Increasing efficiency of photodynamic therapy in in vitro cultured colorectal cancer cells by improved photosensitizer uptake

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Abstract. Colorectal cancer (CRC) is a fatal malignancy with limited therapeutic options and its incidence is on the rise in recent years. Photodynamic therapy (PDT) has emerged as a promising minimally invasive therapeutic modality that employs three fundamentals to induce tumour damage: a photosensitizer (PS), light of a specific wavelength and molecular oxygen. However, PDT has shown undesirable lack of specificity for tumour cells. The aim of this study was to develop a multicomponent nanoparticle-antibody ($ZnPcS_4$ – AuNP-PEG5000-SH-NH_2 - Anti-GCC Ab) based system that is capable of enhanced $ZnPcS_4$ PS delivery within in vitro cultured CRC cells (CaCo-2) to increase the efficiency of PDT. The final conjugate was successfully synthesized and characterized to confirm the efficient binding of the antibody and PS to functionalized gold nanoparticle surfaces. Immunofluorescent results noted that the multicomponent PS nanoconjugate was able to enhance PS uptake in CRC cells, with far more significant cytotoxic responses post-PDT, when compared to PS administration alone. These findings suggest that this synthesized nano-antibody carrier could possibly enhance the in vitro PDT treatment of CRC, however future studies need to confirm its tumor selectivity by comparing its uptake in normal cells.

1. Introduction

Colorectal cancer (CRC) is the world's third most prevalent malignancy and the fourth leading cause of cancer-related mortality [1]. Treatment for CRC continues to be a formidable challenge in oncology due to eminent metastatic incidence and tumour recurrence [2]. Furthermore, currently available CRC conventional treatments such as surgical excision, chemotherapy, radiation therapy, targeted and immunotherapy have rarely yielded appreciable prognosis and are hampered by undesirable side effects in patients [2]. Thus, it remains imperative to develop other therapeutic approaches with increased potency and negligible side effects.

In recent years, photodynamic therapy (PDT) has emerged as a favourable approach to obliterate different cancers [2]. It is based on the uptake of photosensitizer (PS) by tumor cells and subsequent activation of the PS by laser light at a specific wavelength. The activated PS then reacts with molecular oxygen within tumours to generate cytotoxic reactive oxygen species (ROS) [3]. PSs have minimal dark and administration toxicity [4,5]. Preferentially when PSs are administered alone, they tend to passively accumulate more in cancer cells, due to their intrinsic overexpression of low-density

lipoprotein (LDL) receptors [4,5]. However, in most cases this passive accumulation does not render a high enough PS concentration uptakes to produce significant forms of PDT induced cancer cell death [4,5]. Thus, since PDT is non-invasive, with minimal toxicity in comparison to conventional therapies, it is imperative to conduct further research in terms of improving PS accumulation in cancer cells in order to induce significant forms of tumor destruction [4].

Zinc Phthalocyanine Tetrasulfonic acid ($ZnPcS_4$) is a promising metallated second generation PS which has been intensely investigated for PDT cancer applications due to its remarkable photophysical and photochemical properties [6]. It contains a zinc diamagnetic central atom, which produces high quantum ROS yields within the 680 nm wavelength range with minimal dark toxicity [7]. This compound also contains a number of sulfonated thiol groups, which increase its solubility, as well as its distribution in biological tissues and volume of distribution [7]. Studies reported that $ZnPcS_{mix}$ PS and 680 nm laser irradiation at a fluency of 5 J/cm^2, induced a significant 60% of apoptotic cell death within in vitro cultured CRC cells [4]. However, despite the many remarkable attributes PDT has had in oncology, when PSs are administered alone, they inherently are hydrophobic and so tend aggregate when subjected to aqueous conditions [8]. This aggregation profoundly hampers their quantum ROS yields, as well as their bioavailability at target sites [8]. To overcome this issue, nanoparticles (NPs) are often combined with PSs to significantly improve their solubility, through their hydrophilic properties and so enhance PS cellular uptake in cancer cells [8].

Gold nanoparticles (AuNPs) have gained a great deal of attention in PDT applications due to their remarkable photostability, highly modifiable surface chemistry, strong absorption within the near infrared region, high biocompatibility, localized plasmon resonance and high loading capacity [9]. Studies have reported that AuNPs improve PDT treatment outcomes as they protect PSs from biological barriers and so promote passive uptake [6]. Nevertheless, NP PS delivery systems cannot exclusively identify malignant cells and so PS uptake is still often too low, to induce significant forms of PDT tumor cell destruction [10]. Thus, in an effort to enhance PS accumulation in cancer cells, research has focused on the development of antibody NP-based PS delivery platforms [2,8]. Studies by Naidoo et al. (2019) have shown that by developing NP-based PS delivery platforms which have anti-antibody moieties immobilized on their surface (which can identify overexpressed antigens on the surface of malignant cells), PDT tumor cell destruction outcomes are often enhanced, since the selectively bound anti-antibodies promote improved PS uptake and retention [6].

This study combined an efficient cytotoxic oxygen generating hydrophilic tetra sulphonated metal-based Zinc Phthalocyanine Tetrasulfonic Acid PS ($ZnPcS_4$) with CRC-specific targeting antibodies Anti-Guanylate Cyclase (Anti-GCC Ab) on the surface of heterobifunctional PEG amine stabilized AuNPs to enhance PS uptake and retention within in vitro cultured CRC cells, in order to improve PDT tumor cell destruction.

2. Methodology

2.1. Chemical Synthesis of Final Nano Bioconjugate

1 ml of AuNP-PEG5000-SH-NH_2 (Sigma-Aldrich 765309) were added to 1 ml of 125 µM $ZnPcS_4$ (w/v in 0.001M PBS) (Santa Cruz® Biotechnology, sc-264509A) and vortexed overnight at room temperature at 1500rpm to promote spontaneous ligand exchange and absorption. The mixture was then purified by centrifugation (18000rpm for 1hr) and the pellet was resuspended in 1ml 0.001 PBS. To bind the Anti-GCC Ab (ab122404) to the AuNP- $ZnPcS_4$ nanocomposite, an amide bond was achieved using two-step coupling chemistry. The carboxylic terminus of the Anti-GCC Ab was activated with EDC N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and N-Hydroxysulfosuccinimide sodium salt. 200 µg/ml of activated Anti-GCC Ab was then added to 1 ml of AuNP-$ZnPcS_4$ nanocomposite and vortexed overnight to allow for the activated carboxylic moiety succinimidyl ester of the Ab to react with the amine functionalized group on AuNPs so a strong amide bond could be established. Characterization of this final nano bioconjugate (FNBC) was conducted using UV-visible spectroscopy.

2.2. Cell Culture, Dose Response, FNBC and PDT Laser Irradiation Assays

Commercially purchased CaCo-2 CRC cell line CaCo-2 (Cellonex Cat SS1402 CCAC-FL; CCAC-C) was used in this study. Cells were cultured in Dulbecco's Modified Eagle Medium (D5796, Sigma Aldrich) enriched with 10% Foetal Bovine Serum (FBS), 4mM sodium pyruvate, 4mM L-glutamine, 2.5 g/ml amphotericin B, and 100 U Penicillin 100 g/ml streptomycin solution in an 85% humidified condition at 37ºC and 5% CO_2. Control and experimental culture dishes were seeded with cells at a density of 6 x 10^5 cells and incubated for 4hrs to allow for attachment. Dose response studies were performed to determine the lowest concentration of $ZnPcS_4$ PS alone, which could yield 50% cytotoxicity (ICD_{50}), in order to determine the optimum concentration PS loading required within the FNBC. Within FNBC assays control groups received $ZnPcS_4$ or $ZnPcS_4$–AuNP treatments, whereas experimental groups received FNBC treatments. After $ZnPcS_4$ PS dose response or FNBC additions, culture plates were incubated for an additional 20 hrs. Post 24 hrs groups which required PDT laser treatment were irradiated using a continuous semiconductor diode laser (Oriel Corporation) at wavelength of 673 nm, with a fluence of 10 J/cm^2. Following treatment, all control and experimental groups were further incubated for 24 hrs in fresh media, before cytotoxicity and cell death analysis assays were performed.

2.3. Subcellular Localization of FNBC

CaCo-2 cells were seeded at density of 6 x 10^5 cells/ml in culture dishes containing a coverslip and post attachment received $ZnPcS_4$, $ZnPcS_4$–AuNP or FNBC only without irradiation. After 24h of incubation, the cells were stained for 30min with 2 µg/ml ICAM-1 mouse monoclonal IgG1 (AbAB2213 AC: Abcam) and 5 µg/ml Goat anti-mouse IgG-FITC (AB6785 AC: Abcam) on ice, respectively. Thereafter, the cells were stained with µl of 1µg/ml DAPI for 5 min and rinsed with HBSS. The coverslips were then mounted on slides and examined using the filter settings of a Carl Zeiss Axio Z1 Observer immuno fluorescent microscope.

2.4. Cellular Cytotoxicity

CytoTox96® non-radioactive assay (Promega G1780) assay was used to measure lactate dehydrogenase (LDH) released from cellular supernatant cytosol to detect membrane damage prior and post treatment.

2.5. FNBC Cell Death Analysis

Annexin V-FITC/PI cell death detection kit (BD Scientific: BD/556570), was used for detection and quantitation of cells undergoing early or late apoptosis, cells dying from necrosis cells, or cells remaining viable post-PDT treatment using the BD Accuri™ C6 flow cytometer.

3. Results and Discussion

3.1. PS Dose Response Studies

With reference to Figure 1, cells treated with varying doses of $ZnPcS_4$ PS alone, showed no statistical significance in LDH release, when compared to untreated control cells. Irradiated cells incubated with 0.0625 µM to 0.25 µM $ZnPcS_4$ produced significant dose-dependent increases in the amount of LDH released, when compared to untreated control cells. Within irradiated cells groups treated with 0.125 µM $ZnPcS_4$, a significant 45% (indicated by ** in Figure 1) of LDH cytotoxicity was reported and so was the recommended ICD_{50} concentration, which was utilized within FNBC experiments.

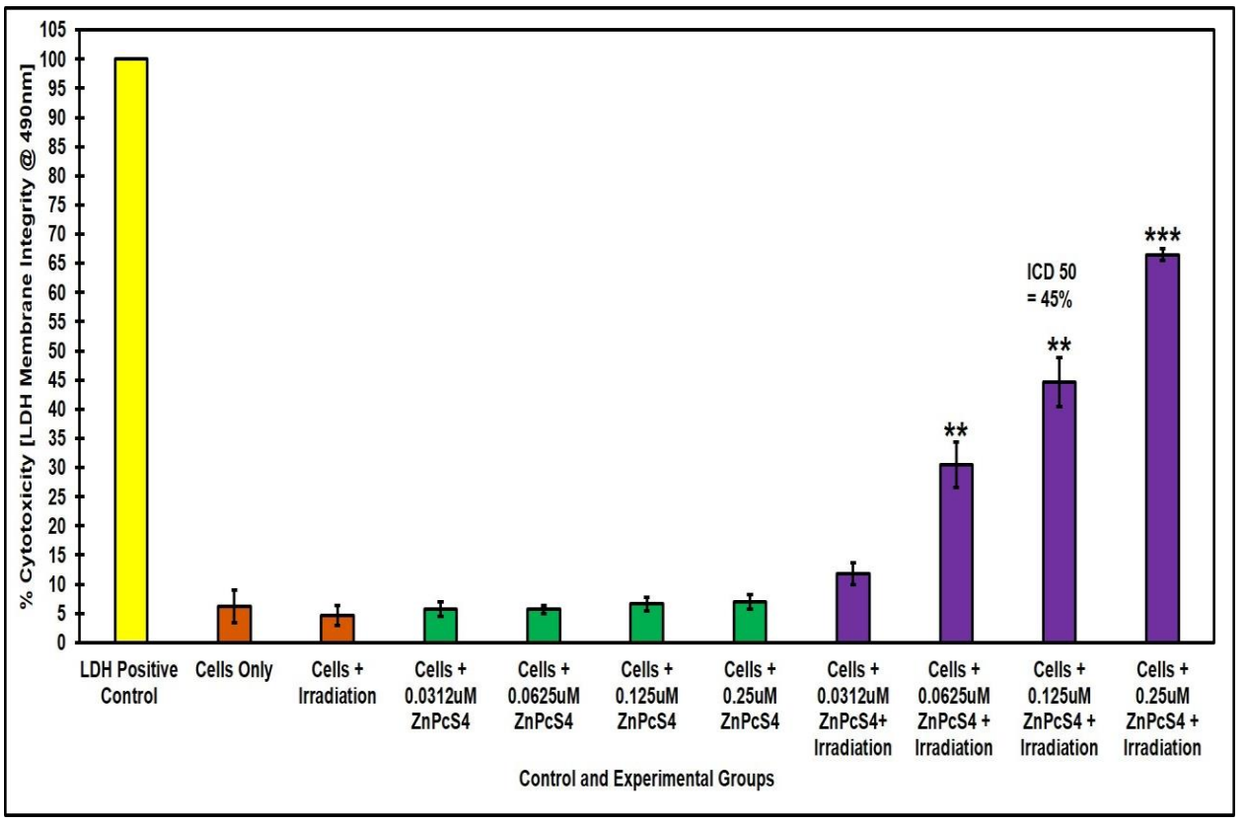

Figure 1. Varying doses of $ZnPcS_4$ administered to CaCo-2 cells with and without laser irradiation showing a dose-dependent increase in the amount of LDH release.

3.2. Characterization of the FBNC

With reference to Figure 2, $ZnPcS_4$ and AuNP-PEG5000-SH-NH_2 retained their respective visible absorption peaks after FNBC conjugation, however with some reduction in absorption, suggesting that these two compounds had successfully bound together. The FNBC noted a slight shift in the resonance peak position of AuNP at 520 nm, signifying that the Anti-GCC Ab had been successfully conjugated to its surface. Moreover, the FNBC retained its prominent $ZnPcS_4$ 673 nm absorbance peak, which meant that the photochemical characteristics of the PS were conserved after conjugation, suggesting that PDT experiments were possible. A slight broadening in the absorption peaks of the FNBC was seen since additional conjugates increased its overall molecular size. Furthermore, the peaks remained smooth suggesting no aggregation occurred. Overall, the FNBC consisted of 0.95 x 10^{15} particles/ml AuNPEG5000-SH-NH bound to 35 µM of $ZnPcS_4$ in 0.001 M PBS (w/v), which was diluted to 0.125 µM $ZnPcS_4$ in relation to the recommended ICD_{50} PS dose concentration that was reported above.

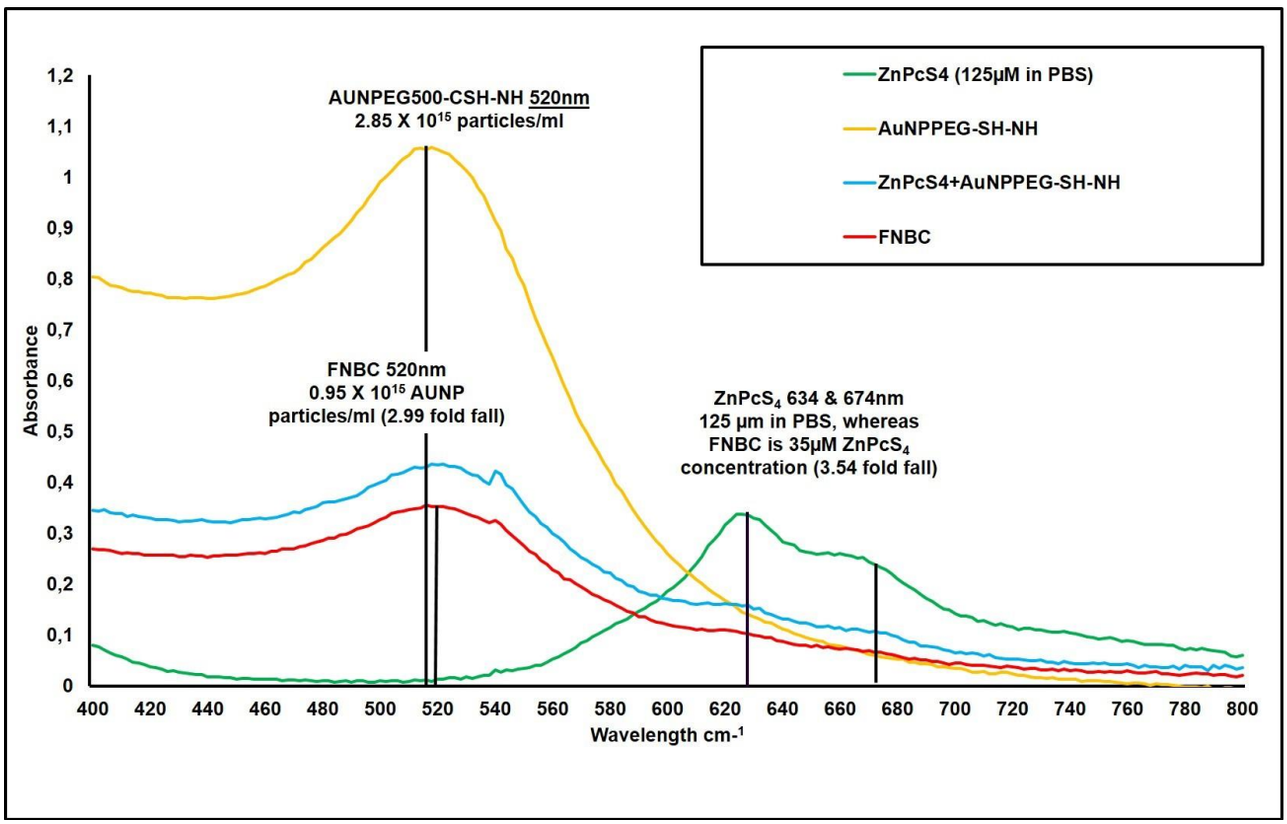

Figure 2. UV-Visible absorption spectral and fluorescence analysis of the FNBC and control groups.

3.3. Subcellular Localization Studies of the FNBC

Live cell immunofluorescent staining and imaging noted a far more improved PS bioavailability in CRC cells which received the FNBC, versus $ZnPcS_4$ PS or $ZnPcS_4$–AuNP alone control administrations (Figure 3). This improved PS accumulation in CRC cells can be observed by the increased red intensity observed in images, which received the FNBC.

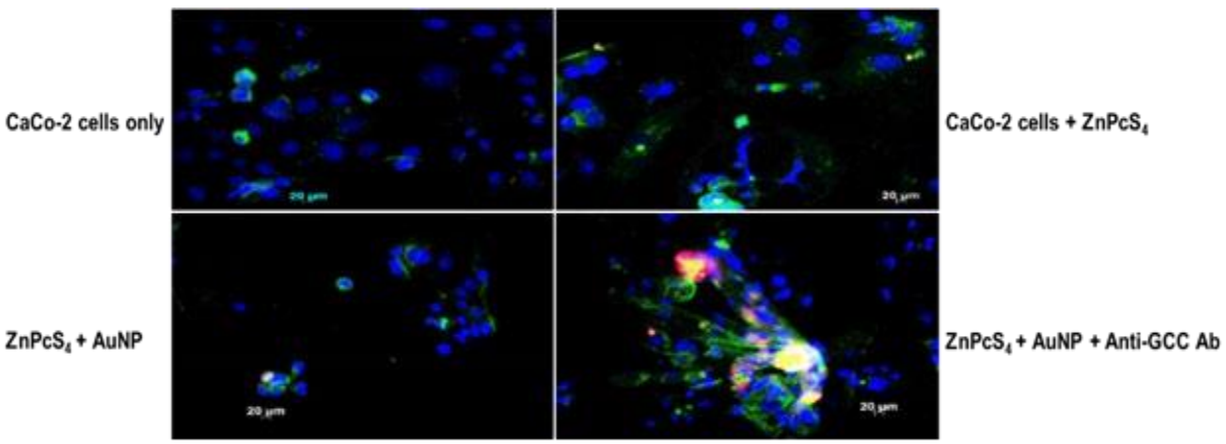

Figure 3. Subcellular localization of $ZnPcS_4$ PS alone, $ZnPcS_4$–AuNP and FNBC within CaCo-2 cells, showing blue stained DAPI nuclei, green stained cellular membrane proteins and red fluorescent $ZnPcS_4$ PS localization.

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These findings suggest that the Anti-GCC Ab within the FNBC enhanced the $ZnPcS_4$ PS accumulation within CRC far more superior than its administration alone. Considering that the only difference within the experimental FNBC group versus the control groups was the fact that it contained Anti-GCC Ab, these results are suggestive that the anti-antibody must have promoted this improved PS uptake. However, in order to confirm if this enhanced FNBC PS uptake in CRC cells was due to selective tumor targeting, future research would need to be conducted in normal cells.

3.4. Cell Death Analysis of the FNBC

Annexin V-FITC/PI cell death detection kit was used differentiate between live, apoptotic, and necrotic forms of cell death in CRC cells. With reference to Figure 4, no significant differences in cell death percentages were noted in control groups which received laser irradiation, $ZnPcS_4$ PS, $ZnPcS_4$–AuNP or FNBC alone when compared to the cells only control, which received no treatment.

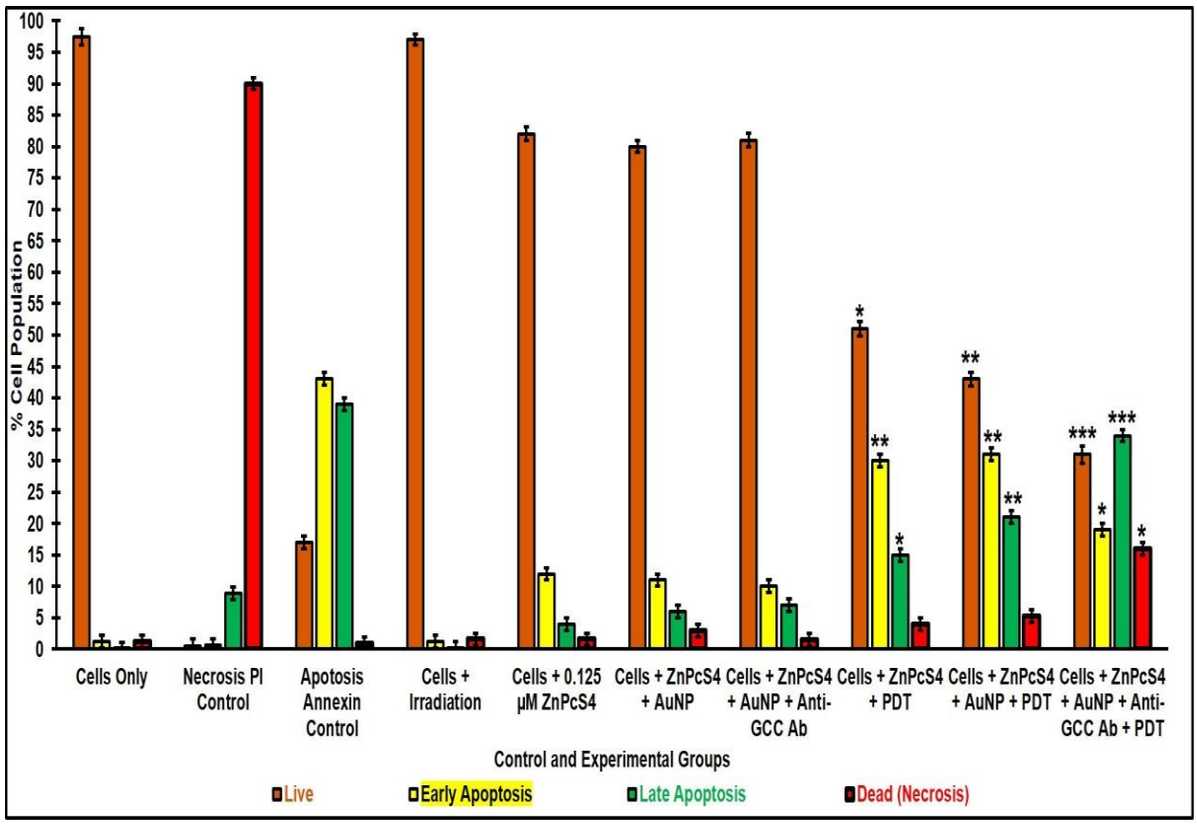

Figure 4. Cell death analysis of control and experimental groups

Irradiated experimental groups which received $ZnPcS_4$ PS, $ZnPcS_4$–AuNP or FNBC noted significant increases in apoptotic cell death, with lowered cellular viability. However, irradiated experimental groups treated with $ZnPcS_4$ PS alone, exhibited less significant late apoptotic cell death (15% - indicated by * in Figure 4), when compared to groups which were treated with $ZnPcS_4$–AuNP (21% - indicated by ** in Figure 4). Furthermore, when comparing these two experimental groups to irradiated FNBC treated cells, a highly significant apoptotic cell death (34% - indicated by *** in Figure 4), with a significant increase in necrotic cell death (16% - indicated by * in Figure 4) was found. Studies have reported that the most favored modes of cell death in relation to successful PDT treatment outcomes are late apoptosis and necrosis, since they destroy cells beyond recovery, unlike early apoptosis, whereby cells can rejuvenate causing potential tumour reoccurrence [6]. Irradiated

experimental groups which were treated with $ZnPcS_4$ PS alone, possibly did not exhibit as much significant late apoptotic cell death (15%), since studies have noted that when this PS is administered alone it tends to aggregate due to its hydrophobic nature, hindering is PS accumulation in cancer cells and so limits it overall PDT cell death outcomes [8]. The significant 21%** late apoptosis observed in irradiated groups treated with $ZnPcS_4$–AuNP, was attributed to the fact that the PS was conjugated to AuNPs and studies have reported that they improve the passive uptake of PSs in cancer cells due to their hydrophilic nature [8]. The highly significant and favorable forms of PDT induce cell death (34%*** late apoptosis and 16%* necrosis) which was found in irradiated groups which received FNBC, was possibly due to the fact that FNBC had Anti-GCC conjugated onto its surface, since previous studies have noted that antibody moieties immobilized on NP surfaces can promote improved PS uptake and retention in cancer cells [6]. However, in order to fully realize and confirm if the improved PS uptake in CRC cells was due to the FNBC selective targeting abilities future studies using comparative normal cells needs to be investigated.

4. Conclusion

$ZnPcS_4$ PS was successfully conjugated onto AuNP-PEG5000-SH-NH_2 NPs surface to increase its solubility and uptake in CRC cells. Furthermore, Anti-GCC Ab was immobilized onto AuNP-PS surfaces to observe if the FNBC was capable of even further enhanced PS retention in CRC cells. Subcellular localization assays revealed that the FNBC significantly increased the intracellular accumulation of $ZnPcS_4$ within CRC cells far more than the administration of $ZnPcS_4$ PS alone or when conjugated to AuNPs. Furthermore, the FNBC post PDT induced a far more significant cell death, when compared to $ZnPcS_4$ PS, or $ZnPcS_4$–AuNP treatment alone. These findings suggest that the conjugation of Anti-GCC Ab to $ZnPcS_4$–AuNPs, enhanced PS delivery in CRC cancer cells. Overall, FNBC showed enhanced cellular PS retention in CRC cells and so improved PDT cytotoxic treatment outcomes enormously. However, this study warrants further investigation to compare the uptake between in vitro cultured CRC and normal cells in order to determine whether the improved PS uptake in CRC cells was due to selective Anti-GCC Ab targeting.

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