, the possible conversion of magnetite to maghemite and ferric hydroxide to goethite and hematite(Sutka et al., 2015) might also take place as shown in Eq. 16 - 17.
The clear and uniform lattice fringes in the high resolution transmission electron microscopy (HRTEM) (Fig. 3.12c) and distinct rings in selected area electron diffraction (SAED) (Fig. 3.12d) images confirmed that the as-synthesized USPIONCs-Goethem were highly crystalline. The measured lattice spacing \( d = 0.253 \text{ nm} \) corresponded to the (311) lattice plane of crystalline magnetite \( (\text{Fe}_3\text{O}_4) \).

The typical XRD pattern of the as-synthesized USPIONCs-Goethem (Fig. 3.12e) showed diffraction peaks at \( 2\theta \) corresponding to (220), (311), (400), (422), (511) and (440) crystallographic planes of cubic inverse spinel magnetite (Lu et al., 2010a; Hou et al., 2003b; Ghandoor et al., 2012) together with two other peaks \( (2\theta) \) at 22.97(110) for goethite (Ford et al., 1997) and 32.78 (104) for hematite (Sahoo et al., 2010).

The result from the zeta potential analysis (Fig. 3.13a) showed that the as-synthesized USPIONCs-Goethem possessed a highly positively charged surface \( (+35.15 \pm 0.47 \text{ mV}) \) which probably explained why the nanomaterial was well-dispersed in water (Fig. 3.13b). The magnetic properties of the as-synthesized USPIONCs-Goethem were evaluated using visual observation and vibrating sample magnetometry (VSM). The visual observation revealed that the as-synthesized nanoparticles exhibited strong magnetic attraction towards external magnetic field both in dried solid form and aqueous medium (Fig. 3.13c-e). The VSM analysis shows that the as-synthesized nanomaterials exist in superparamagnetic state between 100 - 305 K (Fig. 3.14a) with blocking temperature \( (T_B) \) of 55 K (Fig. 3.14b).
3.1.6.3. Separation of neutral red from saline solution using USPIONCs-Goethem

Neutral red is a cell viability probe used for sub-cellular drug localization screening. The capability of USPIONCs-Goethem to separate neutral red from saline solution was evaluated in both acidic and alkaline salt media. In acidic medium (pH 3.80), no visible precipitation occurred probably due to the electrostatic repulsion effect between the protonated neutral red and cationic USPIONCs-Goethem (Fig. 3.15a-b, Scheme 3.4a). Similar observation has been reported by some authors although in the absence of salt (Iram et al., 2010). However, in the alkaline medium (pH 12.27), precipitation of neutral red was highly visible after interaction with the USPIONCs-Goethem (Scheme 3.4b). Mechanistically, the protonated neutral red molecules were deprotonated by the hydroxide ions causing feasible complexation interaction between the nitrogen electron lone pairs and the iron (III) ions of the nanoclusters (Scheme 3.4b). A blue shift was observed in the absorption spectra from 560 to 450 nm with characteristic decrease in absorbance as the neutral red and iron oxide nanoclusters interacted gradually and precipitated out of solution with time (Fig. 3.15c-d). The decrease in absorbance was visualized as yellow sun-flowery-like shell surrounding the brown core of the iron oxide nanoclusters after subjecting the mixture to external magnetic field (Scheme 3.4b). The brown core evolved from the oxidative conversion of magnetite to maghemite during the interactive process (Eq. 5). Furthermore, results from the FT-IR analyses of the residues in the acidic and alkaline media after interaction of the dye with the USPIONCs-Goethem revealed no significant change in the FTIR signals of the USPIONCs-Goethem emanated from the acidic medium (Fig. 3.16a-c) whereas the FTIR signals of the USPIONCs-Goethem from the alkaline medium showed some obvious changes (Fakayode et al.)
The results for the % RE at each interaction time in the alkaline medium are given in Table 3.4. According to Table 3.4, about 86.5 % removal efficiency was obtained within the first 15 min while about 91.9 % was achieved in 45 min.

Table 3.4  Removal efficiency (%RE) in alkaline medium using 1.3 mg USPIONCs-Goethem

<table>
<thead>
<tr>
<th>Time</th>
<th>Absorbance</th>
<th>%RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.431</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0.058</td>
<td>86.54</td>
</tr>
<tr>
<td>30</td>
<td>0.066</td>
<td>84.69</td>
</tr>
<tr>
<td>45</td>
<td>0.035</td>
<td>91.88</td>
</tr>
<tr>
<td>60</td>
<td>0.031</td>
<td>92.81</td>
</tr>
</tbody>
</table>

Table 3.5 shows comparison of the result obtained in this work with some results from the literature. As revealed from Table 3.5, the as-synthesized USPIONCs-Goethem showed high capacity for the removal of neutral red despite the fact that it was employed in a salt medium which had been reported to reduce the removal efficiency of neutral red from aqueous solution (Gholivand et al., 2015; Hu et al., 2013).
Table 3.5 Comparison of removal efficiency of neutral red by different agents

<table>
<thead>
<tr>
<th>Removing agent</th>
<th>Saline conditiona</th>
<th>pH</th>
<th>Capping agent</th>
<th>RE (mg/g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetite nanosphere (Hollow)</td>
<td>-</td>
<td>6</td>
<td>Hydroxyl group</td>
<td>105</td>
<td>(Iram et al., 2010)</td>
</tr>
<tr>
<td>SDS-coated Fe$_3$O$_4$</td>
<td>-</td>
<td>5.7</td>
<td>Sodium dodecyl sulphate</td>
<td>416.67</td>
<td>(Gholivand et al., 2015)</td>
</tr>
<tr>
<td>Magnetic-Carbon nanotubes(Multi-walled)</td>
<td>-</td>
<td>6</td>
<td>Carboxylic acid, hydroxyl group</td>
<td>77.5</td>
<td>(Qu et al., 2008)</td>
</tr>
<tr>
<td>Bamboo shoot hulls</td>
<td>-</td>
<td>4.49</td>
<td>Hydroxyl and carbonyl groups</td>
<td>179.32</td>
<td>(Jiang et al., 2013)</td>
</tr>
<tr>
<td>USPIONCs-Goethem</td>
<td>+</td>
<td>12.27</td>
<td>Gluconic acid</td>
<td>329.16</td>
<td>Present work</td>
</tr>
</tbody>
</table>

aNaCl: Presence (+); absence (-).

3.1.7. SPIONs as proton pump and electrostatic contributor for the simultaneous precipitation of protonated neutral red, Ag$^+$ and chloride ion from aqueous solution

3.1.7.1. Synthesis and characterization of SPIONs (SPIONs 4B)

The SPIONs 4B appeared as a black colloid after interaction of its precursors with ammonium hydroxide solution. The result from the TEM analysis of the as-synthesized SPIONs@gluconic acid showed that the nanomaterials existed as agglomerate clusters (Fig. 3.17a), possibly due to their very small sizes (average: $0.12 \pm 0.04$ nm) which made them relatively unstable resulting to cluster formation which reduced their high surface energies. The HRTEM (Inset in Fig. 3.17a) analysis revealed a clear and distinguishable crystalline lattice space distance ($d = 0.2522$) corresponding to the 311 reflective planes of a typical cubic inverse spinel magnetite. The results from the EDS analysis showed that the nanomaterial contained relatively by atomic composition C (52.28%), O (27.75%), Fe (13.87%), and Cl (3.74%). The presence of Cl may be due to the
Overall reaction

\[
\text{Ag}^+ \text{NO}_3^- + \text{H}_2\text{O} + ^*\text{RN}_4\text{H}^+\text{Cl}^- + \text{Fe}_3\text{O}_4\text{-O-RCOOH} \rightarrow \\
\text{NO}_3^-\text{R}\left(\text{Fe}_3\text{O}_4\text{-O-RCOO}\right)\text{N}_4\text{H}_3^{3+} \text{OH}^- (\text{s}) \text{ (Pink)} + \text{Ag}^+\text{Cl}^- (\text{s}) \text{ (White)} \quad (25)
\]

Note: \(^*\text{RN}_4\text{H}^+\text{Cl}^- = \text{Neutral red hydrochloride}\)

\[
\text{Fe}_3\text{O}_4\text{-O-RCOOH} = \text{Fe}_3\text{O}_4 \left(\text{H-O-CH}_2\text{-}(\text{CH-OH})_4\text{-COOH} = \text{Gluconic acid capped SPIONs (SPIOns 4B)}
\]

Basically, the initial addition of AgNO\(_3\) solution to the neutral red hydrochloride solution caused a decrease in pH from 6.8 to 5.44. This shows that AgNO\(_3\) protonates the system (Eq. 20). The hydrogen ion produced via this protonation caused one of the nitrogen lone pair sites on the neutral red molecule to be blocked, adding additional positive charge to the whole neutral red structure (Eq. 21). Protonation of the remaining nitrogen lone pair site was achieved by addition of SPIOns@gluconic acid (Eq. 22-23) which was evident by a change in the solution pH from 5.44 to 3.78. At this time, the total anion charge of NO\(_3^-\), OH\(^-\) and SPIOn-gluconate ion (Fe\(_3\)O\(_4\)-O-CH\(_2\)-(CH-OH)\(_4\)-COO\(^-\) electrostatically attracted to the protonated neutral red surface balanced the total charge on the protonated neutral red (Eq. 24.). This associative behavior of these anions caused Cl\(^-\) to combine with Ag\(^+\) to form AgCl precipitate (Eq. 24). The formation of AgCl disturbed the equilibrium in Eq. 24, favouring forward reaction, and thus causing continuous precipitation of the protonated neutral red system following Le Chatelier’s principle.
To verify our proposed mechanism, excess AgNO$_3$ crystals, SPIONs@gluconic acid, HCl (aq) and FeCl$_3$.6H$_2$O (aq) were added separately to the solution of neutral red hydrochloride to see which of the materials would induce its precipitation (Fig. 3.19). According to Fig. 3.19, only excess AgNO$_3$ crystals (Eq. 26) was able to precipitate neutral red from solution (Fig. 3.19a).

$$2\text{Ag}^+\text{NO}_3^-(\text{excess}) + 2\text{H}_2\text{O} + \text{'RN}_4\text{H}^+\text{Cl}' \rightarrow$$

$$(\text{NO}_3^-)_2\text{'R N}_4\text{H}_3^{3+}\text{OH}'_{(s)} + \text{Ag}^+\text{Cl}'_{(s)} + \text{Ag}^+\text{OH}' \quad (26)$$

This implied that simultaneous precipitation of protonated neutral red and silver chloride was only feasible in the presence of excess Ag$^+$ ions and hydrogen ions produced in this case by the hydration of the Ag$^+$ ions (Eq. 20, 26). In contrast to the earlier study which involved an insufficient amount of Ag$^+$ (concentration $= 0.01961$ M due to dilution factor in 5 mL neutral red solution) to effect precipitation (Fig. 3.18a) and thus required another protonating species, in this case SPIONs@gluconic acid to induce the final precipitation phase (Fig. 3.18b), excess crystals of AgNO$_3$ in the hypothesis' evaluation was able to generate enough protons to effect the simultaneous precipitation of the three ions. Thus, the hypothesis raised in Eq. (20-24) was found to be true, experimentally. This means SPIONs@gluconic acid can be employed efficiently to simultaneously precipitate lone-pair bearing organic dyes, chloride and silver ions from an aqueous solution containing low amount of Ag (I) ions (Fakayode et al., 2017). Nevertheless, addition of SPIONs@gluconic acid alone could not induce precipitation (Fig. 3.19b). Also, other materials which formed soluble acidic chlorides in water (i.e. HCl and FeCl$_3$.6H$_2$O) were unable to cause neutral red’s precipitation, even in their excess amounts (Fig. 3.19 c-d). Collectively, this means that excess protonation alone was not enough to
Furthermore, the two forms of gold-coated SPIONs were evaluated for core-shell features using transmission electron microscopy. The results are shown in Fig. 3.24a-f. According to Fig. 3.24 a-c, for gold-coated SPIONs under stirred condition, not so much interaction other than being a nanocomposite can be deduced (no visible shell at the edge of the core) (Tintoré et al., 2015; Sabale et al., 2017) whereas for gold-coated SPIONs under unstirred condition, a non-separable one piece of material can be observed with some characteristic features of core-shell nanostructure (Fig. 3.24d-f) (Sabale et al., 2017; Song et al., 2015). Moreover, two forms of the core-shell nanomaterials were observed, viz: the ribbon or doughnut-like form with thin shell lining surrounding a dense core (Fig. 3.2d-e) and the flat smoothly elongated spiral-like form consisting of many fused/blended ribbon forms. (Fig. 3.24f).
3.4. SYNTHESIS OF MESO-TETRAKIS(4-HYDROXYPHENYL)PORPHYRIN

3.4.1. Synthesis and purification

The meso-tetrakis(4-hydroxyphenyl)porphyrin (POP 1) was synthesized via modified Rothemund approach (Rothemund, 1935) (Fig. 3.25). Two methods of purification were employed, viz: solvent extraction using modified Penon et al. approach (Penon et al., 2015) and silica gel column purification using dichloromethane-ethyl acetate solvent mixture (2:1, v/v). However, the result from the solvent extraction purification showed that the obtained porphyrin was not as pure as the one obtained from the silica gel column. Thus, the porphyrin obtained from the silica column was used for the photodynamic therapy evaluation. The colour of each fraction as well as the minimum time of breakthrough is given in Table 3.6. The pure porphyrin fractions were identified using ultraviolet-visible spectrophotometry (UV-Vis) (Fig. 3.26). Furthermore, this procedure was repeated to test the reliability of the approach (Table 3.7, Fig. 3.27). The pure fractions were combined and dried under ambient condition to obtain a dark purple black solid which turned red brown in methanol (Inset, Fig. 3.28a). However, a green dye was obtained after evaporating the methanolic solution in air under ambient condition (Inset, Fig. 3.28a). Finally, the structure of the purified porphyrin was further confirmed using Fourier Transform infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance spectroscopy (\(^1\)H NMR) (Fig. 3.28a-b)(Section 2.2.4.3).
Figure 3.25. Synthesis and purification of crude porphyrin; (a) Pyrrole; (b) 4-hydroxybenzaldehyde; (c) meso-tetrakis(4-hydroxyphenyl) porphyrin; (d-g) column elution; elution solvent: dichloromethane: ethyl acetate (2:1 v/v).

Table 3.6 Evolution of fractions from the silical gel column

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Colour</th>
<th>Time of evolution (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yellow-brown</td>
<td>4.35</td>
</tr>
<tr>
<td>2-3</td>
<td>Brown</td>
<td>5.35*</td>
</tr>
<tr>
<td>4-5</td>
<td>Black-purple</td>
<td>21</td>
</tr>
</tbody>
</table>

*% yield = (Actual mass of pure / Theoretical mass) x 100 = (0.3564/9.9663) x 100 = 3.76.
Table 3.7 Evolution and UV-Vis characteristics of fractions from the crude porphyrin (Repeat silica column purification procedure)

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>UV-Vis characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>Soret dwarf+ undefined Q-bands</td>
</tr>
<tr>
<td>4</td>
<td>Soret medium tall + defined QI, broad QII-QIV; shoulder between soret and QIV</td>
</tr>
<tr>
<td>5-6</td>
<td>Soret taller than 4 + defined QI, broad QII-QIV; shoulder between soret and QIV</td>
</tr>
<tr>
<td>7-8</td>
<td>Broad soret dwarf+ defined QI, broad QII-QIV; two shoulders between soret and QIV</td>
</tr>
<tr>
<td>9-10</td>
<td>Well defined soret; defined QI, broad QII-QIV; high shoulder between soret and QIV</td>
</tr>
<tr>
<td>11-12</td>
<td>Well defined porphyrin structures: soret and QI-QIV; QIV higher in peak than QI</td>
</tr>
<tr>
<td>13</td>
<td>Well defined porphyrin structures: soret and QI-QIV; QIV higher in peak than QI</td>
</tr>
<tr>
<td>14-16</td>
<td>Well defined porphyrin structures: soret and QI-QIV; QIV higher in peak than QI</td>
</tr>
<tr>
<td>17-19</td>
<td>Well defined porphyrin structures: soret and QI-QIV; QIV higher in peak than QI</td>
</tr>
<tr>
<td>20-22</td>
<td>Well defined porphyrin structures: soret and QI-QIV; QIV lower in peak than QI</td>
</tr>
<tr>
<td>23-24</td>
<td>Undefined porphyrin structure</td>
</tr>
</tbody>
</table>
The UV-Vis analysis of the purified porphyrin showed a soret band at 426 nm and QIV-QI bands at 530, 566, 609 and 656 nm respectively (Fig. 3.28a). The QI band showed a narrower band than the other Q bands with characteristic higher absorbance than the QII (Fig. 3.28a). The higher intensity of the Q-band at 650 nm suggested the presence of chlorin (McCarthy et al., 2009) which could be produced during synthesis or drying as a result of exposure to prolonged air oxidation conditions.

**Figure 3.28.** Characterizations of the silica gel column-purified porphyrin. (a) UV-Vis; (b) FT-IR and $^1$H NMR. Insets in (c): (left) digital image of dried porphyrin previously dissolved in methanol (green colour) and dried porphyrin in methanol (red brown colour); (right) molecular structure of meso-tetrakis(4-hydroxyphenyl)porphyrin.
The result from the FT-IR analysis (Fig. 3.28b) revealed that the as-synthesized porphyrin exhibited a characteristic vibrational stretch band peaks for alcoholic (O-H) and amine groups (N-H) at 3381 (range 3682-3126, broad), aromatic C-H group (benzene) at 3100, vinyl C-H at 2932, C=N at 1730, C=C(vinyl) at 1610, C=C(benzene) at 1514, C-N at 1274 and C-O at 1075, in addition to bending peak for C-H(out- of plane ) (Benzene) at 746 cm$^{-1}$ (Fig. 3.28b, Section 2.2.4.3). Moreover, the result of the $^1$H NMR analysis (Fig. 3.28c, Section 2.2.4.3) showed that the as-synthesized porphyrin exhibited a metal-free core which was evident from the sharp singlet peak at -2.90. Also, the phenolic and pyrolic protons were observed between 7.20 and 8.00 $\delta$ and at 8.85 $\delta$ respectively. Lastly, the phenolic hydroxy group protons were observed at 9.97 $\delta$ (Fig. 24). Altogether, these data confirmed the synthesis of pure free base meso-5, 10, 15, 20 - tetrakis (4-hydroxyphenyl) porphyrin. However, the green colour (insert of Fig. 3.28c(left) and the internal NH peaks at -1.6 on the $^1$H NMR spectrum(Fig. 3.28c) suggested the presence chlorin (partially oxidized porphyrin) which could be formed during synthesis and drying due to exposure of the free base porphyrin to air.

3.5. ESTIMATION OF MOLAR EXTINCTION COEFFICIENT OF THE PURIFIED PORPHYRIN (POP 1)

3.5.1. Using methanol and methanol-water mixture

The results for obtaining molar extinction coefficient of the purified porphyrin in methanolic solution is shown in Table 3.8 and Fig.3.29a-b while that obtained from the methanolic-water mixture (1:1 v/v) is displayed in Table 3.9-3.10 and Fig. 3.29c-f). According to Fig 3.29, excellent linear relationships ($R^2 > 0.999$) were achieved between the absorbances and the concentrations of the porphyrin in both methanolic
and methanolic-water solutions leading to molar extinction coefficients of 44156 (for methanol), and 21748 and 15907 L mol\(^{-1}\) cm\(^{-1}\) (for methanol-water mixture (1:1 v/v)) respectively. The last two values were employed for estimating % wt. of porphyrin in PC 1 and PC 2 and PC 5 respectively.

**Table 3.8** Data for estimating molar extinction coefficient of purified porphyrin using methanol solution

<table>
<thead>
<tr>
<th>Sample-ID</th>
<th>Concentration ((\mu g/mL))</th>
<th>Concentration ((Mol/L))</th>
<th>Absorbance @421 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPB</td>
<td>16.6667</td>
<td>2.45557E-05</td>
<td>1.160675</td>
</tr>
<tr>
<td>PPC</td>
<td>5.5556</td>
<td>8.18529E-06</td>
<td>0.45565</td>
</tr>
<tr>
<td>PPD</td>
<td>1.8519</td>
<td>2.72848E-06</td>
<td>0.200024</td>
</tr>
<tr>
<td>PPE</td>
<td>0.6173</td>
<td>9.09493E-07</td>
<td>0.11586</td>
</tr>
<tr>
<td>PPF</td>
<td>0.2058</td>
<td>3.03213E-07</td>
<td>0.090505</td>
</tr>
</tbody>
</table>
Table 3.9 Data for estimating molar extinction coefficient of purified porphyrin using methanol – water solution (Shimadzu UV-Vis spectrophotometer)

<table>
<thead>
<tr>
<th>Sample-ID</th>
<th>conc. (mol/l)</th>
<th>Absorbance@422 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>5.24446E-06</td>
<td>0.102</td>
</tr>
<tr>
<td>C2</td>
<td>6.99261E-06</td>
<td>0.15</td>
</tr>
<tr>
<td>C3</td>
<td>9.32348E-06</td>
<td>0.2</td>
</tr>
<tr>
<td>C4</td>
<td>1.24313E-05</td>
<td>0.266</td>
</tr>
<tr>
<td>C5</td>
<td>1.65751E-05</td>
<td>0.353</td>
</tr>
<tr>
<td>C6</td>
<td>2.21001E-05</td>
<td>0.464</td>
</tr>
<tr>
<td>C7</td>
<td>2.94668E-05</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Table 3.10 Data for estimating molar extinction coefficient of purified porphyrin using methanol – water solution (Perkin Elmer UV-Vis spectrophotometer)

<table>
<thead>
<tr>
<th>Sample-ID</th>
<th>concentration(mol/L)</th>
<th>Absorbance@422 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>2.66778E-07</td>
<td>0.059692</td>
</tr>
<tr>
<td>C2</td>
<td>8.00333E-07</td>
<td>0.07506</td>
</tr>
<tr>
<td>C3</td>
<td>2.401E-06</td>
<td>0.100471</td>
</tr>
<tr>
<td>C4</td>
<td>7.203E-06</td>
<td>0.173888</td>
</tr>
<tr>
<td>C5</td>
<td>2.1609E-05</td>
<td>0.439895</td>
</tr>
<tr>
<td>C6</td>
<td>6.4827E-05</td>
<td>1.086929</td>
</tr>
</tbody>
</table>
In addition, the as-synthesized porphyrin emitted UV light when excited at 300, 350, 630 and 704 nm (Table 3.11, 3.13-3.14), violet light at 630 nm (Table 3.13), indigo light at 300, 350, 704 (Table 3.11, 3.14), blue light at 400, 430 and 808 nm (Table 3.12,3.14) and orange light at 400 nm (Table 3.12) excitations. All excitation wavelengths gave near infrared emission except 808 and 825 nm (Table 3.11-3.14). Moreover, excitation at 400 nm gave the highest red light emission intensity (Fig. 3.30, Table 3.11-3.14). Furthermore, the red emission associated with the near infrared excitations (Fig. 3.31) showed that the as-synthesized porphyrin would be a good potential for deep-seated tumour fluorescence bio-imaging due to the deeper penetration of tissue associated with these excitation wavelengths.

**Figure 3.31.** Photoluminescence (PL) characteristics of the as-synthesized pure porphyrin. Orange red-red-near infrared emissions at 515,550,808 and 825 nm excitations (t,x,y,z = excitation peaks at 515,550,808 and 825 respectively. Ex = Excitation wavelength.
### Table 3.11 Photoluminescence characteristics of the pure porphyrin under UV-light excitations

<table>
<thead>
<tr>
<th>Excitation Source</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Intensity</th>
<th>Emission Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet-light (UV)</td>
<td>300</td>
<td>351</td>
<td>531761</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td>426</td>
<td>38263</td>
<td>Indigo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>615</td>
<td>67816</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>644</td>
<td>132020</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>702</td>
<td>104042</td>
<td>Red-Near infrared</td>
<td></td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>376</td>
<td>169158</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td>431</td>
<td>56775</td>
<td>Indigo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>613</td>
<td>51575</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>650</td>
<td>170131</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>668</td>
<td>74134</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>685</td>
<td>47767</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>862</td>
<td>35274</td>
<td>Near infrared</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.12 Photoluminescence characteristics of the pure porphyrin under violet-indigo light excitations

<table>
<thead>
<tr>
<th>Excitation Source</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Intensity</th>
<th>Emission Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet-light</td>
<td>400</td>
<td>455</td>
<td>14978</td>
<td>Blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>606</td>
<td>299623</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td></td>
<td>651</td>
<td>923524</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>703</td>
<td>105764</td>
<td>Red-Near infrared</td>
</tr>
<tr>
<td></td>
<td></td>
<td>782</td>
<td>23781</td>
<td>Near infrared</td>
</tr>
<tr>
<td></td>
<td></td>
<td>877</td>
<td>38223</td>
<td>Near infrared</td>
</tr>
<tr>
<td>Excitation Source</td>
<td>Excitation wavelength (nm)</td>
<td>Emission wavelength (nm)</td>
<td>Intensity</td>
<td>Emission Region</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------</td>
<td>-------------------------</td>
<td>-----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Green-light</td>
<td>515</td>
<td>620</td>
<td>33073</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>651</td>
<td>156367</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>703</td>
<td>18382</td>
<td>Red-Near infrared</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>617</td>
<td>59920</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>651</td>
<td>168937</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>705</td>
<td>21530</td>
<td>Red-Near infrared</td>
</tr>
<tr>
<td>Red-light</td>
<td>630</td>
<td>349</td>
<td>19346</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>424</td>
<td>1973</td>
<td>Violet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>624</td>
<td>163000</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>640</td>
<td>86643</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>678</td>
<td>390059</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>707</td>
<td>21009</td>
<td>Red-Near infrared</td>
</tr>
<tr>
<td></td>
<td></td>
<td>854</td>
<td>2004</td>
<td>Near infrared</td>
</tr>
</tbody>
</table>
Table 3. 14 Photoluminescence characteristics of the pure porphyrin under red -near infrared light excitations

<table>
<thead>
<tr>
<th>Excitation Source</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Intensity</th>
<th>Emission Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red -Near infrared-light</td>
<td>704</td>
<td>359</td>
<td>15006</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>437</td>
<td>7208</td>
<td>indigo</td>
</tr>
<tr>
<td></td>
<td></td>
<td>618</td>
<td>6547</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650</td>
<td>17146</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>751</td>
<td>4408</td>
<td>Near infrared</td>
</tr>
<tr>
<td>Near infrared-light</td>
<td>808</td>
<td>470</td>
<td>2018</td>
<td>Blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>620</td>
<td>23614</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>651</td>
<td>80931</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>698</td>
<td>9897</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td>825</td>
<td>619</td>
<td>6341</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650</td>
<td>13732</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>698</td>
<td>1907</td>
<td>Red</td>
</tr>
</tbody>
</table>

3.7. SYNTHESIS AND CHARACTERIZATION OF MESO-5,10,15,20-TETRAKIS(4-HYDROXYPHENYL)CHLORIN (POP2) FROM CORRESPONDING PORPHYRIN

Addition of hydrogen peroxide solutions (0.7- 2.3 mL) caused a bathochromic shift in the QI band of the porphyrin from 658 nm to 685 nm with characteristic increase in absorbance (Fig. 3.32, Fig. 3.33a-d) while subsequent addition caused a decrease in absorbance intensity (Inset, Fig.3.33d). Thus, 2.3 mL H₂O₂ produced the optimal yield of chlorin. Critical assessment of the UV-visible absorption spectra showed
Figure 3.33. Monitoring of chlorin (POP 2) formation. Hypochromic shift of QIV band (a,c); Bathochromic shift of QI band(b,d). Inset: Decrease in absorbance after addition of 2.5 mL H₂O₂.

The increase in absorbance with increasing volume of H₂O₂ shows that this approach has the potential to be used for effective quantification of hydrogen peroxide in various aqueous solutions such as environmental waters, pharmaceutical and biological fluids.

3.8. SYNTHESIS OF PORPHYRIN CONJUGATES

Three approaches were employed for the synthesis of porphyrin conjugates, viz: modification of the modified Brust-Schiffrin’s method reported by Penon et al. using tetraoctylammonium bromide–methoxypolyethyleneglycol-thiol (TOAB-mPEGSH) (Brust et al., 1994; Penon et al., 2015), the newly introduced alcoholic solvent
conjugation technique (ASCT) and polar solvent deposition method (PSD). The modified TOAB-mPEGSH approach involved extraction of SPIONs from SPIONs-gold nanomaterials into the toluenic organic phase using TOAB as a phase transfer agent and leaving gold together with methoxy polyethylene glycol-thiol in the aqueous phase. This approach was completely different from the result obtained from Brust and coworkers and Penon et al. (Brust et al., 1994; Penon et al., 2015). In their research, Brust and coworkers extracted gold nanoparticles into the organic phase whereas in this present work SPIONs were extracted into the organic phase at the expense of gold nanoparticles. Similarly, Penon et al. reported porphyrin functionalization of gold nanoparticles in the absence of SPIONs. Moreover, some porphyrin molecules were also extracted into the aqueous phase via their interaction with mPEGSH in the current approach. On the other hand, the ASCT procedure involved mixing alcoholic solutions of two photosensitizers and subsequently evaporating the resulting solution. While, the PSD method involved deposition of hydrophobic porphyrin into the aqueous medium using a polar organic solvent in which the porphyrin was soluble, followed by evaporating the resulting solution. In its simplicity and uniqueness, polar solvent deposition (PSD) approach requires that the porphyrin must be soluble in an organic solvent that is soluble in aqueous solution. Under ASCT and PSD approaches, no stirring was involved, only a few minute moderate shaking (maximum of 10 min), followed by evaporation of the solvents with time. This makes these approaches energy-efficient. The summary of the synthesis of water-soluble porphyrin using the three approaches is shown in Scheme 3.7.
together with the pulling of some of the porphyrin molecules from the organic phase into the aqueous phase via hydrogen bonding.

Moreover, concurrently, as the SPIONs were being drawn up, some porphyrin molecules were being pulled down into the aqueous medium (Scheme 3.8b) possibly via intermolecular hydrogen bonding between the hydroxy group of the porphyrinoid phenols and the oxygen atom of the methoxy group of the mPEGSH, hydroxy group on the citric acid as well as ammonium group on the gelatin (Scheme 3.8, 3.9). Since thiol functional group binds to the surface of gold (Bürgi, 2015; Brust et al., 1994), this binding probably ensured that gold did not have access to the uptake process. In addition, the amino group of the gelatin exists as ammonium ion in the acidic medium, thus making the gelatin capped gold nanoparticles to experience repulsion at the interface between the two liquids where the uptake was taking place (Scheme 3.9). A major difference between the hay yellow and red violet conjugates was that hay yellow was highly magnetic while the red violet was not attracted to external magnetic field. This proved beyond any doubt that SPIONs were successfully abstracted into the organic phase and subsequently combined with the resident TOAB and porphyrin. Nevertheless, both conjugates were soluble in water at room temperature (Chart 3.4).
3.8.2. Synthesis and characterization of white porphyrin-glucose conjugate (PC 3) using PSD approach.

A brownish magnetic methoxyporphyrin (BMMP) conjugate was obtained after shaking glucose solution containing SPIONs with an ethanolic solution of porphyrin. However, a visibly non-magnetic greenish yellow solution was obtained after the BMMP was magnetically separated from its ethanolic solution. After evaporation of the non-magnetic greenish yellow filtrate overnight, white crystals with greenish yellow tint were obtained (Scheme 3.10). The as-synthesized crystals exhibited strong attraction towards external magnetic field and dissolved readily in water and ethanol. After dissolving the crystals in water, a red violet/pink colour was observed which turned yellow after shaking (Scheme 3.10). However, since the solution obtained was clear, no visible magnetization was observed. This may be due to the concentration of the particles being very low. The results of the UV-Visible spectrometry characterizations of the as-synthesized porphyrin crystals and their precursors are shown in Fig. 3.35a-b. According to Fig. 3.35a, the green precursor solution showed characteristic absorption band peaks for SPIONs at 321 nm and porphyrin at 417, 499, 536, 573 and 632 nm for soret, QIV, QIII, QII and QI respectively. On the other hand, the white magnetic porphyrin crystals showed the presence of porphyrin soret at 418 and the SPIONs at 315 nm(Fig. 3.35b).
the broadness but decrease in O-H and N-H groups’ intensity signal relative to those in the uncombined states suggested existence of strong intermolecular hydrogen bonding between the porphyrin and the methylene blue.

The photoluminescence (PL) studies showed that aqueous solution of the hybrid material exhibited intense methylene blue red emissions at 685 nm at excitation wavelengths of 630, 650 and 670 nm while no obvious emission of porphyrin at 650 nm was visible on the spectrum (Fig. 3.39a). The photostability of the methylene blue’s emission in water may be due to its inherent solubility in water (Gao et al., 2012) while the absence of the porphyrin emission may be attributed to solvent quenching effect (Kawasaki et al., 2014). However, excitations at 430, 808 nm and 515, 730 nm gave porphyrin red emission at 650 nm and methylene blue red fluorescence at 685 nm respectively with relatively reduced intensities (Fig. 3.39b). The low emission intensity of porphyrin within these regions may be attributed to solvent quenching effect (Kawasaki et al., 2014), conformational changes (Wang et al., 2011) or aggregation effect (Gao et al., 2012a) while that of the methylene blue could be as a result of poor absorption of light within these regions respectively. In DMSO, all evaluated excitation wavelengths from 350 to 808 nm exhibited strong porphyrin red emissions at 650 nm except 670 nm excitation which exhibited methylene blue fluorescence at 685 nm (Fig. 3.39c-d). Preliminary studies of the pristine porphyrin’s luminescence showed poor emission quality with excitation ranging between 600-700 nm whereas methylene blue gave some better well-resolved emission spectra within this range (Fig. 3.39a,c). This results show that methylene blue acted as a bridging agent within the region where the porphyrin exhibited poor emission quality and make it possible for the excitation regions of the hybrid material to be feasible from ultraviolet (350 nm) to near infrared (808 nm).
aqueous solution of the complex; (c) FT-IR spectra of the complex and precursors; (d) FT-IR spectra of the complex.

In addition, the methylene blue peak was bathochromically shifted from 652 nm to 663 nm (Fig. 3.40a-b). The results from the FT-IR analyses of the complex revealed major characteristic vibrational band peaks at 3423, 3261, 1654, 1414 and 692 cm⁻¹ corresponding to intermolecular hydrogen bonded phenolic hydroxyl group of the porphyrin, ²⁰ ammonium ion (=N⁺-H) group of the protonated methylene blue, C=C_str of porphyrin and methylene blue, O-H_bend of phenols and water (from aqua complex of Fe_{III}) and Fe^{3+}-O_str of aqua complex of Fe_{III} respectively (Fig. 3.40c-d). This confirmed the possible association of the porphyrin, methylene blue and Fe_{III} within the complex. Typically, methylene blue and porphyrin may associate together via π–π electronic stacking, hydrogen bonding and hydrophobic interactions (Tiwari & Singh, 2011; Gao et al., 2012c) (scheme 3.11). Furthermore, aromatic structures such as phenols and pyrroles may have negative charges on their ring structures due to resonance hybridization. These features readily attract the positively charged methylene blue via its ammonium (=N⁺-H) or sulphonium (=S⁺) ion site leading to the assembling of methylene blue molecules on porphyrin’s structure (Scheme 3.11).
detection limit (LOD) of 2.27% (0.7409 M). The latter was estimated using the expression \(3\sigma/s\) (where \(\sigma = \) standard deviation of the blank) and \(s = \) slope of the calibration graph) (Gu et al., 2016; Tiwari & Singh, 2011). Furthermore, the 0-3% linear range could be described using first order exponential expression as shown in Eq. (28) and Fig. 3.42d:

\[
\frac{(P_x)_a}{(P_x)_0} = e^{kc} \quad (28)
\]

which linearized to give Eq. (20-21)

\[
\ln (P_x)_a - \ln (P_x)_0 = -kc \quad (29)
\]

or

\[
\ln \left(\frac{(P_x)_a}{(P_x)_0}\right) = -kc \quad (30)
\]

Where 

\((P_x)_a = \) Mean pixel counts for a given \(\text{H}_2\text{O}_2\) concentration, \((P_x)_0 = \) mean pixel count before decay; \(k = \) first order decay rate constant and \(c = \) concentration of \(\text{H}_2\text{O}_2\) in mol/L (Table 3.15).

A similar observation has been reported by Sulieman and coworkers which observed that the number of applications to achieve uniform tooth whitening effect decreased exponentially with increasing \(\text{H}_2\text{O}_2\) concentration (Sulieman et al., 2004). The 0-3% linear range in this study indicates that this approach has great potential to be used for the quantification of hydrogen peroxide in food drinks and biological fluids which usually accommodate low \(\text{H}_2\text{O}_2\) concentrations (Gu et al., 2016; Tiwari & Singh, 2011).
absence of gas evolution in the 30 % $\text{H}_2\text{O}_2$-labelled pharmaceutical disinfectant solution; (c) RGB colour discrimination of 30 % $\text{H}_2\text{O}_2$ reference solution.

Thus, this study shows that constant routine monitoring of hydrogen peroxide levels in hydrogen peroxide-containing pharmaceutical products such as disinfectant solutions and teeth whitening products is highly essential to ensure reliability of the product’s concentration as deviation from these labels may have occurred over exposure of these products to ambient heat and light conditions. This consequentially may enforce the applicability of these materials over many cycles of time which may lead to toxicity consequences (Naik et al., 2006; Tredwin et al., 2006). In addition, it is essential that, such products should be stored under refrigeration in the dark to avoid accidental degradation.

The proposed mechanism for the detection of $\text{H}_2\text{O}_2$ using the current approach is given in Eq. (31-35).

\[
\begin{align*}
\text{POP}^a\text{-MB}^b\text{-Fe}^{\text{III}} + \text{HO-OH} & = \text{POP}\text{-MB}\text{-Fe}^{\text{II}} + \text{HOO.} + \text{H}^+ \quad (31) \\
\text{POP}\text{-MB}\text{-Fe}^{\text{III}} + \text{HOO.} & = \text{POP}\text{-MB}\text{-Fe}^{\text{II}} + \text{O}_2 + \text{H}^+ \quad (32) \\
\text{POP}\text{-MB}\text{-Fe}^{\text{II}} + \text{HO-OH} & = \text{POP}\text{-MB}\text{-Fe}^{\text{III}} + \text{HO.} + \text{HO}. \quad (33) \\
\text{POP}\text{-MB}\text{-Fe}^{\text{III}} + \text{HOO.} / \text{HO.} & = \text{POOP}^c + \text{MOOB}^d + \text{Fe}^{\text{III}} \quad (34) \\
\text{Fe}^{\text{III}} + \text{HO}. & = \text{Fe(HO)}_3 \quad (35)
\end{align*}
\]

Note:

\(^a\text{POP} – \text{Porphyrin}; ^b\text{MB} – \text{Methylene Blue}; ^c\text{POOP} – \text{Oxidized porphyrin}; ^d\text{MOOB} – \text{Oxidized methylene blue. The evidence of this proposed mechanism is further shown in Chart 3.5a-b.}\)
According to Eq. 22-26, ferric ion oxidized $\text{H}_2\text{O}_2$ to hydro-peroxyl radical (Young et al., 2012) which in turn further reacted with the ferric ion to generate oxygen gas (Alnuaimi et al., 2007; Young et al., 2012) (Fig. 3.43b; Chart 3.5a-b). The ferrous ion generated as a result of this reaction reacted with more $\text{H}_2\text{O}_2$ to produce hydroxyl radical (Young et al., 2012; Alnuaimi et al., 2007; Alnuaimi et al., 2008) which together with hydro-peroxyl radical degraded the colour of the porphyrin-methylene blue on the test strips. Finally, the $\text{Fe}^{\text{III}}$ remaining in the solution precipitated as yellow-orange $\text{Fe(OH)}_3$ as shown in Chart 3.5b. Since the extent of colour degradation depends on the concentration of $\text{H}_2\text{O}_2$ in the test solution, a feasible quantification procedure was established.

### 3.8.5. Synthesis and characterization of carbonated porphyrin powder using polar solvent deposition (PSD) method

Sodium bicarbonate solution was added to 1 mL meso-tetrakis(4-hydroxyphenyl)porphyrin in MeOH to obtain a spontaneous green-yellow precipitate. The solution of the latter was filtered and the residue dried under ambient temperature. The dried porphyrin evolved in two forms, viz: a white powder and
green powder of different solubilities (Table 3.16). These porphyrin powders were then characterized using FT-IR spectroscopy and stored under ambient condition.

**Table 3. 16.** Solubility of the porphyrin powders

<table>
<thead>
<tr>
<th>Porphyrin sample</th>
<th>Water</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>White powder</td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Green powder</td>
<td>Soluble</td>
<td>soluble</td>
</tr>
</tbody>
</table>

The FT-IR analyses of both forms of porphyrin powder are shown in Table 3.17 and Fig. 3.44. According to Table 3.17 and Fig. 3.43, distinct differences were observed in the characteristic absorption of FT-IR spectra at 1661-1784, 1440-1459, 1100-1212 and 590-960 indicating the different orientations of the carbonate groups on the different forms of the porphyrin. In addition, a narrower fused O-H<sub>str</sub> and N-H<sub>str</sub> band was observed for white powder while a broader intermolecular hydrogen-bonded fused O-H<sub>str</sub> and N-H<sub>str</sub> band was observed for the green powder.

**Table 3. 17** FTIR spectra data of the two porphyrin powders

<table>
<thead>
<tr>
<th>Functional groups</th>
<th>White porphyrin powder</th>
<th>Green porphyrin powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H&lt;sub&gt;str&lt;/sub&gt; and N-H&lt;sub&gt;str&lt;/sub&gt;</td>
<td>3484, sharp, free</td>
<td>3355, broad, intermolecular H-bonded</td>
</tr>
<tr>
<td>C-H&lt;sub&gt;str&lt;/sub&gt; Benzene</td>
<td>3101</td>
<td>3007</td>
</tr>
<tr>
<td>H-CO&lt;sub&gt;3str&lt;/sub&gt; (intermolecular H-bonded)</td>
<td>2792-2431</td>
<td>2793-2413</td>
</tr>
<tr>
<td>H-CO&lt;sub&gt;3str&lt;/sub&gt; (intermolecular H-bonded)</td>
<td>2543</td>
<td>2576</td>
</tr>
</tbody>
</table>
The two porphyrin powders had the same composition (i.e. sodium meso-tetrakis(phenolate)porphyrinbicarbonate) as revealed by the FTIR spectra (Fig. 3.44) and Table 3.17, but differed by the orientation (C=Ostr@1661 and 1696 cm\(^{-1}\)) and amount of carbonate ion present (C-Ostr@593 and 646 cm\(^{-1}\)) in their matrix. This explained why the two powders showed different solubilities in water and methanol. For example, in water, both powders were soluble because bicarbonate ion form intermolecular hydrogen bonding easily in water (Table 3.16 and Table 3.17) (Guo et al., 2012; Ruderman et al., 1998). However, in methanol, bicarbonate ions are poor intermolecular hydrogen bonding formation agents. Thus, the white powder with higher carbonate intensity at 593 cm\(^{-1}\) was insoluble in methanol compared with the green powder which exhibited lower transmittance intensity (Table 3.17).

3.8.6. Synthesis and characterization of methoxypolyethyleneglycol SPIONs-gold core-shell meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate (PC 5) and its carbonated white conjugate derivative (PC 6) using PSD approach.

3.8.6.1. Synthesis and characterization

The conjugate (PC 5) evolved as green paint surrounding a densed brown paint on the flat ceramic drying plate. The green paint subsequently turned brown after scraping it out of the plate. On the other hand, a white crystalline mass was obtained after dissolving the green paint or its corresponding brown mass in 1.88% sodium bicarbonate solution, followed by evaporation. Sodium bicarbonate was introduced in order to obtain the brown conjugate in powder for easy handling and storage. Moreover, both the brown conjugate and the corresponding sodium bicarbonate complex exhibited excellent solubility in water. The chemistry behind the sodium crystallite formation is shown in scheme 3.12. According to scheme 3.12, the
intensity for the C-H$_{str}$ and C-O$_{str}$ groups respectively. This showed that the sodium bicarbonate interacted strongly with these functional groups on both the mPEGSH and the phenolic systems.

3.8.6.2. Estimation of % by weight (% wt.) of porphyrin in PC 5

The amount of porphyrin in the conjugate was determined using the data obtained from the calibration graphs employed for the estimation of the molar extinction coefficients reported in section 3.5, Table 3.10. The result of the estimation together with the results for other conjugates, viz: hay yellow (PC 1) and red-violet (PC 2) are shown in Table 3.18. The conjugates were subjected to the same conditions underwent by the pure porphyrin used for the construction of the calibration graphs, i.e. dissolution in methanol-water mixture (1:1 v/v) (4 mL) solution (section 2.2.4.3, section 3.5, Table 3.9-10). According to Table 3.18, the % wt. of porphyrin varied from one conjugate to another due to the different approaches employed for their syntheses.
Table 3.18  Estimation of % wt. of porphyrin in PC 5, hay yellow (PC 1) and red violet (PC 2) conjugates

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Concentration of porphyrin (mol/L)</th>
<th>Molar extinction (L/mol cm$^{-1}$)</th>
<th>$R^2$</th>
<th>Amount of porphyrin (mg/g)</th>
<th>% wt. porphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC 5</td>
<td>9.32564E-06</td>
<td>15907</td>
<td>0.9985</td>
<td>25.32</td>
<td>2.53</td>
</tr>
<tr>
<td>Hay Yellow (PC 1)</td>
<td>1.57532E-05</td>
<td>21748*</td>
<td>0.9991</td>
<td>13.36</td>
<td>1.34</td>
</tr>
<tr>
<td>Red violet (PC 2)</td>
<td>8.55251E-07</td>
<td>21748*</td>
<td>0.9991</td>
<td>1.16</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*molar extinction coefficient estimated from the calibration data obtained from section 3.5, Table 3.9.

3.8.7. Synthesis and characterization of methoxypolyethylene glycol SPIONs-gold core-shell meso-tetrakis(4-hydroxyphenyl)porphyrin conjugate (PC 4) for photodynamic therapy against MCF-7 breast cancer cells.

The SPIONs evolved as dark chocolate brown colloid after precipitation of the precursors in alkaline medium. The result from the TEM analysis showed monodispersed spherical nanoparticles with average particle size of 15.24 ± 5.57 (Fig. 3.47a).
the basis that the shells are unlike active nanoparticles which dissolve or absorb themselves, but mere metallic coating on a surface. Thus, as the particles fused together, they leave behind their gold shells which continuously added to themselves round about the fused nanoparticles. Thus, the shell grew larger while the core decreased in diameter.

The photodynamic nano-drug (gluconic acid capped SPIONs-gold mesotetrakis(4-hydroxyphenyl)porphyrin) (PC 4) was synthesized via a polar solvent deposition (PSD) technique which involved a gentle mixing of methanolic solution of the porphyrin with the aqueous solution of a mixture of methoxypolyethyleneglycol-thiol (mPEGSH) and gluconic acid capped gold-coated SPIONs (core-shell), followed by evaporation of the resulting solution in the presence of air. Methanol was chosen due to its high miscibility with water and because the hydrophobic porphyrin dissolved readily in it. The high degree of miscibility of the two solvents was necessary to ensure the porphyrin was completely transferred into the aqueous environment. As the solvents evaporated, magnetic field-oriented tiny brown specks of different overlaying network densities were observed on the surface of the solution in addition to many rod-like structures (Scheme 3.13a-f).
CHAPTER 4

APPLICATIONS OF MATERIALS FOR PHOTODYNAMIC THERAPY

4.1 SINGLET OXYGEN GENERATION POTENTIALS OF THE CONJUGATES

Singlet oxygen produced during Type II photodynamic reaction which involves the transfer of energy from the excited triplet state of the photosensitizer to molecular oxygen is recognized as dominant species among all the reactive oxygen species responsible for the photodynamic therapeutic actions of photosensitizers against disease cells. Thus, generation of singlet oxygen is used as a qualitative preliminary step for determining whether or not a photosensitizer will be able to eradicate disease cells (Penon et al., 2015; Kawasaki et al., 2014; C. Wang et al., 2011; M. Wang et al., 2014; Tian et al., 2013; Bhattacharyya et al., 2014).

In this thesis, the singlet oxygen generation potentials of five new porphyrin conjugates in addition to the pure porphyrin were evaluated by monitoring decrease in intensity of 1,3-diphenylisobenzofuran (DPBF) at (461 nm / 459 nm) or absorption wavelength (417 nm). DPBF is one of the commonest efficient singlet oxygen sensors (Wang et al., 2016; Dai et al., 2014; Adarsh et al., 2010). The interaction of the DPBF with singlet oxygen often leads to the decrease in the absorbance or emission intensity of the sensor due to the oxidation of the latter to its corresponding diketone by singlet oxygen (Kawasaki et al., 2014). The extent of this decrease in the absorbance or intensity of the sensor indicates qualitatively, the ability of the photosensitizer to generate singlet oxygen while quantitatively, it indicates the relative
photodynamic strength of the photosensitizer. After subjection of the conjugate-DPBF solution in dimethylsulfoxide (DMSO) to light irradiation at 680 nm and 808 nm for deeper red and near infrared excitations respectively, the result showed that the intensity of DPBF decreased with irradiation time up till 2160 s given rise to singlet oxygen quantum yields ranging from 0.07 to 0.26 (Table 4.1) relative to the methylene blue reference (Quantum yield = 0.52) and an absolute relative amount of 0.42 without methylene blue reference (Table 4.1).

**Table 4.1 Singlet oxygen generation potentials of conjugates.**

<table>
<thead>
<tr>
<th>PS Material</th>
<th>PS slope (s⁻¹)</th>
<th>MB slope (s⁻¹)</th>
<th>Absorbance of MB at 680 nm</th>
<th>Absorbance of PS material at 680 nm</th>
<th>Singlet oxygen yield of PS material</th>
<th>DPBF wavelength (nm)</th>
<th>Time of irradiation (s)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure porphyrin</td>
<td>-2.4956</td>
<td>-4.1963</td>
<td>0.043</td>
<td>0.18</td>
<td>0.07</td>
<td>461</td>
<td>200</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>PC 1</td>
<td>-0.5364</td>
<td>-4.1963</td>
<td>0.043</td>
<td>0.092</td>
<td>0.03</td>
<td>461</td>
<td>200</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>PC 4</td>
<td>-13.566</td>
<td>-59.051</td>
<td>0.202797</td>
<td>0.096126</td>
<td>0.25</td>
<td>461</td>
<td>200</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>PC 7</td>
<td>-0.0024</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.42</td>
<td>459</td>
<td>225</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>PC 11</td>
<td>-0.0053</td>
<td>-0.011</td>
<td>0.455987</td>
<td>0.089095</td>
<td>1.28</td>
<td>417</td>
<td>2160</td>
<td>Absorption</td>
</tr>
<tr>
<td>PC 12</td>
<td>-</td>
<td>0.00005</td>
<td>0.110927</td>
<td>0.109022</td>
<td>0.26</td>
<td>417</td>
<td>200</td>
<td>Absorption</td>
</tr>
</tbody>
</table>

*a Quantum yield of MB = 0.52; b excitation wavelength = 680 nm; c New proposed method: \( \Phi_{\text{yield}} = 1 - \exp^{-k_{\text{phox}} \cdot t} \); excitation wavelength = 808 nm.

The decrease in the intensity and absorbance of the DPBF for all evaluated conjugates are clearly shown in Fig. 4.1-4.2. According to Fig. 4.1-4.2, all tested conjugates caused a significant decrease in the intensity or absorbance of the DPBF relatively with time. This shows that all the conjugates have the capability to be used as potential photodynamic therapeutic agents.
Figure 4.1. Singlet oxygen generation. (a-b) pure porphyrin; (c-d) PC 1; (e-f) PC 7 vs. methylene blue using fluorescence method.
Figure 4.2. Singlet oxygen generation; (a-b) PC 4; (c-d) PC 11; (e-f) PC 12 vs. methylene blue using fluorescence and absorption methods respectively.
4.2 NEAR INFRARED-INDUCED SINGLET OXYGEN GENERATION OF TWO-IN-ONE PHOTOSENSITIZER HYBRID (PC 7)

Two photosensitizers, viz: meso-tetrakis(4-hydroxyphenyl)porphyrin and methylene blue were interacted to form a hybrid conjugate while the singlet oxygen generation potential were probed via near infrared excitation at 808 nm. The photo-induced oxidative degradation reaction of 1,3-diphenylisobenzofuran (DPBF) with singlet oxygen was used to describe the singlet oxygen generation potential of the as-synthesized methylene blue-porphyrin conjugate while the singlet oxygen quantum yield was estimated using the newly proposed method (Section 2.2.10.5). This was done since the reference did not absorb at 808 nm. The result of the singlet oxygen generation by this near-infrared induced photodynamic reaction is shown in Fig. 4.3a-b. According to Fig. 4.3a-b, the emission intensity of DPBF at 459 nm decreased linearly with time over exposure to near-infrared light at 808 nm for 225 s. The estimated first order degradation rate constant of DPBF’s fluorescence intensity was $0.0024 \text{ s}^{-1} (R^2 = 0.9986)$ (Fig 4.3a) while the singlet oxygen quantum yield was 0.42 which was higher than some reported values (Table 4.2).
Figure 4.3. Singlet oxygen generation of the as-synthesized methylene blue (MB)-porphyrin conjugate (PC 7). (a) Photo-induced oxidation degradation of DPBF (Inset: time-course curve); (b) Decrease in DPBF emission and photostability of the conjugate emission during the photo-oxidation process; Inset: Photoluminescence of porphyrin@808 nm (red line), methylene blue@670 nm (blue line) and methylene blue@808 nm (purple line) excitations.

The high photostable red emission at 650 nm coupled with the absence of emission at 685 nm (Fig. 4.3b) clearly showed that porphyrin was responsible for the relatively larger degradation rate constant of the conjugate compared to DPBF or DPBF-MB alone (Fig. 4.3a, Table 4.3-4). Furthermore, the photostability of the porphyrin’s emission implied that the conjugate did not interfere with the photooxidative reaction during the interaction period.
Table 4.2 Comparison of singlet oxygen yield of the conjugate with some literature values

<table>
<thead>
<tr>
<th>Photosensitizers</th>
<th>$[^3]O_2$ Quantum yield</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyrin dyad</td>
<td>0.24</td>
<td>9,10-dimethylanthracene</td>
<td>(Milanesio et al., 2001)</td>
</tr>
<tr>
<td>Porphyrin co-polymer</td>
<td>0.15</td>
<td>1,3-diphenylisobenzofuran</td>
<td>(Dai et al., 2014)</td>
</tr>
<tr>
<td>conjugate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromo-cyanine dye</td>
<td>0.046</td>
<td>1,3-diphenylisobenzofuran</td>
<td>(Jing et al., 2016)</td>
</tr>
<tr>
<td>Porphyrin-MB conjugate</td>
<td>0.42</td>
<td>1,3-diphenylisobenzofuran</td>
<td>Present work</td>
</tr>
</tbody>
</table>

Table 4.3 Photodegradation data for the singlet oxygen generation

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>MB+DPBF</th>
<th>Conjugate + DPBF</th>
<th>DPBF alone</th>
<th>Time (s)</th>
<th>MB+DPBF</th>
<th>Conjugate + DPBF</th>
<th>DPBF alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ln I/I₀</td>
<td>Ln I/I₀</td>
<td>Ln I/I₀</td>
<td></td>
<td>Ln I/I₀</td>
<td>Ln I/I₀</td>
<td>Ln I/I₀</td>
</tr>
<tr>
<td>0</td>
<td>2146.6</td>
<td>1852.2</td>
<td>1340</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>2101.7</td>
<td>1715.6</td>
<td>1288.8</td>
<td>25</td>
<td>-0.02114</td>
<td>-0.07661</td>
<td>-0.03896</td>
</tr>
<tr>
<td>50</td>
<td>2062.7</td>
<td>1620.5</td>
<td>1258.2</td>
<td>50</td>
<td>-0.03987</td>
<td>-0.13364</td>
<td>-0.06299</td>
</tr>
<tr>
<td>75</td>
<td>2024.5</td>
<td>1537.4</td>
<td>1247.2</td>
<td>75</td>
<td>-0.05856</td>
<td>-0.18628</td>
<td>-0.07177</td>
</tr>
<tr>
<td>100</td>
<td>1995.7</td>
<td>1451.6</td>
<td>1231.9</td>
<td>100</td>
<td>-0.07289</td>
<td>-0.24371</td>
<td>-0.08411</td>
</tr>
<tr>
<td>125</td>
<td>1970.2</td>
<td>1364.2</td>
<td>1201.3</td>
<td>125</td>
<td>-0.08575</td>
<td>-0.30581</td>
<td>-0.10927</td>
</tr>
<tr>
<td>150</td>
<td>1950.1</td>
<td>1277.2</td>
<td>1184.7</td>
<td>150</td>
<td>-0.096</td>
<td>-0.3717</td>
<td>-0.12318</td>
</tr>
<tr>
<td>175</td>
<td>1932.5</td>
<td>1194.9</td>
<td>1156.7</td>
<td>175</td>
<td>-0.10507</td>
<td>-0.43831</td>
<td>-0.1471</td>
</tr>
<tr>
<td>200</td>
<td>1906.7</td>
<td>1135.9</td>
<td>1132.3</td>
<td>200</td>
<td>-0.11851</td>
<td>-0.48895</td>
<td>-0.16842</td>
</tr>
<tr>
<td>225</td>
<td>1888</td>
<td>1080.9</td>
<td>1109.7</td>
<td>225</td>
<td>-0.12837</td>
<td>-0.53858</td>
<td>-0.18858</td>
</tr>
</tbody>
</table>

MB = Methylene blue; DPBF = 1,3-Diphenylisobenzofuran; $I₀ = DPBF$ intensity at time t = 0; $I_t = DPBF$ intensity at time t. Excitation wavelength = 808 nm. MB solution (2 mL, $1.2505 \times 10^{-6}$ M in DMSO). DPBF solution (2 mL, $1.9729 \times 10^{-6}$ M in DMSO); conjugate solution (2 mL, 0.0004 g/4 mL in DMSO).

Table 4.4 First order degradation rate constants ($K_{phox}$) of conjugate, DPBF and DPBF-MB

<table>
<thead>
<tr>
<th>Material</th>
<th>$K_{phox}$ (s⁻¹)</th>
<th>Equation</th>
<th>$R^2$</th>
<th>First order time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPBF</td>
<td>0.0008</td>
<td>$y = -0.0008x - 0.0121$</td>
<td>0.9868</td>
<td>225</td>
</tr>
<tr>
<td>DPBF-MB</td>
<td>0.0006</td>
<td>$y = -0.0006x - 0.0102$</td>
<td>0.9805</td>
<td>225</td>
</tr>
<tr>
<td>Conjugate</td>
<td>0.0024</td>
<td>$y = -0.0024x - 0.009$</td>
<td>0.9986</td>
<td>225</td>
</tr>
</tbody>
</table>
4.3 PHOTODYNAMIC THERAPY APPLICATION OF SPIONs-GOLD CORE-SHELL MESO-TETRAKIS(4-HYDROXYPHENYL)PORPHYRIN (PC4) AGAINST MCF-7 BREAST CANCER CELLS.

4.3.1 Uptake and dark cytotoxicity

The conjugate (nano-drug) (PC 4, section 2.2.6.3) was well taken up by the cancer cells without significant toxicity in the dark after 24 h post-incubation period (Fig. 4.4a-b). The incubated cells that were exposed to external magnetic field showed enhanced nano-drug uptake than the non-exposed cell group (Fig. 4.4a). This was evident by the higher fluorescence intensity exhibited by these cells than the non-exposed cell group (Fig.4.4a). Contrary to some nano-porphyrin drugs associated with PEGylated SPIONs and gold (sources of fluorescence quenching (Huan et al., 2015; Xue et al., 2013), the nano-drug in this study showed excellent fluorescence imaging property (Fig. 3.5a-d, Fig. 4.4a).
Table 4.5. Magnetic targeting enhancement effect of the nano-drug

<table>
<thead>
<tr>
<th>Cell system</th>
<th>ATP Intensity</th>
<th>LDH membrane disintegration %</th>
<th>% Cell Viability</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell alone (control)</td>
<td>2.06E+06</td>
<td>1</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Cells + 0.5 µg/ml + 10 J/cm²</td>
<td>30119</td>
<td>24</td>
<td>61</td>
<td>***</td>
</tr>
<tr>
<td>Cells + 1 µg/ml + 10 J/cm²</td>
<td>37068</td>
<td>27</td>
<td>54</td>
<td>***</td>
</tr>
<tr>
<td>Cells + 2 µg/ml + 10 J/cm²</td>
<td>1522</td>
<td>32</td>
<td>33</td>
<td>***</td>
</tr>
<tr>
<td>Cells + 0.5 µg/ml + 10 J/cm² + magnet</td>
<td>15218</td>
<td>35</td>
<td>56</td>
<td>***</td>
</tr>
<tr>
<td>Cells + 1 µg/ml + 10 J/cm² + magnet</td>
<td>4864</td>
<td>41</td>
<td>43</td>
<td>***</td>
</tr>
<tr>
<td>Cells + 2 µg/ml + 10 J/cm² + magnet</td>
<td>5253</td>
<td>49</td>
<td>21</td>
<td>***</td>
</tr>
</tbody>
</table>

***P < 0.001.

This trend correlated with the changes in the morphology of the cells, in addition to the results obtained from the lactate dehydrogenase (LDH) and adenosine triphosphate assays (ATP) (Fig. 4.5a-i, Table 4.5). The 2 µg/mL conjugate dose induced 79% reduction in cell viability relative to the control (Table 4.5). Again, incubated cells containing nano-drug that were exposed to external magnetic field showed higher PDT effects than non-exposed cells (Figure 4a-i, Table 4.5). Similar improvement on PDT induced by external magnetic field has been reported for PEGylated-silica-methylene blue-SPIONs on HeLa cells (Zhao et al., 2014), PEGylated-chlorin (Ce6)-SPIONs on Murine breast 4T1 cancer cells (Li et al., 2013) and mitochondrion-targeting peptide-SPIONs on U8Viii malignant brain glioblastoma cells and MD-MB-231 metastatic breast cancer cells (Shah et al., 2014). A similar PDT cellular...
(69.71% and 81.70% ATP decrease relative to the control for non-magnetic and magnetic targeting PDT respectively) (Table 4.6). The relatively high % decrease in ATP values (>50 %) for all experimental groups (non-magnetic field exposed and magnetic field exposed) using 2 µg/mL conjugate concentration relative to the control, also indicated that necrosis might be responsible for the cell death induced by the conjugate in this study (Oleinick et al., 2002).

Table 4.6. Data for % decrease in cellular ATP

<table>
<thead>
<tr>
<th>Conjugate concentration (µg/mL)</th>
<th>% Decrease in cellular ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Cell alone)</td>
<td>0</td>
</tr>
<tr>
<td>0.5 (Non-magnetic PDT)</td>
<td>44.17</td>
</tr>
<tr>
<td>1.0 (Non-magnetic PDT)</td>
<td>50.00</td>
</tr>
<tr>
<td>2.0 (Non-magnetic PDT)</td>
<td>69.71</td>
</tr>
<tr>
<td>0.5 (Magnetic PDT)</td>
<td>50.00</td>
</tr>
<tr>
<td>1.0 (Magnetic PDT)</td>
<td>62.77</td>
</tr>
<tr>
<td>2.0 (Magnetic PDT)*</td>
<td>81.70</td>
</tr>
</tbody>
</table>
5.1. CONCLUSIONS

Several gluconic acid capped SPIONs, SPIONs-gold core-shell, porphyrin derivatives and SPIONs-gold porphyrin conjugates were synthesized using newly developed approaches and via modifications of some existing approaches. The SPIONs were synthesized by a new greener approach without post-synthesis treatments such as centrifugation or toxic organic solvent precipitation. The SPIONs which separated out under gravity from the mother liquor were capped with gluconic acid (biocompatible capping agent) and characterized using ultraviolet-visible spectrophotometry (UV-Vis), Fourier transform infrared spectroscopy (FT-IR), transmission electron microscopy (TEM), high resolution transmission electron microscopy (HRTEM), selected area electron diffraction (SAED), X-ray diffraction (XRD), X-ray energy dispersive spectroscopy (EDS), zeta potential (Z) and vibrating sample magnetometry (VSM). The gluconic acid functionalized SPIONs exhibited a black appearance with excellent UV-visible light absorptions. The TEM analysis revealed a highly monodispersed spherical-shape nanomaterials while the FT-IR analysis revealed characteristic peaks that confirmed the presence of carboxylic acid capped magnetite (Fe₃O₄) nanoparticles. The zeta potential analysis of the SPIONs revealed a highly positively charged surface (+24.94 mV) while the VSM study revealed that, the superparamagnetic characteristic (with absence of hysteresis loop) of the as-synthesized SPIONs
existed at 305 K with a blocking temperature of 46 K. Furthermore, the SPIONs were coated with gold at 74 °C for 7 h to obtain the core-shell nanostructure. The obtained SPIONs-gold core-shell was characterized using UV-Vis, transmission electronic microscopy (TEM) and photoluminescence techniques.

The meso-tetrakis(4-hydroxyphenyl)porphyrin was synthesized via propionic acid-catalyzed condensation reaction of the mixture of freshly distilled pyrrole and 4-hydroxybenzaldehyde, followed by silica-column purification with ethyl acetate: dichloromethane (1:2 v/v) elution solvent mixture. The pure porphyrin fractions were identified by ultraviolet-visible spectrophotometry, Fourier transform infrared spectroscopy (FT-IR), photoluminescence spectroscopy (PL), and proton nuclear magnetic resonance (1H NMR). Furthermore, the as-synthesized porphyrin was conjugated to the SPIONs-gold core-shell nanostructure using thiol-terminated methoxy-polyethylene glycol (mPEG-SH) as hydrophilic and encapsulating agent. The synthesized PEGylated superparamagnetic iron oxide nanoparticles-gold core-shell porphyrin conjugate evolved as magnetic brown rod-like structures which later developed to brown mass with time. The as-synthesized magnetic porphyrin conjugate was characterized using ultraviolet-visible spectrophotometry (UV-Vis), photoluminescence (PL), Fourier transform infrared spectroscopy (FT-IR), transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential (ζ). Moreover, the as-synthesized PEGylated SPIONs-gold core-shell porphyrin conjugate exhibited high solubility in water and strong attraction toward external magnetic field.

The singlet oxygen generation potential of the conjugate was evaluated *in vitro* using 1,3-diphenylisobenzofuran and methylene blue as singlet oxygen sensor and reference respectively. The result showed a singlet oxygen quantum yield of 0.2476,
a value greater than some values reported in the literature and sufficient for the eradication of breast cancer cells. The as-synthesized magnetic porphyrin conjugate was incubated with metastatic breast cancer cells (MCF-7) in the dark to probe its dark toxicity and localization characteristics. The results from the three biological assays employed, viz: Trypan blue, Adenosine triphosphate (ATP) and Lactate dehydrogenase (LDH) showed the conjugate was not toxic to breast cancer cells in the dark. The results from the fluorescence imaging analysis revealed that, the conjugate localized well within the cancer cells. The conjugate was detected within the cells via its self-indicative indigo emission at 358 nm excitation.

To probe the photodynamic efficacy of the as-synthesized magnetic porphyrin conjugate, the cancer cells incubated with the conjugate were further irradiated with light at 673 nm (dose = 10 J/cm²) for 14 min, 51s after 24 h post-incubation in the dark. The results from the three biological assays employed, viz: Trypan blue, Adenosine triphosphate (ATP) and Lactate dehydrogenase (LDH), showed that the conjugate was photo-toxic to cancer cells in dose-dependent manner, the highest effect being associated with 2 μg/mL conjugate concentration. In addition, higher photo-cytotoxicity was observed with cancer cells incubated with the conjugate that were exposed to external magnetic field (68 G) than non-exposed cells. This superiority in photo-toxicity exhibited by the exposed cell group was linked to the rapid concentration of the conjugate within the cells via magnetic response to the applied external magnetic field.

These results showed that the as-synthesized PEGylated superparamagnetic iron oxide nanoparticles-gold core-shell porphyrin conjugate possessed viable chemical, optical and electronic properties efficient for the photodynamic therapeutic
elimination of MCF-7 breast cancer cells and thus may be an optional potential for the treatment of MCF-7 breast cancer disease.

5.2. RECOMMENDATIONS

Following the photodynamic therapy success of the as-synthesized SPIONs-gold core-shell porphyrin conjugate against breast cancer cells, the following are therefore recommended.

- Evaluation of the photodynamic therapy efficacies of the conjugate on other cancer cell lines such as cervical, lungs, prostate, oral and brain in order to fully exploit the photodynamic efficacy horizon of the conjugate.
- Usage of this conjugate for antimicrobial photodynamic therapy to probe its efficacy against bacterial-induced infections such as those caused by gram-positive and gram-negative pathogenic bacteria species.
- Evaluation of the photodynamic therapeutic efficacy of this versatile conjugate in vivo. This is envisaged to pave way for its clinical trial evaluations and possible applications in humans.
- Evaluation of the photodynamic efficacies of derivatives of the porphyrin such as chlorins, bacteriochlorins, metallated forms (e.g. iron, nickel and platinum(II) meso-tetrakis(4-hydroxyphenyl)porphyrins) and the other newly synthesized conjugates against various cancer cell lines.
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