Investigation of a specifically targeted photosynthetic nanoparticle drug delivery system for enhanced photodynamic therapy treatment of metastatic melanoma

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Abstract. Metastatic melanoma (MM) is the 6th most common cancer diagnosed worldwide, with approximately 100, 000 annual related deaths. Photodynamic therapy (PDT) is a photochemotherapeutic cancer treatment that utilizes a photosensitizer (PS) drug that, when activated by laser light at a specific wavelength, yields reactive oxygen species (ROS), which in turn induces cell death. However, due to the passive diffusion of PSs, normal surrounding cells are sometimes affected and their targeted concentrations in cancer cells tends to be minimal, thus limiting the effectiveness of this treatment. Therefore, a multicomponent drug targeting strategy is often applied to improve PS specific delivery and concentration in cancer cells only, which in turn can improve the effectiveness of PDT. Thus, the intention of this study was to improve the photosynthetic drug delivery of Zinc Phthalocyanine Tetrasulfonic acid ($ZnPcS_4$) in MM cells, by enhancing its chemical structure. $ZnPcS_4$ was successfully conjugated to pegylated gold nanoparticles (AuNP) to maximize its solubility and stability, as well as bound to active tumour-associated antibody-antigens (Anti-MIA Ab) to aid in specific targeted PS delivery. Within *in vitro* cultured MM (A375) cells, the final molecular drug conjugate proved to have enhanced subcellular uptake in cells, when compared to $ZnPcS_4$ PS drug delivery alone. Moreover, after conducting *in vitro* PDT experiments a significant amount of 76% cytotoxicity was noted in cells which were treated with final molecular conjugate, versus 48% cytotoxtcity when $ZnPcS_4$ PS drug was administered alone. Suggesting, that overall the final molecular drug conjugation combination of $ZnPcS_4$ with AuNP and anti-MIA Ab, proved to enhance the treatment capabilities of PDT for MM through improved PS drug delivery via active targeting mechanisms.

1. Introduction

Cancer is the rapid abnormal proliferation of cells to produce either benign or malignant tumours.[1] Malignant tumours often spread to surrounding tissues and move throughout the body using circulatory or lymphatic systems, causing metastasis.[2] Melanoma is extremely invasive and due to this it is considered one of the deadliest skin cancers at present.[3] MM is the 6th most common cancer diagnosed worldwide, with approximately 100, 000 annual related deaths.[4] Melanoma originates in the deepest regions of the epidermis and in the beginning of the dermis, where melanocytes which produce the melanin pigment are located.[5, 6] MM is Stage IV of skin cancer and occurs when cancerous cells have metastasized and developed in other organs that are located far from the primary site of occurrence.[5] In the last 50 years the incidence rate of MM has increased more significantly when compared to other types of cancer.[6] It is crucial to detect melanoma in the early stages, as once it metastasized it is difficult

to locate where it originated, and so plays a major role in patient survival rates as surgery is no longer an option due to multiple secondary sites and thus makes it more difficult to treat.[6]

PDT is an unconventional treatment used to treat cancer, which has been investigated for the past 3 decades.[7] It utilizes a PS which is a light sensitive drug that when activated at a specific wavelength causes excitation.[8] This excitation process causes the PS to produce ROS, which induce damage to the cells through photo-cytotoxicity caused by oxidative stress which renders the cells inactive.[9] This is a less invasive form of cancer treatment as the cancerous tumour region is targeted and produces localized destruction with limited side effects when compared to conventional treatments like chemotherapy and radiation. [10]

The PS Zinc Phthalocyanine Tetrasulfonic acid ($ZnPcS_4$) is a second generation metallated phthalocyanine sensitizer that can be used in phototherapeutic applications, with minimal dark toxicity.[11] It contains a zinc diamagnetic central atom, which determines not only the high triplet state quantum yields of ROS, but also the prolonged lifespan of the molecule once excited.[11] The PS also contains various sulfonated thiol groups which makes it hydrophilic and so increases its solubility in terms of cellular uptake.[12] Studies reported post-irradiation that when 5 μM of $ZnPcS_4$ was applied to in vitro cultured human MM cells and PDT light induced at a wavelength of 680nm with a fluence of 10 J/cm^2, more than 50% growth inhibition and apoptotic cell death cytotoxic side-effects were noted.[13]

Passive targeting strategies are often accomplished when the PS that is bound to the nanoparticle (NP) accumulates within the tumour cells, this passive diffusion is due to the characteristics that the NPs have.[14] For effective PDT, functionalized NP platforms need to be used to enhance PS drug delivery and each type has their own individual advantages, whether it may be passively or actively absorbed by tumour cells.[15] The characteristics which NP exhibit are that they have a large surface-area-to-volume ratio; they have simple surface chemistry with the possibility of engineering and functionalization; small dimensions so they can easily accumulate in cells due to the enhanced permeability and retention (EPR) effect; and NPs go undetected by the immune system barriers as they mimic biological molecules and can combine to other molecules such as PSs which improves and enhances drug delivery. [16] AuNPs have been extensively investigated in PDT induced cancer treatments as they have tuneable optics and photothermal properties, which allows for the conversion of laser light into heat improving targeted cellular destruction. [17] NPs can be further functionalized into active targeting molecules through the attachment of molecules which are specifically compatible to targeted receptor tumour sites on the surface of the cancer cell. [16]

Active targeting strategies have been used by incorporating cell-targeting peptides or antibodies (Ab) onto a NP surface is highly desirable in PDT therapeutic applications, as it allows for selective cell tumour cell targeting.[17] The protein Melanoma Inhibitory Activity (MIA) was identified as a key component that was involved in the progression and metastasis of MM.[18] Studies noted that MM cells tend to over express MIA, as it is a melanoma-cell specific antigen, which plays a vital role in carcinogenesis and is not expressed in melanocytes. Thus, MIA has been utilized as a biomarker for diagnosis and detection of MM as it is highly specific and a sensitive marker for MM. [18]

2. Methodology

2.1. Chemical Synthesis of Multicomponent Nanoparticle Drug Based System

Equal ratios of HS Pegylated 500 amine functionalized AuNP (Sigma 765309) were added to 500μM $ZnPcS_4$ (w/v in 0.001M PBS) (CAS 61586-86-5) and vortexed overnight at room temperature, with 15000rpm rotation speed to promote spontaneous ligand exchange and absorption. The mixture was then purified by centrifugation at room temperature (15000rpm for 1hr) and the pellet was re-suspended in 1ml 0.001M PBS. To conjugate the Anti-MIA Ab (ab166932) to the previous AUNP-$ZnPcS_4$ molecular suspension, an amide bond was achieved through EDC N-(3-Dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride and NHS (N-Hydroxysulfosuccinimide sodium salt), through a two-step coupling activation of the carboxylic terminus of the Anti-MIA Ab, which was then purified by

ultrafiltration centrifugation. 200 µg/ml of activated Anti-MIA was then added to 1ml of conjugated AuNP-$ZnPcS_4$ and vortexed overnight. This allowed the activated carboxylic moiety succinimidyl ester of the Ab to react with the amine functionalized group on AuNP and so stable amide bond was induced. Characterization and photostability of the final molecular conjugate were performed using UV-visible spectroscopy.

2.2. Cell Culture

MM cancer (A375) cells were cultured in Dulbecco's modified Eagle's media, 15% foetal bovine serum, 0.1% penicillin-streptomycin and 0.1% amphotericin-B, with incubation at 37°C, 5% CO_2 and 85% humidity. Cells (2.5 X 10^5/ml) were seeded into various experimental and control culture dishes. Dose response studies were conducted to determine the lowest concentration to induce 50% cytotoxicity (ICD50), which is required when administering $ZnPcS_4$ alone, to regulate and compare the effective PDT concentration that was needed within final molecular conjugate PDT experiments which displayed enhanced cellular targeting abilities. The PDT results are not shown; however, it was noted that 2.5µM $ZnPcS_4$ was the most effective in inducing ICD50, in MM cells, thus within final molecular conjugate experiments MM cells received 2.5µM $ZnPcS_4$ + AUNP + Anti-MIA Ab. Laser photo-irradiation experiments were conducted using a continuous semiconductor diode laser (Oriel Corporation) emitting at a wavelength of 673 nm with a fluence of 10J/cm^2. All control and experimental groups after treatment were then additionally incubated for 24 hrs in fresh, drug-free media before cytotoxicity biochemical responses were determined.

2.3. Sub Cellular Localization of Multicomponent Nanoparticle Drug Based System

Immunofluorescent staining was used to determine the successful uptake of ZnPcS4. A375 cells were grown on glass coverslips in culture dishes. An hour prior to observation, cells were stained with ICAM-1 mouse monoclonal IgG, goat anti-mouse IgG-FITC human absorbed fluorescein13, to enhance membrane matrix proteins for observation, and then fixed with paraformaldehyde for nuclei counterstaining with 40-6-Diamidino-2-phenylindole (DAPI). Slides were examined using the Carl Zeiss Axio Z1 immunofluorescent microscope, to observe blue DAPI counter stained nuclei and green FITC ICAM-1 membrane proteins in cells, while the Cy5 auto fluorescent signal of $ZnPcS_4$ was noted to detect enhanced cellular uptake of the final conjugate in cells.

2.4. Cytotoxicity Biochemical Assays

The non-radioactive CytoTox96® assay (Promega G1780) was used to measure lactate dehydrogenase (LDH) supernatant released from cells cytosol upon cellular membrane damage before and after treatment in 96 micro well plates. Absorbance was read using a Victor^{3}microplate reader (Perkin-Elmer) at 490nm and results interpreted quantitatively.

3. Results and Discussion

3.1. Characterization of Multicomponent Nanoparticle Drug Based System

The final molecular conjugate was found to be photostable over its period of experimental utilization. With reference to Figure 1, the AuNPPEG-CSH-NH and $ZnPcS_4$ visible absorption spectrum peaks were not affected after conjugation and remained prominent, however lowered in absorption and so a final conjugate concentration of 227µM $ZnPcS_4$ was found to be bound to 0.89 X 10^{15} AuNP particles/ml, which could effectively be diluted to 2.5µM $ZnPcS_4$ in relation to ICD50 dose response assays. Moreover, the $ZnPcS_4$ absorption spectrum peak at 673nm remained present after final conjugation, suggesting the PS properties were preserved for PDT effective ROS and singlet oxygen yield production. Lastly, the peaks of the final molecular conjugate did slightly broaden, however remained smooth, suggesting an increase in molecular size due to bonding with additional components with minimal

aggregation and the slight shift in resonance peak position of AuNP after conjugation indicated the Ab and PS had been successfully conjugated to its surface.

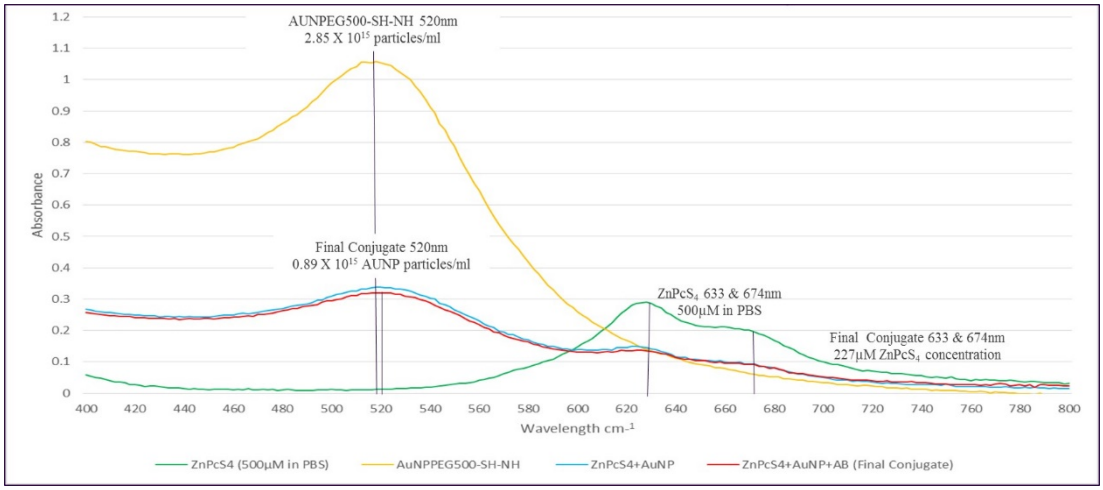

Figure 1: UV-visible spectrophotometry absorption and fluorescence spectra of final molecular conjugate and controls.

3.2. Sub Cellular Localization of Multicomponent Nanoparticle Drug Based System

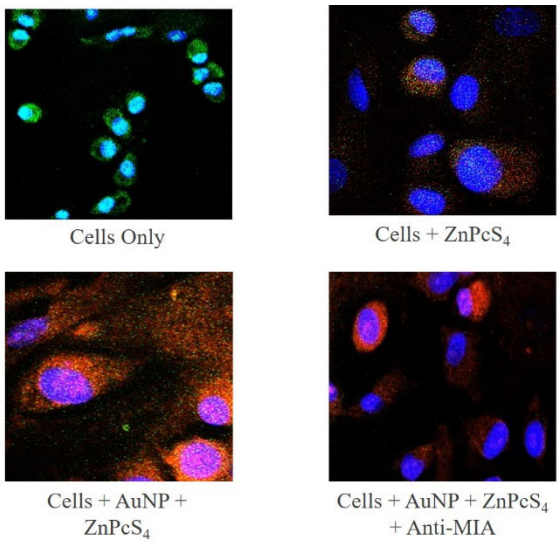

Figure 2: Immunofluorescent Staining of final molecular conjugate and controls.

Live cell imaging immunofluorescent staining was used to determine subcellular localization and uptake of the PS ($ZnPcS_4$) alone versus the final molecular conjugate (AUNPPEG500-SH-NH + $ZnPcS_4$ + Anti-MIA Ab) within in vitro cultured MM cells (Figure 2). MM cells showed poor uptake of $ZnPcS_4$ PS drug alone, however when conjugated to the AuNP and Anti-MIA Ab, improved and concentrated PS drug uptake was observed in cellular cytoplasm and nuclei. These findings suggest that the targeting affinity of the PS drug conjugate in relation to Anti-MIA Ab biomarker specify for MM A375 cells was functional, and so improved the subcellular localization and concentration uptake within these cells.

3.3. Cytotoxicity Biochemical Assays

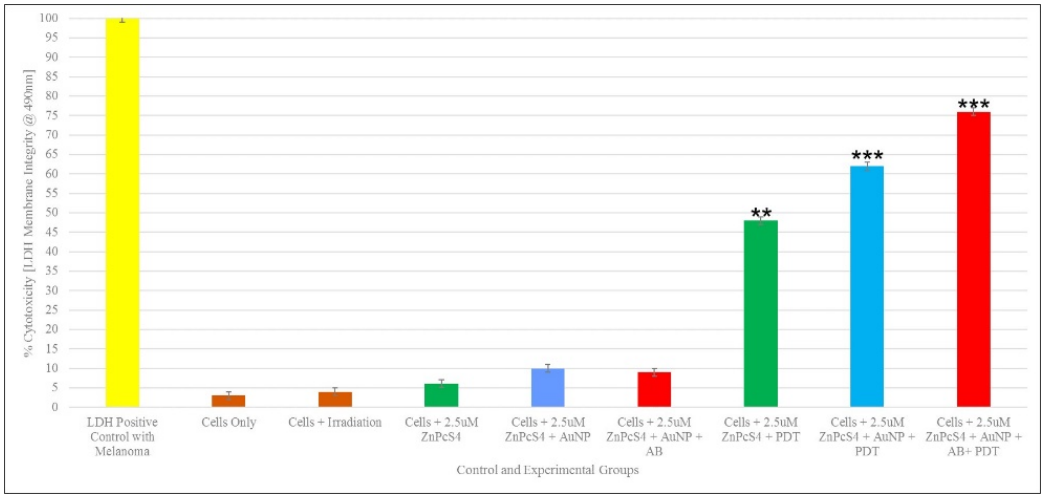

Figure 3: Cellular Cytotoxicity of Final Molecular Conjugate before and after PDT

With reference to Figure 3, the LDH positive control represents 100 % cellular cytotoxicity in MM cells, due to cell lysis and so was used as a standard reference to calculate percentage cytotoxicity in all control and experimental groups. The control group of cells only, was used as a standard reference for viable MM cells, which obviously reflect no cellular cytotoxicity, for statistical comparison in all other control and experimental groups. In control groups, which received laser irradiation only, no significant increase in cellular cytotoxicity was noted. This indicates that laser irradiation at 673 nm with a fluency of 10 J/cm^2 has no phototoxic side effect on cultured MM cells. Within control groups which received 2.5 µM of $ZnPcS_4$ PS drug only, no significant cytotoxicity was noted. These results suggest that the administration of PS drug to MM cells in the absence of light has no dark toxic side effects. Additionally, there was no significant cellular cytotoxicity observed in MM control groups, which received control 2.5 µM $ZnPcS_4$ – AuNP-PEG5000-SH-NH_2 or experimental 2.5 µM $ZnPcS_4$ – AuNP-PEG5000-SH-NH_2 – Anti-MIA Ab final molecular PS drug conjugate administration alone. These findings indicate that even when the $ZnPcS_4$ PS drug was bound to either AuNP-PEG5000-SH-NH_2, as well as Anti-MIA Ab, it remained stable pre-PDT and had no cytotoxic effects on cells. The results from these control groups were used as a reference so that the findings from the final molecular conjugate PDT experimental groups could be compared to these control references, making it possible to identify significant increases in cellular cytotoxicity ($P < 0.05$*) ($P < 0.01$**) ($P < 0.001$***).

All PDT treated experimental groups showed a highly significant cellular cytotoxicity values when compared to the control of MM cells only, however these significant increases percentage values varied over the different groups. Within PDT experimental groups which received 2.5 µM $ZnPcS_4$ PS drug only and 10 J/cm^2 laser irradiation, a significant increase of 45 % (***) cellular cytotoxicity was noted. Whereas, within PDT experimental groups which received 2.5 µM $ZnPcS_4$ – AuNP-PEG5000-SH-NH_2 and 10 J/cm^2 laser irradiation an even higher significant increase of 59 % (***) was found. These findings suggest that the binding of $ZnPcS_4$ PS drug to AuNP-PEG5000-SH-NH_2 promoted its passive uptake in cultured MM cells and so when subjected to laser irradiation their PDT induced cytotoxicity significantly increased. However, the most significant increase of 76 % (***) in cellular cytotoxicity was found in PDT experimental groups, which received 2.5 µM $ZnPcS_4$ – AuNP-PEG5000-SH-NH_2 – Anti-MIA Ab. Overall, the final molecular $ZnPcS_4$ PS drug conjugate induced far more significant cytotoxicity (76%) in MM cells after PDT treatment, when compared to PS drug alone PDT treatment

(48%) at the same PS concentration. These findings suggest that the conjugation of Anti-MIA Ab to $ZnPcS_4$ – AuNP-PEG5000-SH-NH_2, within the final molecular conjugate actively, as well as specifically enhanced PS drug uptake in MM cancer cells and so improved PDT cytotoxic treatment outcomes enormously.

4. Conclusion

$ZnPcS_4$ PS was successfully conjugated to PEG500-SH-NH, to maximize its solubility and stability, as well as bound to active tumour-associated antibody-antigens (anti-MIA Ab) to aid in specific targeted PS delivery. Within in vitro cultured MM (A375) cells, this final molecular drug conjugate proved to have far more enhanced and concentrated cellular uptake of $ZnPcS_4$ PS, than when just administered without drug delivery modification. Moreover, the final molecular $ZnPcS_4$ PS drug conjugate induced far more significant cytotoxicity (76%) in MM cells after PDT treatment when compared to PS drug alone PDT treatment (48%) and the same PS concentration was applied.

This suggests that the multicomponent AuNP PS drug-based system did have specific MIA antigenic targeting abilities for the active and far more concentrated photosynthetic drug delivery of $ZnPcS_4$ in MM cancer cells. Thus, the final molecular conjugate did in fact enhance PDT treatment in MM cells, though increased theranostics, as well as far more specific and targeted PS drug delivery, improving cellular uptake and overall cytotoxicity cell death induction. In relation to this studies increased specificity of PDT treatment, future investigative approaches will include the use of another normal cell line in order to be able to compare efficiency result outcomes.

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