FABRICATION AND CHARACTERISATION OF METHYLENE BLUE- LOADED GRAPHENE OXIDE AND REDUCED GRAPHENE OXIDE FOR PHOTODYNAMIC THERAPY APPLICATION

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by

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LIST OF SYMBOLS

$^1O_2$	Singlet oxygen
$\Phi\Delta$	Quantum yield of singlet oxygen
Φf	Quantum yield of fluorescence emission

LIST OF ABBREVIATIONS

AA	Ascorbic acid
ABMDMA	9,10-anthracenediyl-bis(methylene) dimalonic acid
AuNCs	Gold nanocages
BBB	Blood brain barrier
Ce6	Chlorin e6
CO_2	Carbon dioxide
CuS	Copper sulfide
D_2O	Deuterium oxide
DCIS	Ductal carcinoma *in situ*
DHA	Dehydroascorbic acid
DL	Drug loading
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy Dispersive X-ray
EE	Encapsulation efficiency
EPR	Enhanched permeability and retention effect
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FRET	Förster resonance energy transfer
FT-IR	Fourier Transform Infrared Spectroscopy
GI	Gastrointestinal
GO	Graphene oxide
GQDs	Graphene quantum dots
HA	Hyaluronic acid
HCl	Hydrochloric acid
HER-2	Human Epidermal Growth Factor Receptor 2

HNO_3	Fuming nitric acid
HPPH	2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-alpha
IBC	Inflammatory breast cancer
ICG	IndoCyanine Green
IDC	Invasive ductal carcinoma
ILC	Invasive lobular cancer
$KClO_3$	Potassium chlorate
$KMnO_4$	Potassium permanganate
MB	Methylene blue
miRNA	microRNA
MPS	Mononuclear phagocyte system
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NIR	Near-infrared
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PET	Positron emission tomography
PI3K	Phosphatidylinositol-3 kinase
PNA	Peptide nucleic acid
PPa	Pyropheophorbide-a
PTT	Photothermal therapy
PVP	poly(*N*-vinyl-2-pyrrolidone)
rGO	Reduced graphene oxide
ROS	Reactive oxygen species
SD	Standard deviation
SE	Spinach extract
SEM	Scanning Electron Microscope
SERM	Selective oestrogen receptor modulator
SI	Selectivity index

SOG	Singlet oxygen generation
TDDS	Targeted drug delivery system

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PEMBUATAN DAN PENCIRIAN GRAFENA OKSIDA DAN GRAFENA OKSIDA TERTURUN BERMUATAN BIRU METILEN UNTUK APLIKASI TERAPI FOTODINAMIK

ABSTRAK

Kanser payudara merupakan kanser yang biasa dijumpai dalam golongan wanita. Pilihan rawatan adalah penting untuk mengurangkan morbiditi dan kadar kematian. Terapi fotodinamik ialah salah satu teknik yang diperkenalkan untuk merawat penyakit seperti kanser dengan penggunaan molekul diaktifkan cahaya yang dikenali sebagai fotosensitizers untuk menghasilkan spesies oksigen reaktif dan seterusnya menyebabkan penghapusan sel kanser. Oleh sebab terapi fotodinamik ialah rawatan tersasar dan tidak invasif, ia memberi manfaat seperti mengurangkan kesan sampingan serta meningkatkan kecekapan rawatan. Bahan berasaskan grafena semakin mendapat perhatian dalam bidang farmaseutikal disebabkan sifat fizikokimia yang menarik seperti luas permukaan yang tinggi serta mudah untuk dimodifikasi untuk pemuatan molekul aktif bagi tujuan penghantaran ubat tersasar. Fotosensitizer sendiri tidak mampu manyasarkan kanser payudara secara khusus tanpa menyebabkan kesan toksik yang ketara terhadap sel yang sihat. Oleh itu, ejen yang sesuai untuk memuatkan fotosensitizer dan mampu mengawal pelepasan fotosensitizer terhadap kanser payudara perlu diteliti dan dikaji. Kajian ini meneroka keberkesanan grafena oksida (GO) dan grafena oksida terturun (rGO) untuk memuatkan biru metilen (MB) dan juga ketoksikan sel secara ujian *in vitro* untuk GO-MB dan rGO-MB terhadap sel kanser payudara. GO telah dihasilkan melalui kaedah Hummers' yang diperbaik manakala rGO pula dihasilkan menggunakan kaedah penurunan kimia. Sifat fizikokimia kompleks GO dan rGO telah dicirikan dari segi saiz, caj permukaan,

analisis inframerah, sifat termal dan pelepasan dalam larutan penampan pH7.4 dan pH4.5. GO, GO-MB, rGO dan rGO-MB telah berjaya dihasilkan dengan saiz 1.52±0.10, 3.10±0.23, 2.37±0.38 dan 5.91±0.5 µm serta caj permukaan -34.1±1.61, -29.7±0.77, -29.6±2.02 dan -22.5±0.99. Analisis inframerah menunjukkan bahawa GO telah dioksidakan daripada grafit manakala rGO telah diturunkan daripada GO. GO-MB dan rGO-MB telah berjaya dihasilkan berdasarkan peralihan dalam jalur serapan inframerah dan ini menunjukkan bahawa π-π interaksi berlaku antara GO/ rGO dengan MB. Pelepasan *in vitro* menunjukkan bahawa MB telah dilepaskan sepenuhnya daripada GO-MB dan rGO-MB dalam 24 jam dan 20 minit pada pH4.5. Kajian sel secara *in vitro* menunjukkan bahawa GO ialah pilihan yang lebih baik untuk memuatkan MB berbanding rGO untuk menyasarkan kanser payudara dengan ketoksikan sel yang lebih rendah terhadap sel fibroblas. Kesimpulannya, kajian ini telah menunjukkan potensi GO-MB dan rGO-MB dengan ciri yang baik dan kajian sel telah menunjukkan keselamatan GO sebagai pembawa molekul aktif dengan kesan toksik yang diperlukan terhadap kanser payudara.

FABRICATION AND CHARACTERISATION OF METHYLENE BLUE-LOADED GRAPHENE OXIDE AND REDUCED GRAPHENE OXIDE FOR PHOTODYNAMIC THERAPY APPLICATION

ABSTRACT

Breast cancer is a common type of cancer diagnosed in women. The choice of treatment is important to reduce the morbidity and mortality rate. Photodynamic therapy (PDT) is one of the techniques introduced to treat diseases such as cancers by employing light-activated molecules known as photosensitisers to produce reactive oxygen species and subsequently inducing cancer cells apoptosis. As PDT is a non-invasive and targeted treatment, it provides advantages including lowering the potential side effects while improving the treatment efficiency. Graphene-based materials are getting more attention in pharmaceutical applications due to its attractive physicochemical properties including large surface area and easy modification for drug loading and targeting purposes. As the photosensitiser alone was unable to specifically target the breast cancer without causing cytotoxicity towards the normal cells, a suitable drug carrier to be able to control the release of the drug towards the breast cancer should be investigated. This study explores the effectiveness between graphene oxide (GO) and reduced graphene oxide (rGO) in the loading of methylene blue (MB) as well as the *in vitro* cytotoxic effect of GO-MB and rGO-MB on breast cancer cells. In this study, GO and rGO were synthesised through improved Hummers' method and chemical reduction method respectively. The physicochemical properties of the GO and rGO-based complexes were characterised in term of particle size, surface charge, infrared analysis, thermal properties and drug release in buffer pH7.4 and pH4.5. GO, GO-MB, rGO and rGO-MB were successfully produced with a particle size of

1.52±0.10, 3.10±0.23, 2.37±0.38 and 5.91±0.5 µm and a surface charge of -34.1±1.61, -29.7±0.77, -29.6±2.02 and -22.5±0.99 respectively. Infrared analysis showed that GO was successfully oxidised from graphite and rGO was successfully reduced from GO. GO-MB and rGO-MB were successfully formed with a shift in the absorption bands, suggesting the possible π-π interaction occurred between GO/ rGO with MB. *In vitro* drug release for GO-MB and rGO-MB showed that the encapsulated MB was completely released within 24 hours and 20 minutes respectively at pH4.5. Moreover, *in vitro* cell study suggested that GO was more favourable in the delivery of MB compared to rGO in targeting breast cancer cells with a lower cytotoxicity in human fibroblast cells. This study has managed to show the potential of MB-loading on GO and rGO with good characteristics and the *in vitro* cell study indicates the safety of the GO carrier whilst at the same time showed cytotoxicity in breast cancer cells.

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Cancer is a leading cause of death worldwide and the burden of cancer incidence as well as the mortality rate has increased rapidly (Sung et al., 2021). Breast cancer is one of the chronic and non-communicable diseases found especially among female. It is the most common type of cancer found in women and the second most common cancer overall in 2018 (*Breast Cancer Statistics*, 2022). However, in 2020, breast cancer became the most commonly diagnosed cancer worldwide, surpassing the number of lung cancer diagnosed for the first time. According to the Global Cancer Statistics 2020, an estimated of 2,261,419 (11.7%) new cases of female breast cancer was reported worldwide and the mortality rate was 684,996 (6.9%) (Figure 1.1) The chances for female in getting breast cancer is higher as compared to male (Sung et al., 2021).

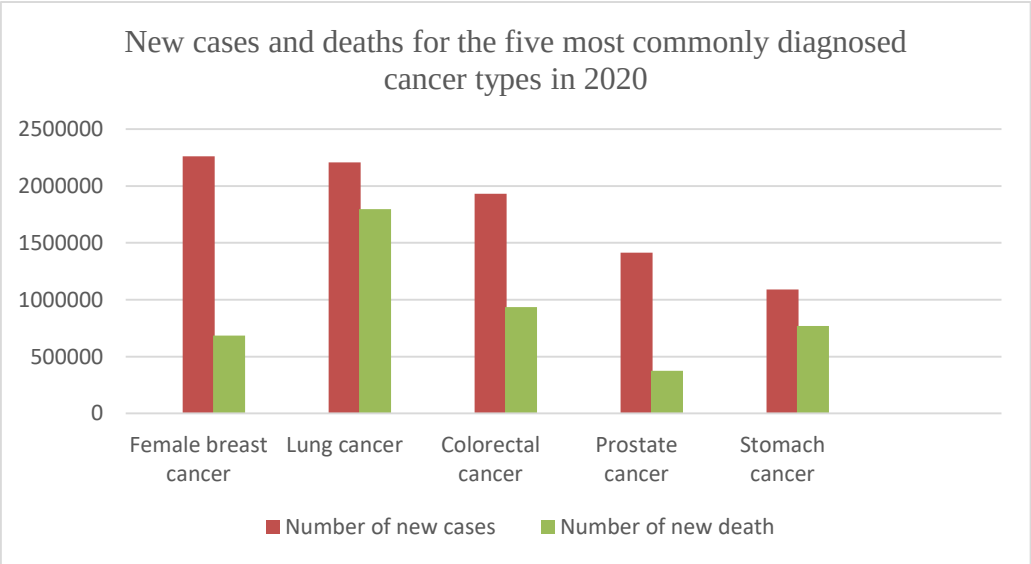

Figure 1.1 Number of new cases and deaths for the five most common diagnosed cancer worldwide in 2020 for both sexes, all ages (Sung et al., 2021)

In Malaysia, breast cancer is the most common form of cancer found among females and around 1 in 20 females are at risk. According to Globocan 2020 and National Cancer Registry of Malaysia, there were 8,418 new cases of breast cancer diagnosed in 2020 which comprised of 17.3% from the overall number of new cancer cases reported in the country (Figure 1.2). It was ranked first for the most frequent cancer diagnosed among female (Sung et al., 2021). The number of deaths recorded was 3,503 cases, which was 11.9% from the overall number of deaths due to cancer in both sexes and all ages (Figure 1.3). The common causes of breast cancer include genetic mutation (*BRCA* and HER-2), environmental factors such as radiation exposure and lifestyle due to alcohol consumption and obesity (Rock et al., 2020).

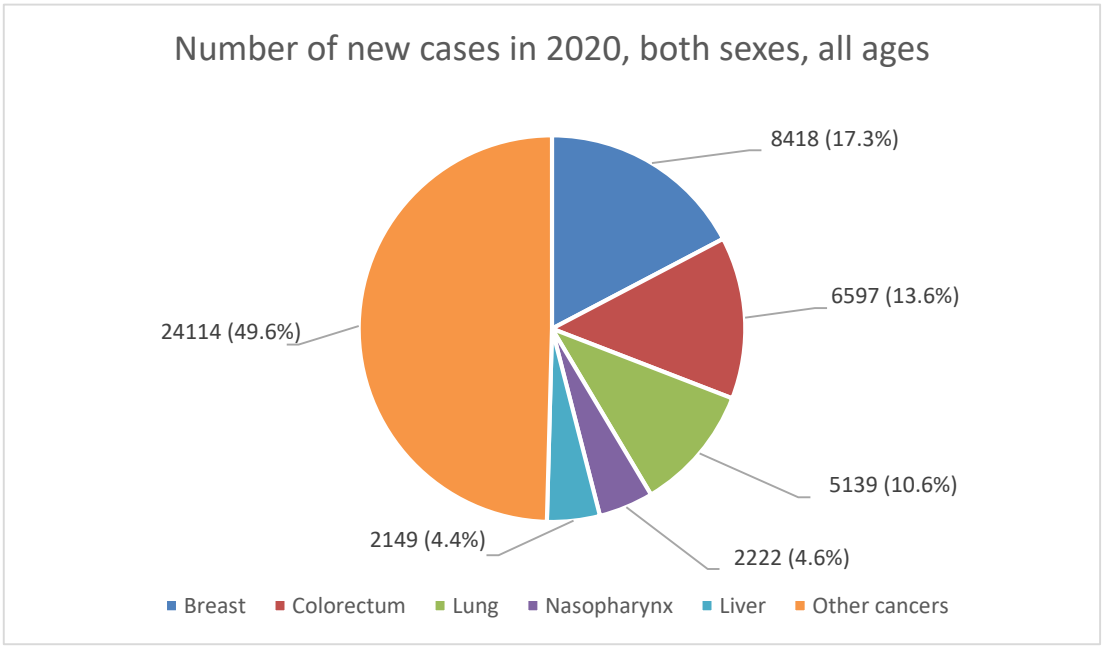

Figure 1.2 Number of new cases found in Malaysia for both sexes and all ages (Sung et al., 2021)

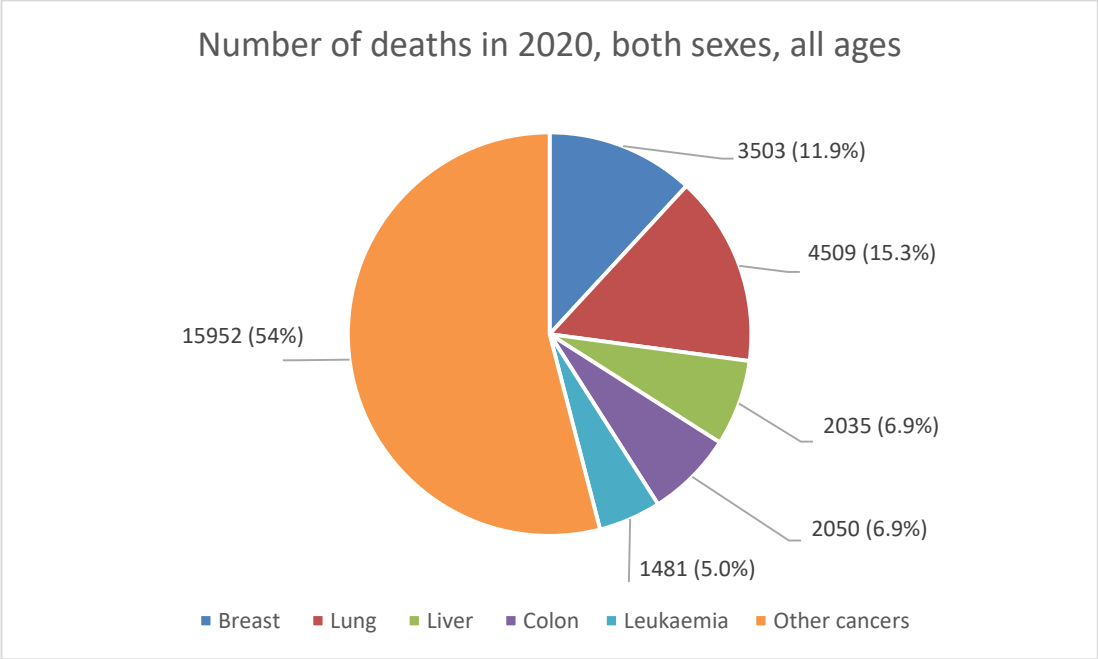

Figure 1.3 Number of deaths for cancer found in Malaysia 2020 for both sexes, all ages (Sung et al., 2021)

There are several types of breast cancer including ductal carcinoma *in situ* (DCIS), invasive ductal carcinoma (IDC), invasive lobular cancer (ILC), triple negative breast cancer, inflammatory breast cancer (IBC) and metastatic breast cancer. According to WHO, the percentage of breast cancer that arises from the epithelium of the ducts is 85% while from lobules in the glandular tissue of the breast consists of 15% (WHO, 2021). These cancer cells would invade to the surrounding breast tissue and may further spread through lymphatic vessels or blood vessels to the lymph nodes, bones, lungs, brain and liver (Chye et al., 2008).

The common treatment for breast cancer includes chemotherapy, radiation therapy, surgical removal and medication such as targeted drug therapy and hormonal therapy. However, these treatments may eventually lead to unwanted side effects such as nausea and cellular toxicity after chemotherapy while post-operative complications, fatigue, pain and swollen in breast area may occur following radiation therapy.

The low specificity of chemotherapeutic agents has long been a problem in anticancer therapy, which has led to side effects such as hair loss and reduction in immunity level. To improve the treatment specificity, techniques including PDT are being explored for cancer treatment. PDT that involves three main components – photosensitiser molecules, light at specific wavelengths and oxygen molecules, has the potential to reduce chemotherapy-induced side effects as the activation of the photosensitiser is done only in the presence of the light. PDT is a non-invasive treatment for cancer cells by employing external light at specific wavelength to photo-activate the photosensitiser to produce singlet oxygen and reactive oxygen species (ROS) which are considered cytotoxic. This would eventually lead to the apoptosis of cancer cells.

1.2 Graphene

Graphene is a type of carbon-based material, made up of single layer of sp^2 carbon atoms linked by covalent bonds to form a honeycomb structure. The concept of graphene was first proposed by Wallace in 1947 and the electronic properties of graphene was studied using tight-binding model (Wallace, 1947). However, the study of graphene remains at the level of theory for several decades (Song et al., 2014). In 2004, Konstantin Novoselov and Andre Geim proposed the separation of single graphene layer from graphite *via* simple mechanical peeling method, following which the interest on graphene for various science and technology applications increases (Novoselov et al., 2004).

Graphene has found specific applications in different areas such as in engineering for surface coating and strain sensor technology (Nassef et al., 2020). Due to its unique properties, graphene was also being introduced for biomedical and

pharmaceutical applications such as in the delivery of therapeutics against cancer (Smith et al., 2019; Geetha Bai et al., 2017; Sharma et al., 2017). The unmodified graphene consists of free surface π electrons which allows the formation of π-π interactions for drug loading and covalent modifications (Priyadarsini et al., 2018). The structure of graphene is shown in Figure 1.4.

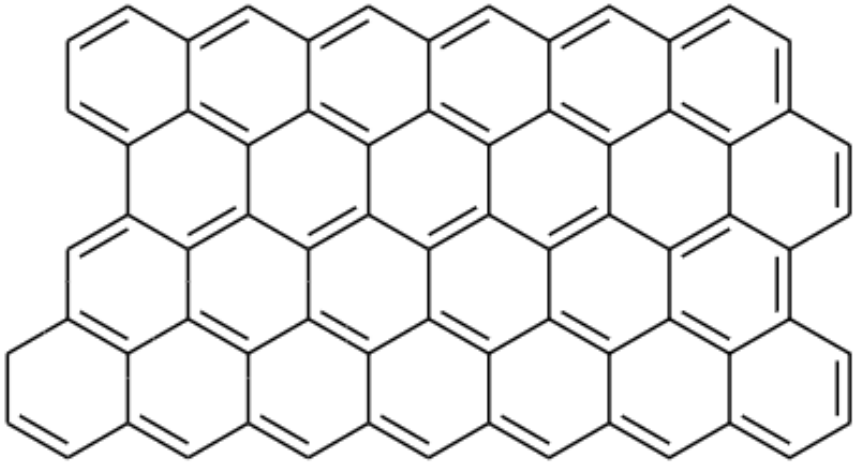

Figure 1.4 Proposed molecular structure of graphene (Bai et al., 2019)

In 2008, graphene ability to load hydrophobic drug (SN38) *via* van der Waals interaction was firstly reported (Liu et al., 2008). From that time onwards, the research on graphene-based materials for pharmaceutical and biomedical areas have advanced, including in drug delivery applications. Unfortunately, the insoluble properties of graphene limits the use in biomedical applications such as in cell study and hence, the development of GO and rGO has opened up the possibility to exploit graphene-based materials for drug and gene delivery, especially since they are more hydrophilic in nature as compared to graphene (Hoseini-Ghahfarokhi et al., 2020).

In general, there are two ways for graphene synthesis, either the bottom-up or top-down approach. The bottom-up method usually starts from simple carbon molecule such as ethanol while for the top-down method, graphene is produced from graphite as the starting material. Both methods can produce graphene with good

quality. However, the top-down method *via* chemical oxidation and reduction is preferable for graphene production as it is more cost effective and able to give a high yield of product (Chua & Pumera, 2013).

Graphene derivatives such as GO and rGO are being highly focused on for the drug delivery application, which can be seen from a number of publications describing the preparation of GO and rGO-based carriers for the delivery of anticancer drugs such as doxorubicin (DOX) and cisplatin (Zhou et al., 2014; Pei et al., 2020). Another potential application of GO-based material is as a carrier for antibacterial and antimicrobial agents (Oliveira et al., 2022; Pan et al., 2019; Hoseini-Ghahfarokhi et al., 2020).

1.3 Graphene oxide (GO) and reduced graphene oxide (rGO)

GO was first introduced by Schafhaeul in 1840 followed by Brodie in 1859 (Sharma et al., 2017). It is a highly oxidised form of graphene with sp^2 carbon sheet consisting oxygenated functional groups on its basal surface with single atom thick sheet and lateral dimensions from a range within a several nm to μm (Tiliakos et al., 2015). The oxygen containing epoxy groups help GO to disperse in water, making it hydrophilic (Sharma et al., 2017; Thakur & Karak, 2015). Due to its hydrophilicity, the availability of large surface area and ease of surface functionalisation as well as good cytocompatibility, GO has attracted interest for its use in anticancer therapy (Hosseinzadeh et al., 2017; Yaghoubi et al., 2022).

The loading of drugs on GO can be accomplished *via* electrostatic interaction, hydrogen bonding, π-π stacking, and others. The modification of its surface allows specific cell targeting or controlled delivery and this would eventually improve therapeutic efficacy on the targeted diseased cells, example being in cancer therapy

(Y. Li et al., 2015). GO drug carrier also helps to improve drug bioavailability and solubility (Oliveira et al., 2022; Maulvi et al., 2021; Laurenti et al., 2019).

A study by Yaghoubi et al. (2022) mentioned that GO allowed the transport of hydrophobic drug such as curcumin which has low absorption and solubility as well as fast systemic elimination in the body towards cancerous cells. The cell proliferation in human multiple myeloma cells were inhibited significantly by GO loaded with DOX as compared to DOX alone (Yaghoubi et al., 2022; S. Wu et al., 2014). Another study reported that co-loading of multiple drugs such as curcumin and paclitaxel onto polymer-functionalised rGO is effective in the elimination of breast cancer cells (MDA-MB-231) and lung cancer cells (A549 cells) (Yaghoubi et al., 2022; Muthoosamy et al., 2016).

rGO on the other hand differs from GO as it consists of less oxygen-containing functional groups. Its lattice structures are re-established from GO but with a reduced oxygen content and increased hydrophobicity (Smith et al., 2019; Sharma et al., 2017). According to the previous study, the application of rGO as a drug carrier was challenging due to the strong hydrophobicity of rGO which tend to agglomerate irreversibly and they might restack into graphite through van der Waals interactions in the absence of stabiliser, causing rGO to be difficult for further processing (Wei et al., 2012).

The 2D planar structure of graphene allows a high loading of drug not just hydrophilic but also hydrophobic drug (Zainal-Abidin et al., 2020). The choice of the drug to be loaded depends on several factors such as the polarity of the drug and the presence of functional groups which allow the conjugation of the drugs with the reactive oxygen groups on the drug carrier (Oliveira et al., 2022). However, a study by Wei et al. (2012) revealed that the adsorption of water-insoluble drug (hydrophobic)

onto the planar GO (hydrophilic) depends mainly on weak hydrophobic interactions, thus resulting in a lower drug-loading ratio. In other words, the results reported could also be used to explain the binding of hydrophilic drug onto hydrophobic rGO *via* weak hydrophobic interactions, which may also cause a lower drug-loading ratio. The study also proposed that the drug loading for hydrophobic drug onto rGO was relatively higher as compared to GO through π-π stacking interactions (Wei et al., 2012). In general, this property means that rGO may be more suitable for the loading of hydrophobic drugs.

For both GO and rGO, they are useful in neurological applications to treat Alzheimer's and Parkinson's diseases as they are able to pass through blood brain barrier (BBB) and reach the brain (Oliveira et al., 2022; M. Li et al., 2012; Xiong et al., 2021). Study reported by Xiong et al. (2021) revealed that the lactoferrin functionalised GO has the potential as a carrier for puerarin to treat Parkinson's disease as it can binds with the vascular endothelial receptor in the BBB and thus allowing the transport of this drug across the barrier *via* a receptor-mediated transport. In addition, according to Mendonça et al. (2015), rGO is able to pass through BBB and it is also useful in delivering drugs to treat brain disorders that are usually unresponsive to conventional treatment due to BBB impermeability.

The loading of photosensitisers on GO and rGO has been reported in the literature. The complex produced showed pH responsive property in which the release of loaded photosensitisers can be controlled under a certain range of pH conditions (Hosseinzadeh et al., 2017). In tumour cells whereby the pH may be acidic, the release of photosensitisers was found to be higher as compared to neutral condition. This suggested the potential of such complex to be used in tumour site-specific targeted drug delivery application.

1.4 Types of photosensitisers for photodynamic therapy (PDT)

PDT is a type of non-invasive treatment for cancer cells by employing external light at specific wavelength to photo-activate the photosensitiser and subsequently producing singlet oxygen ($^1O_2$) and ROS which are cytotoxic. These ROS would eventually lead to the apoptosis of cancer cells. PDT was accidentally discovered by Oskar Raab and Hermann von Tappiener in year 1900 when they observed that acridine orange-stained *Paramecium spp*. protozoas was killed after light exposure (Moan & Peng, 2003; St. Denis et al., 2011; Abrahamse & Hamblin, 2016).

In 1841, the first photosensitiser was introduced when haematoporphyrin was extracted following the removal of iron from dried blood (Kou et al., 2017). Photosensitisers usually tend to be deeply coloured due to the extensive electron delocalisation. Because of this factor, only a low energy is required to excite the electrons and therefore the absorption bands are in the longer wavelength region, considering the high possibility of excitation (St. Denis et al., 2011).

There are several types of photosensitisers available for PDT and they can be classified into different generations. The first generation of photosensitisers are known as haematoporphyrin derivative which is a water soluble mixture of porphyrin. Photofrin is the purified form of the haematoporphyrin derivative, which contains porfimer sodium as its ingredient (Abrahamse & Hamblin, 2016). It has been used in the treatment of early stage oral cavity and laryngeal malignancies (Schweitzer, 2001). However, there are some limitations for photofrin such as low light absorption at 630 nm and complex composition. The low light absorption at 630 nm means that the amount of light penetrating through the skin into deep-seated tumour cells is limited as most of the light is blocked on the skin surface, which would subsequently cause

photosensitive toxicity to the skin (Kou et al., 2017; Abrahamse & Hamblin, 2016). Therefore, second generation photosensitisers were introduced for PDT application.

The second generations photosensitisers can be grouped based on the tetrapyrrole structure which consists of porphyrin, chlorin, bacteriochlorin and phthalocyanine (Abrahamse & Hamblin, 2016). Most of the structure for the second generation are based on porphyrin such as benzoporphyrins, protoporphyrin IX (PpIX), purpurins and others. The structure is simpler as compared to the first generation photosensitisers and the photosensitivity as well as tissue selectivity have been improved. Most of the photosensitisers employed for anti-cancer treatment consist of tetrapyrrole backbone which is similar to protoporphyrin in haemoglobin (Abrahamse & Hamblin, 2016). PpIX is a precursor of heme and it showed a longer wavelength absorption in erythroleukemia cells (Kou et al., 2017). 5-aminolevulinic acid is another commonly used photosensitiser which is a biological precursor of PpIX that can be applied topically or orally (Abrahamse & Hamblin, 2016). There are some other photosensitisers based on chlorin structure such as temoporfin and chlorin e6 (Ce6), based on bacteriochlorin such as BC19 and based on phthalocyanine such as silicon phthalocyanine (Kou et al., 2017; Agostinis et al., 2011; Abrahamse & Hamblin, 2016).

Synthetic dyes such as phenothiazinium and squaraine as well as natural products including hypericin and curcumin were also employed as photosensitisers for PDT. As for MB, it is a type of synthetic dye which is categorised under phenothiazinium salt (Abrahamse & Hamblin, 2016; Wainwright & Crossley, 2002). MB is also known as methylthioninium chloride (*PubChem,* 2019). It is a type of hydrophilic dye which is used as photosensitiser with light absorption at 660 nm. When MB is exposed to light excitation, it reacts with oxygen to produce $^1O_2$ and ROS. The

selective uptake of MB by cancer cells would eventually induce apoptosis and causing cell death. Table 1.4 summarised several examples of photosensitisers for PDT application.

Table 1.4 Examples of second generation photosensitisers for PDT application (Abrahamse & Hamblin, 2016)

Class	Photosensitiser	Absorption peak	References
Porphyrin	ALA-induced protoporphyrin IX (PpIX)	635 nm	Dirschka et al. (2012)
Chlorin	Ce6	660 nm	Biswas et al. (2014)
Bacteriochlorin	BC19	732 nm	Huang et al. (2010)
Phthalocyanine	Silicon phthalocyanine (PC4)	675 nm	Anderson et al. (1997)
Phenothiazinium salt	Methylene Blue	660 nm	Wainwright & Crossley (2002)
Perylenequinone	Hypericin	570 nm	Theodossiou et al. (2009)

Moreover, to improve the precise targeting of photosensitisers, control localisation and minimise phototoxicity effect on normal tissue especially skin, chemical modification of photosensitiser with various targeting vehicles has been conducted that led to the development of third generation photosensitisers (Kou et al., 2017; Nishiyama et al., 2009). PDT can specifically target cancer cells by conjugating photosensitisers with antibodies, peptides and ligands with specific cellular receptors. Table 1.5 showed several examples of photosensitisers and the targeting ligands that have been reported in the literature for targeted PDT application.

Table 1.5 Examples of photosensitiser-targeting ligand conjugates for PDT application (Abrahamse & Hamblin, 2016)

Class	Ligand	Photosensitiser	Target	References
Monoclonal antibody	OC125	Ce6	Ovarian cancer	Goff et al. (1994)
Peptide	Octreotide	Ce6	Somatostatin receptor	Kaščáková et al. (2014)
Serum protein	Transferrin	Haematoporphyrin	Transferrin receptor	Hamblin & Newman (1994)
Steroid	Oestradiol	Pheophorbide-a	Steroid receptor	El-Akra et al. (2006)

An ideal photosensitiser should has low level of dark toxicity to both humans and experimental animals, low incidence of administrative toxicity such as hypotension or allergic reaction and an absorbance peak between 650 and 800 nm (deep red spectral region) as the level of penetration of light into tissue is high in this wavelength range (Castano et al., 2004; Abrahamse & Hamblin, 2016). The absorption of photons with wavelengths longer than 800 nm was unable to provide enough energy to excite oxygen to its singlet state and does not have the capacity to form adequate yield of ROS upon irradiation (Agostinis et al., 2011; Juzeniene et al., 2006). Ce6 which has a strong near-infrared (NIR) light absorption at 650-800 nm has been shown to be able to enhance the ROS production at a longer wavelength (Kou et al., 2017; Lee et al., 2013).

1.5 Photodynamic therapy (PDT) mechanism

The photo-activation of a photosensitiser can be divided into two mechanisms, during which the excited photosensitiser would produce both singlet and triplet species. During the excited singlet state, photosensitiser is relatively unstable and would tend to release energy either *via* emission of light or heat generating. As for the triplet state, it is less energetic and considered as more stable form than singlet state with a longer lifetime (St. Denis et al., 2011; Abrahamse & Hamblin, 2016). The excess triplet

energy can be either transferred through electrons (Type I mechanism) or energy (Type II mechanism) (Tardivo et al., 2005; Ochsner, 1997). For electron transfer, photosensitiser would directly transfer an electron or donate proton to oxygen, producing superoxide anion ($O_2^{\cdot -}$) followed by the formation of other ROS such as hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) which are considered harmful towards nucleic acids, enzymes and cellular membranes. In Type II mechanism, the energy used to excite the photosensitiser to the triplet state is transferred to oxygen to form $^1O_2$. This form of oxygen is considered reactive, unstable and short-lived and this would eventually lead to strand-breaking in nucleic acid, causing DNA damage (Ochsner, 1997; St. Denis et al., 2011; *Iarc Monographs*, 2018). Type II mechanism is simpler as compared to Type I mechanism in generating ROS and most photosensitisers applied for anti-cancer PDT are believed to generate ROS *via* Type II rather than Type I mechanism (Abrahamse & Hamblin, 2016). The overall processes were summarised in Figure 1.5.

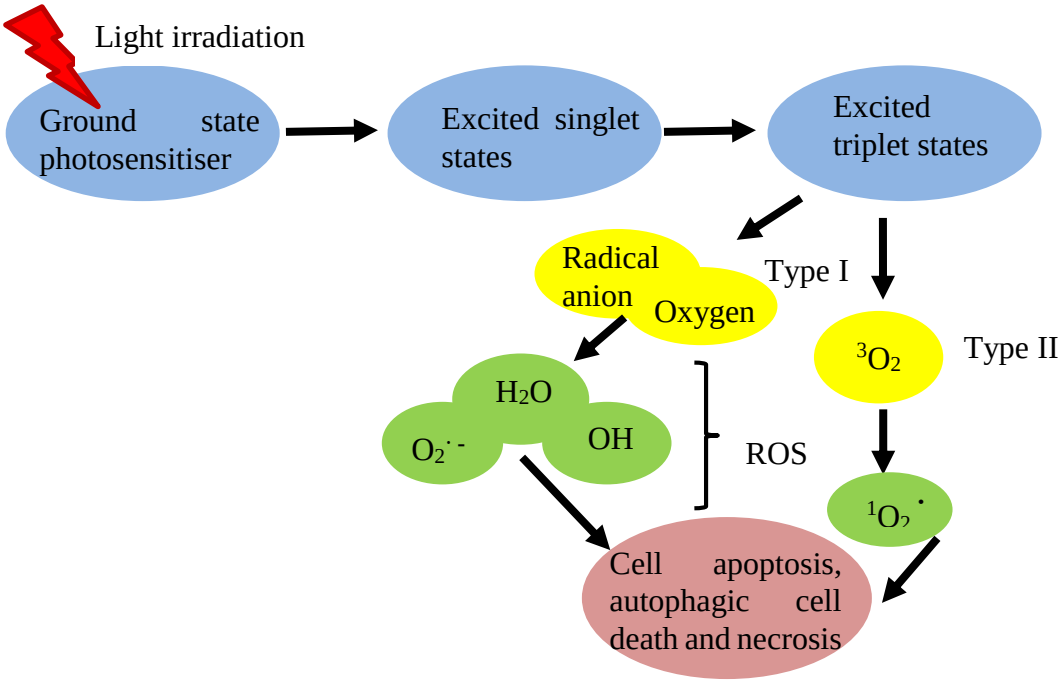

Figure 1.5 Schematic illustration of photodynamic therapy involving the light activation of photosensitiser to generate ROS and singlet oxygen *via* Type I and Type II mechanisms (Kou et al., 2017; St. Denis et al., 2011)

1.6 Problem Statement

There are several ways of treatment available to cure breast cancer. The most common treatment applied is chemotherapy. However, the untargeted nature of chemotherapy may cause unwanted side effects, leading to the death of normal cells. According to the literature, the conventional methods such as radiotherapy and surgical removal might induce postoperative complications as well as prone to recurrence while chemotherapy would cause serious toxicity and side effect such as nausea (Ma et al., 2020). It is challenging to eradicate cancerous cells specifically by just using the anticancer drugs alone, therefore in order to induce cellular toxicity in a more efficient way, targeted treatment such as PDT may be a potentially useful approach to minimise the off-target delivery of anticancer drugs.

Graphene-based drug delivery has recently been studied extensively due to its biocompatibility and large surface area which allows the loading of drug in a more efficient way. Anticancer agent loaded on GO and rGO has been reported to be useful in improving the elimination of cancerous cells. The ability of GO and rGO to control release the anticancer drug under acidic pH condition makes them favourable as a drug carrier since the tumour environment is more acidic as compared to the normal tissue. It is important to evaluate the dark cytotoxicity of MB loaded GO and rGO before proceed to PDT in order to confirm the effectiveness in targeting breast cancer cells without inducing much toxicity towards the healthy cells.

1.7 Research Objectives

The aim of this study is to develop a drug delivery system employing GO and rGO as the drug carrier against breast cancer cells (MCF-7). The research objectives are stated as shown below:

1. To synthesise GO and rGO microparticles *via* improved Hummers' method and chemical reduction method respectively followed by characterisation through Fourier transform infrared spectroscopy (FT-IR), Scanning electron microscope (SEM), Energy dispersive X-ray (EDX), Differential scanning calorimetry (DSC), particle size and surface charge
2. To load MB onto GO and rGO and to evaluate the physicochemical characteristics of the complexes produced
3. To investigate the *in vitro* release profiles of GO-MB and rGO-MB under pH 7.4 and pH 4.5
4. To evaluate the dark cytotoxicity of GO-MB and rGO-MB in human fibroblast cells (Hs27) and human breast cancer cells (MCF-7) through MTT assay for further PDT application
5. To investigate the differences in efficacy between the two types of microparticles, whether the rGO microparticles have any advantages over the GO microparticles in terms of drug delivery and cancer cells elimination

1.8 Scope of Study

The main purpose of this study is to synthesise GO and rGO followed by the loading of MB. The physicochemical characteristics of the complexes would be characterised. The release profiles and cytotoxicity towards human fibroblast cell lines (Hs27) and human breast cancer cells (MCF-7) would also be determined. GO is synthesised through improved Hummers' method by employing strong acid and $KMnO_4$ as the oxidising agent. The rGO is produced through chemical reduction method by using ascorbic acid (AA) as the reducing agent.

GO-MB and rGO-MB would be characterised by UV-Vis spectroscopy, FT-IR, SEM, EDX, DSC, particle size, surface charge and product yield. A standard curve of MB at an absorbance of 663 nm was plotted to determine the drug encapsulation efficiency, drug loading percentage and cumulative release of MB from drug carrier for the drug release assay. The comparison between the effectiveness of GO and rGO in carrying MB to target breast cancer was investigated.

As for the *in vitro* release study, the main objective is to investigate the effect of physiological and acidic pH towards the rate of drug release from the loaded GO-MB and rGO-MB. The dark cytotoxicity of GO, rGO, MB, GO-MB and rGO-MB would be evaluated on Hs27 and MCF-7 cell lines. The IC_{50} values and statistical analysis between the samples would be analysed through GraphPad Prism 8 software to indicate the significance of the results obtained.

1.9 Thesis Organisation

This thesis comprised of five chapters including Introduction, Literature Review, Materials and Methods, Results and Discussion as well as Conclusion and Future Recommendations. Chapter 1 briefly introduces the background and statistics for breast cancer and also the current treatment and issues in treating breast cancer. The properties of GO, rGO and MB are also briefly described as well as the mechanism and application of PDT in anticancer therapy.

Chapter 2 consists of literature review regarding the background of breast cancer, the causes and current available treatment. The structure and properties of GO, rGO and MB as well as the common application of PDT are also included in this chapter.

Chapter 3 describes the materials and methods used throughout this study. This includes the synthesis method for GO and rGO, the analysis and characterisations conducted, the drug release assay and the cytotoxic study.

Chapter 4 reveals the results obtained from the research conducted. Each result was analysed, explained and discussed in comparison to previously reported studies. The percent yield, drug loading percentages and encapsulation efficiency percentages were calculated. The release of MB from GO and rGO as well as cytotoxicity of the GO-MB and rGO-MB complexes are also described.

The final chapter, Chapter 5 summarises the results and concludes the study. Future recommendations are also described in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

2.1.1 Breast cancer

Breast cancer is one of the non-communicable diseases which is more commonly diagnosed in women. Most breast cancer cells are carcinomas which means that the tumour begins in the epithelial cells of the tissues. These carcinomas are known as adenocarcinoma when they start to form in ducts or lobules (milk glands) (American Cancer Society, 2021c).

According to the Global Cancer Statistics 2020, there were 11.7% of new cases for female breast cancer found worldwide and the mortality rate was 6.9%. More than 2.3 million women were diagnosed with breast cancer worldwide and around 685,000 died from breast cancer. Breast cancer is the most commonly diagnosed cancer followed by lung cancer and it is the second leading cause of cancer death among women (Sung et al., 2021).

The common causes of breast cancer include genetic mutation (*BRCA* gene and HER-2 gene), environmental factors such as radiation exposure and lifestyle due to alcohol consumption, smoking and obesity (American Cancer Society, 2019). The chances of getting breast cancer is also dependent on gender and age as it is mostly found among women and usually would develop at the age above 40. Only around 0.5-1% of breast cancer cases are found in men (WHO, 2021).

Breast cancer can be divided into *in situ* or invasive type. *In situ* breast cancer refers to cancer cells which are non-invasive and usually is detected as early-stage breast

cancer. For invasive breast cancer, it is usually known to spread into the surrounding breast tissues (American Cancer Society, 2021c). There are several types of breast cancer including DCIS or IDC, ILC, triple negative breast cancer, IBC and metastatic breast cancer (American Cancer Society, 2019).

DCIS is a non-invasive cancer found at the cell lining of the duct and is usually in the early stage that is treatable while for IDC, it is an invasive cancer found in the ductal area which comprises about 70-80% of all breast cancer cases. ILC begins in the lobules of the breast, and it can infiltrate through the walls of glands where they are originated and continue to grow in the surrounding tissue (American Cancer Society, 2019). However, ILC is not easily be detected as compared to other types of breast cancers. For triple negative breast cancer, it occurs in around 10-20% of diagnosed breast cancers. Due to the lack of three types of receptors on cancer cells known as oestrogen, progesterone and the HER-2/neu gene, this type of cancer cannot be treated with hormone-based therapy and is considered difficult to treat as compared to other types of breast cancer.

IBC is an aggressive type of breast cancer which comprises of about 1-5% of diagnosed breast cancer cases. It is usually refers to stage 3 breast cancer. This inflammatory breast cancer is able to infiltrate the lymph vessels and skin, often causing no obvious lump within the breast (National Cancer Institute, 2016). It requires treatment such as chemotherapy, radiation or hormone therapy as well as surgical removal in some cases. Metastatic breast cancer on the other hand refers to breast cancer cases that are being diagnosed at a late stage (stage 4), which has spread to other parts of the body such as liver, bones, brain and lungs.

The common signs and symptoms for breast cancer are abnormal lump formation without pain, alteration in appearance of breast and redness in the skin. Breast

screening (mammogram) is important for early detection. Tissue sampling (biopsy) can be done to confirm whether the lump is malignant breast cancer or benign cysts (WHO, 2021). Gene expression profiling is useful to understand breast cancer heterogeneity and predict the risk of recurrence after surgery and hormone therapy as well as to optimise the treatment for each individual (Chen et al., 2021).

2.1.2 Common treatment for breast cancer

2.1.2(a) Chemotherapy

Chemotherapy is one of the most common treatment modalities against breast cancer. It is useful especially for triple-negative breast cancer. Due to the lacks of three receptors as mentioned previously, the treatment options available are limited to chemotherapy since hormonal therapy is not effective. However, recurrence tends to happen more often as compared to other types of breast cancers even though it responds well to initial chemotherapy (Jhan & Andrechek, 2017). Chemotherapy drug would also cause toxicity on normal cells. The side effects of chemotherapy include nausea, hair loss, easy bruising, vomiting and weakening of the immune system. Drug resistance is a risk, therefore a combination of drugs might be required depending on individual patient's conditions (Yaghoubi et al., 2022).

2.1.2(b) Surgery

Surgery removal of breast cancer can be divided into two types- (1) breast conserving surgery or also known as partial mastectomy (lumpectomy) with sentinel lymph node biopsy (removal of one or more lymph nodes) or axillary lymph node dissection and (2) mastectomy (Czajka & Pfeifer, 2023). Breast conserving surgery

involves the removal of partial part of the breast which contains the cancer cells. For mastectomy, the breast tissue is completely removed and it is applied when the breast cancer cells are found in most of the lymph nodes. Although breast conserving surgery may be able to conserve most of the breast, it may require radiation after the surgery to prevent recurrence of breast cancer. For mastectomy especially in early stage cancers, radiation is less likely required after the surgery. However, breast reconstruction surgery might be needed after a mastectomy.

Recurrence of breast cancer may happen in the same breast or in surgery scar, in the lymph nodes nearby as well as distant metastasis. Patients without recurrence in five years would be considered as having a low risk of recurrence (Omidvari et al., 2013). Several factors such as age, stage of diagnosis, hormonal receptors and genetic variants may affect the risk of getting recurrence. Early recurrence usually happens in females with tumours larger than 2 cm and lymph node metastasis while for females with oestrogen and progesterone receptor positive as well as human epidermal growth factor receptor 2 (HER-2) negative would have a higher chance of late recurrence even though survived from breast cancer previously (Chen et al., 2021; Wangchinda & Ithimakin, 2016).

2.1.2(c) Radiotherapy

Radiotherapy is a type of treatment which employs high energy rays to destroy cancer cells (Baskar et al., 2012). It is usually done after breast conserving surgery or mastectomy to reduce the chances of recurrence and is also applicable when the cancer has metastasised. Radiation therapy can also help to avoid mastectomy for women with early stage breast cancer (WHO, 2021). There are two types of radiotherapy which are (1) external beam radiation therapy and (2) brachytherapy.

External beam radiation therapy requires a machine which focuses the radiation on the breast area affected by cancer cells (Baskar et al., 2012). It can be applied for either whole breast radiation or accelerated partial breast irradiation. Whole breast radiation is given to females who have had breast conserving surgery and cancer that has not spread to lymph nodes. However, when the cancer returns after whole breast radiation or surgery, accelerated partial breast irradiation is chosen by giving larger doses within a shorter period, with the beam focuses on specific parts of the breast. However, there might be some possible side effects for external beam radiation such as swelling in the breast area and redness of the skin.

For brachytherapy, it is an internal radiation which employs radioactive pellets placed inside the breast tissue (Baskar et al., 2012). It can be used as accelerated partial breast irradiation for females who had breast-conserving surgery. The possible side effects includes breast pain, damage to fatty tissue in breast area and redness of the skin.

2.1.2(d) Photodynamic therapy (PDT)

PDT is a relatively newer option for anticancer treatment. It was first introduced in early 1900s and is known as the first drug-device combination approved by the United States food and drug administration (FDA) (Agostinis et al., 2011). It is not as common as the other treatment options because there is a limited number of studies reported on its safety and efficacy in treating cancers (Dos Santos et al., 2017). Even though PDT is still an emerging therapeutic modality, it has been clinically proven for the treatment of several types of malignant diseases (Gazzi et al., 2019; Agostinis et al., 2011).

PDT is a type of non-invasive way of treatment for anticancer treatment. During the process, irradiation of a photosensitiser molecule would be conducted at a specific

light wavelength, which would lead to the transfer of the photon energy to the surrounding oxygen molecules, inducing the formation of ROS such as singlet oxygen. The singlet oxygen molecules would help to eradicate cancer cells (Wojtoniszak et al., 2013). PDT can be applied either before or after chemotherapy, radiotherapy or surgery without compromising the efficacy of the other modalities (Agostinis et al., 2011).

There are three mechanisms involved for PDT in eradicating cancers including direct phototoxicity towards cancer cells, destruction of the vascular system of tumour cells and also immune-mediated inflammatory damage towards tumour cells, leading to cancer cells apoptosis (cellular shrinkage), necrosis (cellular death due to lacking of blood flows) or autophagic cell death (mediated by autophagosomes) (Kou et al., 2017; Buytaert et al., 2007; Kim et al., 1999; Abrahamse & Hamblin, 2016).

PDT has been applied as an experimental treatment modality for several types of cancers (Dos Santos et al., 2017; Agostinis et al., 2011; Simone et al., 2012). Photosensitiser such as hypericin was employed to target human breast adenocarcinoma MCF-7 and MDA-MB-231 cells (Kou et al., 2017; Kimáková et al., 2017). According to some studies, PDT revealed promising results in treating high recurrence cancer types. Recently, it was employed together with surgery in orthotopically implanted human pancreatic cancer in nude mouse models, which was shown to be efficient in preventing local and metastatic recurrence as well as able to remove microscopic disease found in the post-surgical tumour bed (Dos Santos et al., 2017; Maawy, Hiroshima, Zhang, Garcia-Guzman, et al., 2015; Maawy, Hiroshima, Zhang, Heim, et al., 2015).

PDT offers several advantages such as minimising the damage to the surrounding normal tissues and able to reduce long term morbidity as compared to other types of treatment such as chemotherapy (Dos Santos et al., 2017). PDT is also a preferable treatment to eliminate possible drug resistance, as there is also no obvious

mechanism for gaining resistance towards PDT (Agostinis et al., 2011; Mizuno et al., 2011; Kou et al., 2017). However, PDT has shown several side effects including pain in the lesions sites and inflammatory reactions on the skin at the cancer sites (Dos Santos et al., 2017).

2.1.2(e) Hormonal therapy

Hormone therapy is used to treat breast cancer that are affected by hormones such as oestrogen and progesterone. It can help to reduce the chances of recurrence, besides being effective and selective to the breast area. It can also be used for the treatment of tumours in other parts of the body with positive hormone receptors (Burstein et al., 2016; American Cancer Society, 2021a).

There are several types of drugs for hormone therapy. One of the common drugs is tamoxifen which is a selective oestrogen receptor modulator (SERM) that helps to block the oestrogen from binding to its receptor expressed by the cancer cells. This would slow down the cancer cell growth or even stop the growth of breast cancer cells. However, this drug might cause some side effects including changes in the menstrual cycle, formation of blood clots and increases the risk of getting endometrial cancer especially for menopausal women (American Cancer Society, 2021a).

2.1.2(f) Immunotherapy

Immunotherapy is a treatment which uses medicine such as monoclonal antibodies and PD-1 inhibitor (pembrolizumab) that helps to boost or suppress the immune response to fight against breast cancer cells and eventually would induce the destruction of tumour (Gupta et al., 2022). It can be used together with chemotherapy to treat triple negative breast cancer. However, there are possible side effects such as