Photodynamic Therapy to Treat Triple Negative Breast Cancer in Vitro

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PHOTODYNAMIC THERAPY TO TREAT TRIPLE NEGATIVE BREAST CANCER IN VITRO

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
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ABSTRACT

Triple negative breast cancer (TNBC) is the most resilient form of breast cancer, being one of the leading causes of death for women and making up 7% of all cancer deaths. Photodynamic therapy (PDT) offers a minimally invasive solution to TNBC as a passive-targeting treatment that reduces the need for other well established yet harsh treatments that can be taxing on the patient. PDT involves the use of a high-energy red light on the area of a tumor injected with photosensitizers (PS) that generate reactive oxygen species (ROS) in the tumor, triggering cell death. The PS tetra(hydroxyphenyl)chlorin (m-THPC) was used in this study due to its high singlet oxygen yield activated by 660-670 nm. Its hydrophobic nature requires the use of the hydrophilic carrier dextran-grafted-polyacrylamide (D-g-PAA) which allows for systemic circulation of m-THPC. D-g-PAA's pH sensitivity encourages the release of m-THPC from D-g-PAA's active amide group when in the presence of low extracellular pH typical of tumors. Gold nanoparticles (GNPs) were also used in this study as they are effective in causing tumor cell death when in the presence of the thermodynamic changes in the area of the tumor during PDT treatment.

The goal of this study was to determine the best concentration of PDT nanocomposite comprised of D-g-PAA encapsulated with GNP loaded with m-THPC for PDT treatment of the TNBC cell line, MDA-MB-231, with minimum damage to the human breast epithelial cell line, MCF10A. PDT nanocomposite concentration 7.2 µg/mL D-g-PAA+GNP loaded with 0.72 µg/mL m-THPC was determined to be the most effective in treating TNBC after statistical analysis of cell counts and MTT assay,
determining that this PDT nanocomposite concentration was twice as effective as the other concentration also considered, 6.0 µg/mL D-g-PAA+GNP loaded with 0.6 µg/mL m-THPC. TUNEL assays were used to confirm that apoptosis was the mode of cell death initiated by nanocomposites. Immunohistochemistry (IHC) staining showed activation of cleaved caspase-9, cleaved caspase-3, and BCL2. All three are in pathways for cell death and initiated following the nanocomposite activation with red light. This study supports that PDT with nanocomposite offers an effective treatment for TNBC in vitro.
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<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
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<td>PDT</td>
<td>Photodynamic Therapy</td>
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<td>PS</td>
<td>Photosensitizer</td>
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<td>m-THPC</td>
<td>Tetra(hydroxyphenyl)chlorin</td>
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<td>D-g-PAA</td>
<td>Dextran-grafted-polyacrylamide</td>
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<td>GNP</td>
<td>Gold nanoparticle</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>Cas9</td>
<td>Caspase-9</td>
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<td>Cas3</td>
<td>Caspase-3</td>
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<tr>
<td>PNIPAM</td>
<td>Poly(N-isopropylacrylamide)</td>
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<td>Ce6</td>
<td>Chlorin-e6</td>
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<td>DLI</td>
<td>Drug-to-light interval</td>
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<tr>
<td>PTT</td>
<td>Photothermal therapy</td>
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<tr>
<td>EPR</td>
<td>Enhanced permeability and retention effect</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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CHAPTER ONE: REVIEW OF LITERATURE

PHOTODYNAMIC THERAPY

Chemotherapy is a common component included in the cycle of care for patients with cancerous tumors. Unfortunately, chemotherapy’s common characteristic of killing the fast-multiplying cells of tumors also results in many side effects as other cells in the body that are naturally designed to multiply quickly, such as hair follicles, are also the target of the same drugs (Dreno et al., 2013). Photodynamic therapy (PDT) is a solution for this as it allows the targeted activation of a photosensitizing compound at the tumor site without it being activated peripherally to cause the negative side effects typical of other chemotherapies, such as alopecia. PDT is minimally invasive and more precise in its application than surgery and radiotherapy as well, minimizing side effects even further (Qin et al., 2021). PDT is designed to work in conjunction with a photosensitizer (PS) which is activated with high energy light applied to the area of the tumor. In this two-step procedure, the PS is injected intravenously to circulate in the blood stream until it reaches the site of the tumor to be activated. A period of time known as the drug-to-light interval (DLI) helps in determining when the light should be applied depending on the characteristics of the drug used (Dąbrowski & Arnaut, 2015). It is suggested that this be within the range of a few minutes or hours after administration while PS is still within the blood vessels albeit within proximity of the tumor (Abrahamse & Hamblin, 2016). A heightened inflammatory response occurs as there is guaranteed necrosis which causes inflammation, thus stimulating the immune system to be activated in the site of the tumor.
PDT treatments that involve PSs that primarily employ apoptosis have also been shown to be highly immunogenic and can stimulate antitumor immunity (Abrahamse & Hamblin, 2016). This makes apoptotic PS’s ideal for PDT treatments as there is less damage to normal cells while the killing of tumor cells is maintained. PS kill tumor cells after being taken up into the cells by causing reactive oxygen species (ROS) to form which damage tumor cells (Fig. 1) as they attempt to go through the cell cycle, rendering the tumor cells inert (Yang et al., 2011). This interaction of PSs and ROS is validated as studies have shown that oxygen quantum fields from ROS decrease when oxygen is removed from solution when PDT treatment with PSs is induced (Dąbrowski & Arnaut, 2015). Cancers will be more sensitive to this form of treatment as they tend to divide more often than the typical normal cell depending on the region of the body.

The delivery of the high-energy light required for PDT is delivered either superficially or via a microfiber probe integrated with a laser system (Li et al., 2013). This is ideal as several effective PS activate with shorter wavelengths of red, high-energy light (600-700 nm) which penetrate from 0.5 to 1 mm into the skin when applied superficially before losing 37% of its intensity (Zein et al., 2018). This and the higher wavelength near-infrared light (700-900 nm) have seen extensive use in the relief of pain as the red lights encourage blood flow to the dermis and all the way to the bone respectively when used superficially in the therapeutic setting (Zein et al., 2018).
**GOLD NANOPARTICLES**

Enhancing the environment for anti-cancer PSs, GNPs make the area of the tumor hyperthermal when introduced to high energy light. Patients with malignant tumors who undergo treatment involving GNPs or similar components are typically combined with radiation and/or chemotherapy (Solopan et al., 2011). In this way, GNPs are used to enhance cancer treatments via their radiosensitive surface structure and electronic properties (Wang et al., 2013). This is considered a hybrid form of PDT known as photothermal therapy (PTT) (Yan et al., 2017). Because GNP electrical surface properties can be manipulated through a variety of factors, they are capable components in cancer imaging once delivered to the tumor with the use of MRI and photoacoustic imaging (Jang et al., 2011; Yang et al., 2011). Along with this, the unique characteristics of nanoparticles in general also make them an advantageous option, comprising of a very small particle size, a high surface area, and the possibility of surface modification (Li et al., 2013). GNPs range between the sizes of 10-60 nm and have a maximum capacity for being functionally active with wavelengths near 520-540 nm (Pustovalov et al., 2008).
GNPs also vary in shape. The different shapes they are typically used in for thermal applications are gold nanoshells, hollow gold nanospheres, nanocages, and nanorods (Kang et al., 2016; Lin et al., 2013). Once activated, their properties allow them to enhance cancer targeting of treatments, such as PDT, with strong absorption and scattering properties (Kang et al., 2016). These scattering properties facilitate the making of reactive oxygen species which further damage the cancer cell so to halt proliferation (Wang et al., 2013).

GNPs require a means to target tumor cells in order for them to be effective in enhancing the effects of anti-cancer therapies. This is why many studies concentrate on the attachment of GNPs to a carrier in order for GNPs to be taken into tumor cells. One such option for GNP carriers takes advantage of the Warburg effect with the attachment of glucose molecules so that tumor cells will be more likely to take up GNP along with the raw materials due to tumor cells’ relatively high rates of metabolism (Shinoda et al., 2020; Wang et al., 2013; Li et al., 2014). Wang et al., 2013 found that tumor cells have a greater response to larger GNPs as radiosensitizers and in regards to uptake due to enhanced permeability and retention effect (ERP). GNPs can be taken up via receptor-mediated endocytosis with the glucose carriers and are encapsulated into endosomes where GNPs are eventually processed for excretion once merged with lysosomes (Wang et al., 2013; Li et al., 2014). It is in this respect that EPR applies as larger GNPs are harder to excrete, leading to a direct correlation of GNP size to GNP uptake (Wang et al., 2013). There is a significant difference between how glucose conjugated GNPs can be applied to cancers that are liquid tumors and those that form solid tumors. A liquid tumor
(ex. monocytic leukemia) is better treated with glucose bound GNPs in conjunction with radiotherapy than a solid tumor (ex. epithelial breast cancer) as more GNP is taken up by the liquid tumor (Hu et al., 2015). This allows for the treatment of liquid tumors to exceed traditional radiotherapy treatment of solid tumors, even after the addition of GNP despite solid tumors’ relatively enhanced sensitivity in the early stages of radiotherapy (Hu et al., 2015). The uptake of glucose bound GNPs is also dependent on the starvation of the cancer cells themselves, meaning the less energy sources the cancer cells have taken up the more likely they are to take up this form of GNPs. With starvation times being optimal up to 3 hours, it will be a tradeoff of whether the patient be starved of nutrition before treatment, thus making it more likely for normal cells to take up the glucose bound GNPs as well, or if they should not be starved prior to treatment, meaning the uptake of glucose bound GNPs will not be as optimal for the anticipated results (Hu et al., 2015). The tendency for carcinomas to have a heightened presence of glucose transporters might show promise for similar cancer types with the specificity of glucose bound nanoparticles, though, some amount of consideration of the individual might need to be considered with this practice in vivo (Li et al., 2014).

The use of glucose bound nanoparticles has seen success with other molecules besides GNPs. For example, ionically crosslinked carboxymethyl chitosan is able to effectively deliver anti-cancer drugs such as paclitaxel, improving solubility, stability, and specific targeting to ER+ epithelial breast cancer (Calavia et al., 2018; Guo et al., 2010; Li et al., 2014). This, at the very least, shows that there is merit in the regular use of glucose bound nanoparticles in the clinical setting. The surface composition of cancers
such as glioblastoma also have a characteristic of having an upregulation of integrin-$\alpha_v\beta_3$-receptor which can then be used as a marker (Wu et al., 2017). Nanoparticles integrated with a cyclic peptide, c(RGDfK), are able to use this integrin as a marker for tumor specificity, facilitating penetration of the tumor with what is attached to the c(RGDfK) (Wu et al., 2017).

Besides radiotherapy, GNPs are effective in the process of magnetized photothermal radiation. GNP in the form of nanorods are used for photothermal radiation because the wavelength tunability from their oval shape in addition to the other properties of GNP discussed earlier allow for them to be the most effective type of GNPs for magnetized thermal applications (Kang et al., 2016; Wang et al., 2013). Iron oxides are another form of nanoparticle used in this same fashion by inducing hyperthermia in order to induce necrosis (Bubnovskaya et al., 2014).

**PHOTOSENSITIZERS**

PSs are the active agent in the PDT process that are purposed to kill cancer as they are activated and generate apoptosis inducing ROS. PSs typically require the use of a hydrophilic carrier due their hydrophobic nature. The two photosensitizers focused on will be different forms of chlorin: chlorin-e6 (Ce6) and temoporfin (m-THPC). Chlorins are a popular choice for PS due to their high extinction coefficient in red light region and high singlet oxygen quantum yield (Yan et al., 2017). Photosensitizers are not toxic until illuminated when in cancer or other diseased cells, avoiding damage to normal tissues (Kuntsche et al., 2010). The focus of many recent studies involve second-generation PSs
which are defined as well-defined photosensitizers with optimized physiochemical and optical properties (Kuntsche et al., 2010). It is through the development of second-generation PSs that PDT owes its status as a legitimate clinical practice today (Kuntsche et al., 2010).

Ce6 is derived from naturally occurring chlorophyll and is considered to be the most commonly used form of chlorin as it can be activated by NIR light and eliminated rapidly from the body (Yan et al., 2017). Ce6 even has the potential of being used as a red fluorescence imaging dye while being irradiated with wavelengths of 650-900 nm, avoiding interference with endogenous chromophores in the body (Zhang et al., 2015). Ce6 is noted to be solely dependent on its carrier for the targeting of tumor cells. Some of the options for Ce6 carriers are liposomes, glucose molecules, polymer carriers, and polymer + GNP composites (as are the focus of recent studies) (Zhang et al., 2015). Ce6 conjugated with another form of sugar, mannose, was shown to have equivalent effects to that of Ce6 conjugated glucose and had greater cellular uptake and distribution in glioblastoma cells than an established second-generation PS, talaporfin sodium, which is approved for use in PDT treatments for lung, brain, and esophageal cancer (Shinoda et al., 2020). Ce6 added to polymer, poly(dopamine), nanospheres injected directly into hepatocellular carcinoma cell lines were required to be dissolved in PBS and 10% fetal bovine serum before they out performed free Ce6 in the speed and number of ROS production given all the same parameters (Zhang et al., 2015). In addition to this method of PS delivery’s specific requirements to function, their requirement of activation via high energy light was also noted with the hepatocellular cancer cell line as even high
doses of the Ce6-polymer conjugates (8 µg/mL) left the cancer cells at 90% viability after a 24-hour incubation (Zhang et al., 2015). The combination of this concept with PTT from GNP allows for the enhanced cellular damaged and tumor destruction. This is evidenced by the comparison of Ce6 with only polymer, polyethyleneimine, being out performed by Ce6 conjugated to gold nanorods via polymers polyethyleneimine and polystyrene sulfonate which included a significant spike in temperature (Yan et al., 2017).

m-THPC is more hydrophobic than most other PSs, being one of the most potent generation II PSs, necessitating the use of a carrier with a log P of 9.24 (Yang et al., 2011; Mattioli et al., 2022). It is activated at a wavelength of 652 nm with light penetration depth of around 1 cm (Yang et al., 2011). In addition to this, drug doses for m-THPC are typically prescribed for 0.1-0.15 mg/kg with tumors illuminated with 652 nm wavelengths with a total dose of 10-20 J/cm² (Dąbrowski & Arnaut, 2015). m-THPC is noted as having relatively intense absorption bands at longer wavelengths compared to previously developed PSs which allow PDT treatments to penetrate deeper below the skin as well as decrease the time needed for treatments (Dąbrowski & Arnaut, 2015). It is a resilient PS with a terminal plasma half-life of 65 hours (Dąbrowski & Arnaut, 2015).

Just like Ce6, m-THPC is effective when used with liposome carriers. The first example of m-THPC loaded liposomes were made to release the m-THPC when its environment is just above body temperature (Kuntsche et al., 2010). The hydrophobic PS m-THPC is set in the lipid bilayer of the liposome, altering the physiochemical properties of the liposome carrier which requires modification of the liposome not only in respects
to targeting and release but also in its stability (Kuntsche et al., 2010). Micellar structures are not seriously considered as they significantly decrease the amount of m-THPC that can be loaded (Kuntsche et al., 2010). Pegylated phospholipids are used to modify the surface of the liposome to increase circulation time as the polyethylene glycol on the surface of what is typically dipalmitoylphosphatidylcholine with dipalmitoylphosphatidylglycerol added for stability which all together allow for effective m-THPC delivery (Kuntsche et al., 2010). m-THPC loaded liposomes are effective in other applications as well, such as the killing of MRSA after being delivered via a peptide modified liposome in the process of PDT (Yang et al., 2011). The liposomes equipped with antimicrobial peptides have to be the part that targets the bacteria in this case as m-THPC does not have any affinity for bacteria itself and will infiltrate surrounding normal cells with its tendency toward integrin ligands (Yang et al., 2011). At the very least, this offers an answer to increasing antibiotic resistance (Yang et al., 2011). This means that if m-THPC can continue to be potent towards bacteria without its resistance to the PS, there is promise for tumor cells to also have continued sensitivity to the PS. Though, it is a question if m-THPC resistance would be an issue in the first place as it shows potential in requiring less applications in order to get the same results that other cancer treatments hope to achieve.

POLYMER CARRIERS

With the typically hydrophobic nature of photosensitizers and poor uptake of both PSs and GNPs, a suitable carrier be used in tandem with them. Combined block polymers have great potential in the role of polymer carriers as different polymers with different
characteristics can be jointly combined to cater to the most ideal mode of activation given the target (Cook et al., 2017). Joined polymers that react to different stimuli can function as more than just hydrophilic carriers but also provide a complex system of activation that encourages the specific release of their attached drug given the particular conditions of all polymers involved are met. One example of this is with the potential of a block of a hydrophobic polymer (poly(dimethylsiloxane)) used as a linker between two blocks of hydrophilic polymer, poly(N-isopropylacrylamide) (PNIPAM) (Cook et al., 2017). This nanocomposite formed well defined aggregates for a structurally sound polymer carrier compound (Cook et al., 2017). This was due to the opposing natures of the bound polymers that could then be activated to release a given component attached to the carrier by the increase of temperature as a result from PNIPAM’s thermosensitive nature.

Dextran-graft-polyacrylamide (D-g-PAA) copolymers are both efficient in the carrying and entrapment of various micromaterials, some of which being nuclear material such as uranium which is known to be quite toxic given the right conditions and setting a health hazard in environments of nuclear waste storage or nuclear terrorism (Bliznyuk et al., 2022). When used as a carrier, D-g-PAA enhances the stability of nanoparticles by introducing steric and/or electrostatic repulsions between them (Naumenko et al., 2017). Polyacrylamide (PAA) can also be used as a biocompatible nanohydrogel that carries anticancer materials, such as GNP, for PDT treatments which has proven to enhance uptake compared to those materials being unembedded (McLean et al., 2020). PAA allows for photodynamic properties to be retained so properties like two-photon absorption and singlet oxygen efficiency can be preserved (McLean et al., 2020).
Modifying the PAA with other polymers like PEG and guided tumor targeting cell
penetrating peptides allow for PAA carriers to target tumor cells themselves for even
better efficiency of its load and reduced toxicity to the rest of the body for conjugated PS
(Wang et al., 2012).

CANCER AND PHOTODYNAMIC THERAPY

Cancers can require a variety of different treatments or those that can be harsh to
the person in both its application and the recovery process. PDT’s effectiveness has been
tested on these fronts for a variety of cancers, giving promise to a possible universal
treatment for cancer.

Skin cancer is the most well studied rendition of tumors for PDT as the main
disadvantage of PDT having a limited penetration depth is somewhat mitigated by
superficial nature cancers of the skin can have. The permeability available for skin cancer
even allows for the consideration of using natural sunlight as an activator for the
photosensitive PS (Galvão et al., 2017). The advantage of skin cancers that are candidates
for this, such as the precancer actinic keratoses, is that the PS can be easily applied via
either topically or a direct injection. Aminolevulinate and methylaminolevulinate, PS’s
that would be used in topical applications, still require a standard for a light source which
makes topical PS application dependent on the region of the world a patient is being
treated in with low latitude areas being the most ideal due to more direct and frequent
sunlight (Galvão et al., 2017). Injected PS can also be activated by sunlight even with the
intention of PS activation being through a controlled light with a specific wavelength.
Such is the case for Ce6 which reportedly kept its photosensitivity 3-7 days after injection into melanoma in vivo (Beack et al., 2015). This offers another one of the advantages and disadvantages of PDT for skin cancers as there is an increased likelihood of PS reactivity due to the general location of PS but this apparently decreases potential for modulated activation of PS which runs the risk of unintentionally hurting cells that are not cancerous.

Few esophageal cancer patients benefit from even multimodality therapies of traditional treatments such as surgery and radiation combined with chemotherapy, even when the cancer is localized (Li et al., 2013). It is for this reason that the photothermal effect of GNP has been utilized in PDT tests that do not involve PS. GNPs can be designed to have maximal resonance at a particular wavelength so that the electronic oscillations of the particle surface are converted into heat by infrared light (Li et al., 2013). In this instance, biocompatible chitosan was used as a carrier for GNP injected directly into the tumor site where the specialized GNP nanocomposite showed selectivity for the esophageal cancer (Li et al., 2013). The delivery of the light source to the tumor has more potential issues than the GNP delivery as the microfiber optic probe has potential to damage surrounding tissues if it gets too close or makes direct contact with epithelium (Li et al., 2013). This is a potential problem PDT to deep tissues will have to consider with future applications.

More than 1 million women per year are known to be diagnosed with breast cancer worldwide with several thousand succumbing to the disease, making it the most prevalent cancer women are diagnosed with (Tao et al., 2014). It is suggested that the
occurrence of breast cancer stems from the heightened activity of miR-21 with upregulation which in turn downregulates the tumor suppressor gene, PTEN (Tao et al., 2014). With this, mutations are more likely to occur, the most common that bring about breast cancer being BRCA1 and BRCA2 (Tao et al., 2014). BRCA1/2 occur as dominant gene mutations (congenitally acquired) along with TP53 (Tao et al., 2014). These tend to effect patients less than 45 years old while other modes of breast cancer occurrence, such as those originating from single nucleotide polymorphisms, tend toward the other side of the spectrum (Tao et al., 2014). Cell surface expression helps classify them into five subtypes dependent on the expression of estrogen receptors (ER), progesterone receptors (PR), and Her2 oncogenes (Tao et al., 2014). So far, cell lines with multidrug resistance (ex. MCF-7) can have viability decreased by 53.5% with a combination of PSs to enhance the effectiveness of PDT with increased fluorescence and targeting based on the metabolism of the tumor cells (Zeng et al., 2015). MCF-7’s were also found to have enhanced proapoptotic p53 and Bax reactions with suppressed antiapoptotic BCL-2 with PDT treatments (Fickova et al., 2014).

Even the most aggressive metastatic subtype of breast cancer, triple negative breast cancer (TNBC), is killed off effectively with limited PDT. GNP loaded to thiogluucose for systemic transport and biocompatibility are able to react with wavelengths of 490 nm to significantly lower the viability of the TNBC cell line MDA-MB-231 with the effects of PTT as well (Wang et al., 2013). This suggests that a resilient and aggressive breast cancer like TNBC will likely require specialized nanocomposites and possibly multimodality treatments in order to be effectively treated.
PD-L1 and HDAC8 have been considered for TNBC targeting; however, they either are a rare occurrence for this type of cancer or still have a long road ahead before their use is practical in application respectively (Mittendorf et al., 2014; Menbari et al., 2020). Compared to ER & PR-positive cells (MCF7s), TNBCs do express PD-L1 significantly more with 20% of the TNBC being estimated to overexpress this marker for the potential for CD8⁺ T-cell targeting via its PD-1 receptor (Mittendorf et al., 2014). This does give an answer for some TNBC that happen to express this receptor, but this leaves 80% of the rest of TNBC cases that will need to benefit from alternative forms of treatment. Inhibition through HDAC8 is dependent on the antitumoric effect of miR-483-3p mediated by the Wnt/β-catenin signaling pathway (Menbari et al., 2020). This means of targeting will likely require a specialized form of delivery not mentioned above, but its execution for clinical treatment is yet to be investigated. Other subtypes of TNBC’s based on potentially targetable phenotypes including two basal-like (BL1 and BL2), an immune-modulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL), and a luminal androgen receptor (LAR) type (Denkert et al., 2017). BL1 is reported with having the most response rate compared to the others, though, is not the most prevalent as LARs take up the most cases of TNBC with 16% (Denkert et al., 2017). These markers can at the very least be used to help in the diagnosis of TNBC even though none of these are used in clinical diagnosis currently as there 2188 genes that must be considered with suggestions that there is potential to cut down this number to 101 in the near future (Denkert et al., 2017).
G₂/M phase is the phase of the cell cycle that is the most effected in treatments that encourage a photothermal environment, such as in radiotherapy when paired with GNPs (Wang et al., 2013). The decreased expression of p53 and cyclin A paired with the increased expression of cyclin B1 and cyclin E causes G₂/M phase arrest (Hu et al., 2015). The additional activation of CDK kinases induces both G₀/G₁ cell cycle acceleration and cell accumulation in G₂/M phase, thus, speeding up the process of arrest of mitosis in cancer (Hu et al., 2015). The S phase where the mass of DNA replication occurs in the cell cycle is vulnerable to the damage of ROS from treatments, causing this phenomenon so the cell is unable to progress effectively through the M phase. There is a threshold for ROS dosage in order for it to be effective, but concentrations of 1-10 mM have been reported to cover a variety of cases (Dąbrowski & Arnaut, 2015). There are potential treatments that trigger cell arrest at the G₀/G₁ phase such as with the miR-483-3p/HDAC8 HDAC8 premiR-vector which would involve active cell targeting (Menbari et al., 2020).

CONCLUSIONS OF LITERATURE

PDT/PTT is shown to be a highly effective treatment in cancer therapy, outpacing many other forms of cancer treatment if not mitigating further complications with the application of combined treatments. An amalgamation of GNPs, PS, and polymer carrier seems to be a promising mode of treatment based on the evidence of current research. With PDT, the shape GNPs take does not seem to hinder their effects in any way. The intense absorption bands at longer wavelengths make m-THPC more ideal for treatment of deeper tumors with reduced times for light exposure. Dextran-graft-polyacrylamide
copolymers with the combined characteristics of two polymers combined with the two aforementioned components has promise for making an effective cancer killing machine.
CHAPTER TWO: IN VITRO TREATMENT OF TNBC

INTRODUCTION

Breast cancer will develop in every 1 in 8 women in the United States during their lifetime according to the American Cancer Society (Tao et al., 2014). Triple negative breast cancer (TNBC), is the most aggressive metastatic subtype of breast cancer despite it comprising of only 15% of all epithelial breast cancers (Menbari et al., 2020; Denkert et al., 2017). It is characterized by the lack of expression of estrogen receptors (ER) and progesterone receptors (PR), and lack of amplification or overexpression of human epidermal growth factor receptor 2 (HER2) which are the three primary biomarkers typically used to identify breast cancers (Denkert et al., 2017). The absence of these receptors makes TNBC resistant to most cancer treatments that target the individual receptors. Instead, treatments such as chemotherapy, radiation, and excision are the best measures against TNBC but can be more taxing on the patient such as traditional chemotherapy’s tendency to target all fast-growing cells, not just tumor cells (Denkert et al., 2017; Dreno et al., 2013). For this reason, another means of localized treatment is being developed that has promise of mitigating the other harsh and invasive treatments typical for TNBC patients.

Photodynamic therapy (PDT) is a high-energy, deep red-light procedure that produces a localized effect on cancer tumors. The high-energy light activates photosensitizers (PS) which generate reactive oxygen species so that the cells of the tumor will undergo necrosis/apoptosis (Rapozzi & Jori, 2015). Temoporfin (m-THPC) is a preferred PS given its high singlet oxygen yield and reactivity only under wavelengths
of light of 660-670 nm (Beack et al., 2015). m-THPC has integrin targeting properties. Cancer cells typically express higher levels of integrin allowing m-THPC to interact with tumors more than healthy cells (Yakavets et al., 2019). Just like other PS’s, a drawback to the use of m-THPC in the body is that it is hydrophobic (Wu et al., 2017). Therefore, the use of a hydrophilic, thermostensitive polymer, dextran-polyacrylamide (D-g-PAA), is crucial to allowing transport of the PS through the body as m-THPC is bound to it and will not be readily released until D-g-PAA is near the tumor where lower pH levels typical of cancer allow for m-THPC release (Cook et al., 2017). The D-g-PAA will release m-THPC from its active amide group and allow m-THPC to be taken up by cancer cells so they might be killed off when m-THPC is activated (Kutsevol et al., 2008). The combined block polymer structure of D-g-PAA pH levels to be within a range to activate both polymers so its contents can be released (Cook et al., 2017).

Gold nanoparticles (GNPs) must be in the presence of high-energy light for them to activate through energetic changes to damage tumor cells when in the area of the light. These require polymers or other carriers such as D-g-PAA for significant specificity to tumors in order to carry out their effects of photothermalysis (Pustovalov et al., 2008).

The aim of this study was to determine ideal concentrations of D-g-PAA, GNPs, and m-THPC to make a photodynamic compound that kills TNBC cells with minimal death of normal breast cells using a standard PDT protocol.
MATERIALS & METHODS

Cell Lines and Culture

The cell lines MDA-MB-231 (TNBC cells) and MCF10A (human breast epithelial cells) used in this study were from the ATCC (Manassas, VA, USA). TNBC cells’ culture media was comprised of DMEM (Corning, NY, USA; Thermo Fisher Scientific Waltham, MA, USA) enhanced with 10% FBS (Corning, NY, USA) and 1% antibiotic/antimycotic solution (AA; Thermo Fisher Scientific, Waltham, MA, USA). MCF10A culture media was comprised of DMEM enhanced with 10% FBS, 1% AA, and MEGM SingleQuots (Lonza, Walkersville, MD, USA) comprising of 0.5 mL insulin, 0.5 mL hydrocortisone, 0.5 mL GA-1000, 0.5 mL hEGF, and 2.0 mL BPE. Cells used in this study were incubated at 37°C with 5% CO₂.

Photodynamic Therapy Materials

Dextran-graphed polyacrylamide (D-g-PAA) compounded with GNP diluted in DI water, and m-THPC used in this study were provided by Dr. Valery Bliznyuk from Clemson University’s Department of Environmental Engineering and Earth Science. Preparation of PDT solutions were performed in dim light so to control the reactions of the different components to ambient light while maintaining the minimum amount of light needed to adequately handle the materials. Stock PDT solutions were diluted in PBS (Corning, NY, USA). The different concentrations of PDT solutions used to treat cells were comprised of one or more of the stock solutions of D-g-PAA+GNP or m-THPC with the addition of DMEM. After the addition of DMEM, PDT solutions were placed on
a Fisherbrand Multi-Platform Shaker stir plate (Thermo Fisher Scientific) for 30 minutes at 60 rpm to allow PDT solutions with D-g-PAA or D-g-PAA+GNP to affix to m-THPC if combined in the same solution. Solutions that had a combination of D-g-PAA/D-g-PAA+GNP and m-THPC always had a 10:1 concentration to encourage adherence of the two constituents. All PDT solutions were used within 24 hours of being compounded.

**Photodynamic Therapy Protocol**

All procedures involving PDT treatment were performed in dim light so to minimize any reactions of PDT constituents with ambient light. All PDT experiments included an even number of plates with the same array of concentrations to account for their effects with and without red light exposure. TNBC and human breast epithelial cells were seeded in well plates and grown to 70% confluency prior to PDT. Culture media was removed and replaced with either culture media as appropriate to the cell line or one of the different concentrations of PDT solutions as previously described with a minimum of three samples each. Cells were incubated at 37°C for 90 min before a PBS wash was performed on all cells three times. With the third dose of PBS on the cells, half of the plates per experiment were exposed to red light from a 660 nm Deep Red LED Light Bulb (ABI Inc., MD, UDA) with a power density of 93.6 mW/cm² and light dose of up to 28.1 J/cm². The other plates were controls with no exposure to red light. The red light was set in a cell culture hood and suspended 5 inches above a plate of cells which were exposed to the red light for 10 min without the plate’s lid. The red-light source and plate of cells were surrounded by aluminum foil so the cells would receive the full effect of red light and to validate its effects without ambient light. PBS was then removed from all
plates and replaced with the appropriate culture media per cell line. Cells were then incubated at 37°C for at least 24 hours before further evaluation.

**Cell Counts and Morphology**

TNBC and human breast epithelial cells were seeded separately at a density of 2x10^5 cells/well in two 24 well plates with each cell line comprising half of each plate. After PDT protocols with varying concentrations and constituents were implemented, the cell morphology was imaged with a Zeiss Axiovert 40 CFL inverted microscope and collected with a Zeiss AxioCam camera and AxioVision software (Pleasanton, CA, USA). Cell counts were performed manually using a hemocytometer after being washed with PBS and detached with 0.05% trypsin (Corning, Manassas, USA) neutralized with cell culture media. A timepoint of 72 hours was first analyzed before switching to a timepoint of 24 hours for the remainder of experiments.

**MTT Assay**

TNBC and human breast epithelial cells were seeded separately at a density of 5x10^4 cells/well into two 96 well plates with n=3 for each sample. PDT protocols with varying concentrations of D-g-PAA+GNP combined with m-THPC were implemented. Thermo Fisher’s (Oregon, USA) CyQUANT™ MTT Cell Proliferation Assay Kit was used according to the manufacturer’s guidelines to determine cell viability. Absorbance was measured at 540 nm as according to the protocol and analyzed via a BioTek Synergy Plate Reader with Gen5 Software.
**Apoptosis Assay**

PDT protocols were followed prior to TUNEL apoptosis assays with cells grown to confluency in 24 well plates and treated with 6.0 μg/mL D-g-PAA+GNP and 0.6 μg/mL m-THPC or 7.2 μg/mL D-g-PAA+GNP and 0.72 μg/mL m-THPC. The control groups did not receive any nanocomposite. Click-iT™ Plus TUNEL Assay (Thermo Fisher Scientific, Waltham, MA, USA) with stock solutions for Alexa Fluor™ 488 picolyl azide dye prepared according to the manufacture’s guidelines were used to help analyze the apoptotic effects of PDT. The experimental protocol for cells grown on coverslips by the manufacturer was followed. Stained cell images were acquired using an EVOS® FL Auto Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence excitation was approximately 495 nm and emission was 519 nm.

**Immunohistochemistry Staining**

Cells were grown to confluency in 24 well plates and treated with 7.2 μg/mL D-g-PAA+GNP and 0.72 μg/mL m-THPC if not the control group. After PDT protocols, cell culture media was removed and cells were fixed for 10 minutes with 4% paraformaldehyde in preparation for immunohistochemistry (IHC) staining. They were then rehydrated via washing them with PBS twice followed by blocking 3% hydrogen peroxide (MPL, USA) for 5 minutes at room temperature. Cells were washed twice again with PBS before applying 0.5% Tween-20 (SIGMA, USA) in PBS for 5 minutes at room temperature. Cells were washed twice again with PBS before applying 5% goat serum (Corning, NY, USA) in PBS for 10 minutes at room temperature. The primary antibodies
used in this study were cleaved caspase-9 (Cas9), cleaved caspase-3 (Cas3), and BCL2 (Cell Signaling Technology, Danvers, MA, USA) were applied separately on three separate rows per plate with a 1:100 dilution in PBS. Plates were then incubated overnight at 4°C. The cells were then washed thrice with PBS before the secondary antibodies (goat anti-rabbit HRP) with a 1:200 dilution in PBS was applied for 45 minutes at room temperature. Cells were washed thrice with PBS before DAB (Vector, CA, USA) was prepared according to manufacturer guidelines and applied for 10 minutes at room temperature. Cells were washed thrice more with PBS before counterstained with 100% hematoxylin (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes at room temperature. Cells were then rinsed with water thrice for 5 minutes before 70% EtOH was applied for 1 minute then 100% EtOH was applied for 3 minutes at room temperature. Xylene was then applied to the cells twice, 5 minutes each at room temperature. Coverslips were then placed on each sample using Permount mounting media. Images of cells were taken with an EVOS® FL Auto Imaging System (Thermo Fisher Scientific, Waltham, MA, USA).

**Statistical Analysis and Figures**

Statistical analysis for samples with one variance was performed using pair of two samples for a means and statistical analysis for samples with more than one variance were performed with single factor analysis of variance. Tests and plots were generated using Excel (Microsoft, Washington, USA). Experiments had a minimum n=3 per group. Date reported shows the mean value ± the standard deviation. A p-value of ≤ 0.5 was considered significant. Images of TUNEL assay (Fig. 5) were edited for exposure to
further remove background noise via Photoshop (Adobe, California, USA). All images were compiled using Word and PowerPoint (Microsoft, Washington, USA).

RESULTS

Cell Counts and Morphology

All graphs shown have data normalized with percent control. MCF10A cells without red light treatment are represented by dark green bars and those that were exposed to red light are represented by light green bars. MDA-MB-231 cells without red light treatment are represented by dark red bars and those that were exposed to red light are represented by light red bars.

Figure 2 contains data from the application of the varying components of the nanocomposite 72 hours after PDT treatment to verify the effects of these components. The polymer carrier was applied assuming that it would not elicit a significant reduction in cell count relative to the control; therefore, the larger concentration of D-g-PAA used for this experiment was implemented by itself (2.4 µg/mL D-g-PAA). This resulted in a significant increase of MCF10A cells without light treatment (Fig. 2A) but a significant decrease in MDA-MB-231 cells with the same treatment (Fig 2B) likely due to errors in passaging. All other cell counts in Figure 2A are either increased or not significantly reduced. The D-g-PAA+GNP only and 2.4 µg/mL D-g-PAA+GNP and 0.24 µg/mL m-THPC samples for Figure 2B have a significant decrease also likely due to errors. This means that the reduction in cells for Figure 2B’s 0.12 µg/mL m-THPC and 0.24 µg/mL m-THPC samples could also be a result of factors other than PDT.
Figure 2: Cell counts with different nanocomposite components 72 hours after PDT treatment. A) MCF10A cells that did not receive light are denoted with dark green and MCF10A cells that did receive light are denoted with light green (n=3), B) MDA-MB-231 cells that did not receive light are denoted with dark red and cells that did receive light are denoted with light red (n=3). Error bars indicate the standard deviation. Statistical significance is denoted with *$p \leq 0.5$, **$p \leq 0.05$ versus the control groups.

The potency of PDT with m-THPC was tested to deduce the concentration of m-THPC that is best to use for the nanocomposite with MDA-MB-231. There is a significant difference between without red light and with red light for all samples save for the control group and the 1.00 µg/mL m-THPC samples. Without a hydrophilic carrier
for m-THPC, it is expected that the PS itself will not be enough to kill the cancer unless it is applied in concentrations too toxic when combined with the polymer carrier to facilitate its distribution. The concentration of 0.35 µg/mL had a significant difference between cells without red light treatment and those with red light treatment. The 0.48 µg/mL and 0.60 µg/mL concentrations of m-THPC resulted in a significant reduction in cells for those not treated with red light. The concentration after those, 0.72 µg/mL, is a better representative of the expected result for this experiment with there being a significance between the single variable of light exposure while there is no significance between the cell counts with those of the control groups. There is a drastic increase of cells for the 0.84 µg/mL concentration. The 0.96 µg/mL concentration of m-THPC saw a significant difference between without light and with light while both variables for light exposure were reduced for the 1.00 m-THPC concentration.

**Figure 3: Cell counts with different m-THPC concentrations 24 hours after PDT treatment.** MDA-MB-231 cells that did not receive light are denoted with dark red and cells that did receive light are denoted with light red (n=3). Error bars indicate the standard deviation. Statistical significance is denoted with *p≤ 0.5, **p≤0.05 versus the control groups.
The cell counts for both MCF10A cells and MDA-MB-231 cells declined with nanocomposite applications with neither control group being significantly different per cell line as seen in Figure 4. MCF10A cells that received 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC are shown to have significantly declined by nearly 50%; however, those not exposed to the red light have the most variation of sample sizes in Figure 4 according to its error bar. Both samples of MCF10A that were given 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC declined significantly with MCF10A cells that were not exposed to the light dropping by 65% and MCF10A cells that were exposed to the light decreased by 70%. MCF10A that received 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC were also noted to be significantly different from one another.

Both the without light and with light MDA-MB-231 samples that received 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC were significantly decreased by 25% and just above 50% respectively. For MDA-MB-231 cells that were treated with 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC, those that did not receive light were actually more numerous than those with the lesser nanocomposite concentration but those that did receive light decreased significantly by 50% according to the control group and its counterpart that did not receive light.
Changes in MCF10A and MDA-MB-231 cell morphology 24 hours after PDT treatment and with different nanocomposite concentrations, without and with light, can be seen in Figure 5. All cells in the control groups expressed normal cell morphology with and without light irradiation, being spindle shaped and closely linked to one another. Throughout Figure 5A, the majority of MCF10A cells can still be seen attached to the bottom of the wells. MCF10A cells that did not receive light and were treated with nanocomposites retained normal cell morphology for all but a few cells. MCF10A cells that received both nanocomposite and light start to show some signs of damage with light treated MCF10A cells with 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC have more cells that are rounded compared those in the same sample without light irradiation. MCF10A cells with 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC that were exposed to light begin shrink in general, even for those that are attached. In Figure 5B, MDA-MB-231 cells can be seen becoming more and more round with increasing
concentrations of nanocomposite with some having noticeably spaced apart with increasing concentrations of nanocomposite. MDA-MB-231 cells that are left became rounded for both nanocomposite concentrations, without and with light. The degree of space increase is more prevalent for MDA-MB-231 cells that received light than those that did not. The 7.2 µg/mL D-g-PAA+GNP and 7.2 µg/mL m-THPC concentration for MDA-MB-231 cells with light exposure had the most noticeable loss of spindle morphology and spacing compared to all other samples in Figure 5. The same concentration of nanocomposite for MDA-MB-231 cells that did not have light irradiation were still more spindle shaped and linked together than MDA-MB-231 cells treated with 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC and light.

![Figure 5: Cell morphology 24 hours after PDT treatment with select nanocomposite concentrations. Different PDT treatment concentrations for A) MCF10A and B) MDA-MB-231. Those that received light are on top and those that did not receive light are on the bottom.](image)

**MTT Assay**

Living cells’ conversion of MTT into formazan crystals was measured with the MTT assay within 24 hours of PDT as shown in Figure 6 (Meerloo et al., 2011).

Increased absorbance is directly related to mitochondrial activity, indicating the viability of cells with increased absorbance from MTT conversion to formazan crystals (Meerloo
et al., 2011). Decreased absorbance is associated with a decrease in a cell’s ability to function to proliferate and stay alive. MCF10A cells and MDA-MB-231 cells are represented in the same manner as they are in the cell count experiments.

Neither control group were significantly different from each other per cell line. A drop in cell conversion of MTT was seen with nanocomposite application for all cells. The effect of the full PDT treatment of red light and nanocomposite is evident with samples that did receive nanocomposite consistently have a more significant difference compared to control. A significant difference was observed between MDA-MB-231 cells exposed to 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC that did not receive light irradiation and those that did. This was also evident for MDA-MB-231 cells given 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC. The only case that there was a significant difference between MCF10A samples exposed and not exposed to light was in those that were given 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC.
Figure 6: Cell viability based on absorbance [540 nm] 24 hours after PDT treatment with select nanocomposite concentrations. MCF10A cells are shown in green and MDA-MB-231 cells are shown in red. Darker shades of each denote cells that did not receive light and lighter shades of each denote cells that did receive light. Error bars indicate standard deviation. Statistical significance is denoted with *p≤ 0.5, **p≤ 0.05, ***p≤ 0.005 versus the control groups.

**Apoptosis Assay**

A TUNEL assay was used to evaluate DNA fragmentation, indicative of apoptosis, 24 hours after PDT and nanocomposite treatments. Figure 7 shows TUNEL assay-treated MDA-MB-231 cells with a control group and two different concentrations of nanocomposite. The enzyme, terminal deoxynucleotidyl transferase (TdT) mediated dUTP conjugated with the fluorescent dye, Alexa Fluor™ 488 picoly azide was selective for DNA strand breaks so to detect apoptotic cells. Normal DNA shows as light green
while bright green represents cleaved DNA. DAPI is used to mark the nucleus of the cells.

Figure 7A shows the control group had hardly any cleaved DNA when not exposed to light as when the DAPI and cleaved DNA images are combined, very little green is able to show past the competing fluorescence of DAPI. Control group samples irradiated with light have slightly more green fluorescence showing in both the cleaved DNA and in the combined image.

Figure 7B shows the 6.0 μg/mL D-g-PAA+GNP and 0.6 μg/mL m-THPC treated MDA-MB-231 while Figure 7C shows the 7.2 μg/mL D-g-PAA+GNP and 0.72 μg/mL m-THPC treated MDA-MB-231 cells. Both have an evident increase in light green relative to the control group in Figure 7A. Some DAPI is still able to show through in the combined image for 6.0 μg/mL D-g-PAA+GNP and 0.6 μg/mL m-THPC while the 7.2 μg/mL D-g-PAA+GNP and 0.72 μg/mL m-THPC samples have equivalent amounts of bright green and DAPI. It is apparent in both Figure 7B & C that MDA-MB-231 cells are not present in samples that received light, meaning that most have likely already been killed with the PDT treatments. Cell nuclei also appear to shrink more with increased nanocomposite concentrations.
Figure 7: MDA-MB-231 cells tested for signs of apoptosis via TUNEL assay 24 hours after PDT treatment with select nanocomposite concentrations. Green fluorescence denotes where DNA has been cleaved. A) Control, B) 6.0 μg/mL D-g-PAA+GNP and 0.6 μg/mL m-THPC, C) 7.2 μg/mL D-g-PAA+GNP and 7.2 μg/mL m-THPC.
**Immunohistochemistry Staining**

The apoptotic pathways occurring as a result of PDT treated MCF10A and MDA-MB-231 cells was evaluated with IHC staining using apoptosis associated proteins cleaved Cas9, BCL2, and cleaved Cas3. Immunoreactivity is evidenced by dark, brown staining where areas where the protein of interest in each case.

Figure 8A shows MCF10A and MDA-MB-231 cells that did not receive any nanocomposite and Figure 6B presents samples of each cell line that received a concentration of 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC. In all cases in Figure 8, activation of proteins associated with apoptosis are hardly seen in MCF10A samples while all cases of MDA-MB-231 with light show the activation of these proteins. Less cells are seen for samples that did receive nanocomposite and light (Fig. 8B); however, the activation of apoptosis associated proteins is not seen for MCF10A cells. There are a few MDA-MB-231 cells in Figure 8A that show reactivity with the IHC staining of each of the apoptosis associated proteins for those that were exposed to light. In Figure 8B, some MDA-MB-231 cells that were not irradiated with light but still received nanocomposite show reactivity while a majority of those that were given both nanocomposite and light treatment reacted with the apoptotic proteins. Based on these images, MDA-MB-231 cells appeared to have the most reactivity of cleaved Cas9 and the least reactivity with BCL2.
Figure 8: IHC staining for cleaved Cas9, cleaved Cas3, and BCL2 24 hours after PDT treatment with select nanocomposite. Activation of proteins associated with apoptosis can be seen where there are black blotches in images. A) Control, B) 7.2 µg/mL D-g-PAA+GNP and 7.2 µg/mL m-THPC.

DISCUSSION

Triple negative breast cancer (TNBC) is the most resilient and aggressive form of breast cancer, being one of the leading causes of death for women, making up 7% of all
cancer deaths, despite only making up 15% of all epithelial breast cancers (Menbari et al., 2020; Denkert et al., 2017). The lack of unique markers to target TNBC makes it difficult to not have other, healthy cells caught up in the process of treating TNBC as the general area, if not the entire body, must be exposed to harmful treatments in order to kill TNBC with traditional therapies. This is why this study aimed to evaluate PDT treatment’s capabilities in treating TNBC with minimum damage to human breast epithelial cells. A hybrid nanocomposite was developed that comprised of D-g-PAA, GNP, and m-THPC with the capability to use passive targeting to kill TNBC cells. This is the first known study to utilize a nanocomposite using these specific components on MCF10A and MDA-MB-231 cell lines.

Incubation time of cells with nanocomposite remained the same throughout this study. A red-light lamp that with 660 nm wavelength red light was used for PDT treatments and suspended 5 inches above cell cultures for 10 minutes with the lid of the plate cells were cultured in off. Different concentrations of nanocomposite were evaluated as part of this process. A 24-hour time point after PDT treatment was chosen in this study due to the MDA-MB-231 cell line capability of proliferating quickly in vitro, meaning longer wait times could lead to discrepancies in results (Wang et al., 2013). It was concluded that this allowed for too much variance and supported that repeated treatments will be necessary for total treatment of TNBC in the future due to there being a lot of variances after 72 hours post-PDT treatment (Fig. 2). Extended time after PDT treatment allows for any remaining cells that might survive to proliferate and have excessive amounts of growth in cell culture, subsequently leading to necrosis due to
overcrowding as cell begin to compete for space and nutrients. With the individual components tested in Figure 2, D-g-PAA was supported to not have any necrotic/apoptotic effects on its own. Significant decreases of cells that did not receive light relative to the control and groups with sizable error bars are likely due to issues in passaging with maintaining two different cell lines. The large increase in Figure 2A for the MCF10A without light containing 2.4 µg/mL D-g-PAA was a result of a significant outlier.

m-THPC has been reported to be effective 24 hours after application (Lange et al., 2019; Kukcinaviciute et al., 2017). The incubation of cells with m-THPC is said to result in sufficient uptake from 3 to 45 hours after application (Teiten et al., 2001; Matteoli et al., 2022). Cells were incubated with nanocomposite for 90 minutes as combined nanocomposite components are shown to have a decreased uptake time (Riley & Day, 2017; Chyan et al., 2018). Removing the nanocomposite after incubation validates that the cells take up the nanocomposite and any effects from PDT treatments is a result of the nanocompoite’s activation.

Analysis of varying concentrations of m-THPC without a polymer carrier or GNP resulted in a concentration that resulted in the decrease of both MDA-MB-231 cells without light to a significant margin and cells with red light treatment as seen with the 1.00 µg/mL concentrtration (Fig. 3). The 0.96 µg/mL of m-THPC had a significant increase in TNBC cells not exposed to light and a significant decrease of cells exposed to light, but if 0.96 µg/mL of m-THPC is to be added in with a polymer carrier it would likely be to potent to be able to control between its effects on cancer and normal cells.
The m-THPC concentration, 0.84 µg/mL, was too indecipherable with increases in cell count for TNBC cells treated with this; therefore, 0.72 µg/mL m-THPC was chosen to be further tested along with the lesser concentration, 0.6 µg/mL m-THPC, to determine the effects of both on MDA-MB-231 cells and MCF10A cells. There were inconsistencies. Cell counts for both MCF10A cells and MDA-MB-231 cells decreased with the addition of nanocomposite (Fig. 4). None of the control groups for cell counts were significantly different. MCF10A cells that received 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC and did not have light irradiation significantly declined but also had the most variation of sample sizes in Figure 4 according to the size of its error bar. This is most likely the result of outliers in the data and possibly Figure 4A containing too few sample sizes. Figure 4B has an n=6 and is likely more precise because of that. Figure 4B displays that there is significant reactivity to the nanocomposite at the concentration 6.0 µg/mL D-g-PAA+GNP and 0.6 µg/mL m-THPC both without and with light but only the light exposed 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC MDA-MB-231 cells had a significant change while those not exposed to light did not. This shows that there is some uptake and reactivity of GNP and/or m-THPC even without the red light. The more acidic pH environment of cancer cells would explain MDA-MB-231’s reaction with nanocomposite without light, but there is also the possibility of dim, ambient light exposure being enough to result in the effects of the nanocomposite. There is also the possibility that nanocomposites at the concentrations of those used in this study are able to act passively.
Cell imaging, on the other hand (Fig. 5), reveals striking evidence that the nanocomposite concentrations used do have effectiveness in regards to killing off MDA-MB-231 cells while leaving MCF10A relatively intact. Distortion to MDA-MB-231 cell morphology is increased relative to MCF10A cell’s morphology in treatment concentrations in terms of loss of cells’ spindle shape and space where cells are not linked together (Luke & Silverman, 2011). This suggests that more MDA-MB-231 cells are being killed off than MCF10A cells with PDT treatments.

Though relatively close, MCF10A cells do not drop below MDA-MB-231 cells in viability (Fig. 6), suggesting that 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC is the maximum concentration of nanocomposite that can be used for TNBC treatment while killing less human breast epithelial cells. This concentration has significant effects on both cell lines based on data discussed so far. The fact that it keeps from having any more of an effect on MCF10A cells’ viability than that of MDA-MB-231’s still makes it feasible to be used in TNBC treatment.

The TUNEL assay clearly shows that apoptosis is evident with the use of nanocomposite. The lack of cells in Figures 7B & C where cells are exposed to red light whereas those in these figures not exposed to light had a similar density of cells compared to the control group (Fig. 7A) evidences that there was likely cell death before the components of the assay were applied. This means that PDT is quick in its effects. Further tests need to be done to validate the mode of nanocomposite in this way.
The lack of activation of apoptotic associated proteins for MCF10A cells shows that apoptosis will only be evident in MDA-MB-231 cells when presented with nanocomposite with light. MCF10A cells did wash away with PBS just as what was shown in Figure 4A; however, cells that are still present would be able to recover and proliferate according to Figure 8 as MDA-MB-231 cells would not be able to recover seeing that apoptotic associated proteins are activated. The apoptosis pathway which this IHC stain evaluated is activated when the cell experiences stress, which in this case would be a result of GNP and m-THPC (McIlwain et al., 2013; Kessel & Oleinick, 2018). This results in the release of cytochrome-c from the mitochondria as a result of Bax which allows for the activation of caspase-9 into its active form, cleaved caspace-9 (Lange et al., 2019; Green, 2000; Löw et al., 2011; Oleinick et al., 2002). The presence of BCL2 indicates the inhibition of apoptosis so that Bax is not released to subsequently initiate cleaved caspase-9 action (Campbell & Tait, 2018; Jürgensmeier et al., 1998; Kluck et al., 1999). This would make sense for the results of Figure 8B as BCL2 is the protein that MDA-MB-231 cells have the least immunoreactivity with as apoptosis is encouraged. Cas3 is activated later in the apoptosis cascade by Cas9 so that cleaved caspase-3 can cleave caspases-6, 7, and 9 so to break down apoptotic cells (Kahyap et al., 2021; Kessel & Oleinick, 2018). The lack of IHC reactivity with the proteins tested in MCF10A cells shows that necrosis and not apoptosis is likely the means that any of this cell line died. The MDA-MB-231 cells having cleaved caspase-9 activated the most within them suggests that apoptosis that did occur was more progressed with cleaved caspase-3 already have enhancing cleaved Cas9 activity and the mechanisms of BCL2’s
inhibition of apoptosis was likely too late in resisting apoptosis for the majority of MDA-MB-231 cells.

CONCLUSION

PDT treatment of TNBC in vitro with nanocomposite comprised of D-g-PAA, GNP, and m-THPC was supported to be effective in the killing the TNBC cell line MDA-MB-231 while showing potential in not having as harmful of an effect on the human breast epithelial cell line, MCF10A. TNBC is a very resilient and aggressive form of breast cancer which typically requires treatments that have potential of causing an assortment of side effects due to the harsh nature of those treatments. PDT shows the potential of having localized effects on tumors without side effects, if any, arising anywhere else in the body. The proposed nanocomposite for this study is designed to allow for the modulated release of tumor fighting components GNP and m-THPC when in the area of a tumor and in the presence of high-energy red light. Though, the concentration of nanocomposite 7.2 µg/mL D-g-PAA+GNP and 0.72 µg/mL m-THPC effected both MDA-MB-231 and MCF10A cell lines when used in PDT treatments, the signs of apoptosis from apoptotic assays and IHC staining suggests that human breast epithelial cells have better potential of recovering from PDT treatment in this way than TNBC cells.
CHAPTER THREE: FUTURE DIRECTIVES

For future research of PDT treatment of TNBC with nanocomposite, further studies should look directly at how PDT treatment compares to other specific forms of cancer treatments. Acceptable results were produced in this study to support further studies into MDA-MB-231 treatment with the PDT protocol. Future studies could attempt to understand what a more optimal dosage of nanocomposite and its components could be in hopes of killing even more TNBC than normal cells. Exact rate of uptake of nanocomposite and its initial point of action are also specifics that need to be better understood.

Only two different cell lines were used in this study, meaning other cell lines of TNBC and human breast epithelial cells ought to be evaluated to ensure the same effect in PDT treatments for TNBC. Other constituents for nanocomposite should also be considered for the enhancement of modulated release of PS. Mixed cultures are to be evaluated with both MDA-MB-231 and MCF10A in order to test the selectivity of the nanocomposite towards cancer.

PDT protocols remained consistent throughout this experiment besides the variation in concentrations tested. This leaves room for further optimization of nanocomposite exposure, red light exposure, and incubation time to name a few. A timepoint should be tested for the most effective light activation of nanocomposite.

Cell counts in this study saw the most variability and would benefit from larger sample sizes and the evaluation of other means of cell counting with other
hemocytometers. More contrast such as the use of trypan blue in brightfield imaging may also help in visualizing the effects of PDT treatment.

Other apoptosis associated proteins need also to be tested in order to identify the exact timeframe of apoptosis in TNBC cells with PDT treatment. The evaluation of the exact effects that nanocomposite has on normal cells as well should be evaluated with proteins involved prior to BCL2 to see if there is any apoptotic effects before the 24-hour timepoint.

This study was conducted strictly in vitro and experiments in vivo would help to evaluate how PDT with the nanocomposite used in this study might bode for actual patients. The penetration of tissues is one of the main concerns for PDT as previously mentioned and future studies would benefit from evaluating if m-THPC or GNP might enhance the range that red light used in PDT is effective. The testing of mice with TNBC tumors will be the first mode of in vitro testing where peripheral circulation of nanocomposite and its elimination can also be evaluated in determining an optimal timepoint for red light exposure.

Determining if and how many repeated treatments of PDT with nanocomposite are necessary for TNBC as this study only utilized a 24-hour timepoint. There are many modes of cancer treatments that are combined with others in order to enhance the effects of both. The combination of other treatments with PDT could be beneficial in bypassing some of the downsides PDT can have such as the use of PTT so that there is further penetration of treatments for deeper tumors (Zhang et al., 2015). With there still being
room for growth for PDT treatment of TNBC, it can be expected that the optimization of PDT treatments will lead to even more selective therapies for breast cancer.
LITERATURE CITED


