Towards optimisation of surface enhanced photodynamic therapy of breast cancer cells using gold nanoparticle-photosensitiser conjugates

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Abstract

Gold nanoparticles (AuNPs; *ca.* 4 nm) were synthesised and functionalised with a mixed monolayer of polyethylene glycol (PEG) and one of two zinc phthalocyanines (ZnPcs), the difference between the two molecules was the length of the carbon chain that connects the Pc to the gold core. The chain was composed of either three (C3Pc) or eleven (C11Pc) carbon atoms. The C11Pc photosensitiser displayed higher fluorescence emission intensity than the C3Pc in solution. By contrast, the C3Pc photosensitiser exhibited higher fluorescence when bound to the surface of the AuNPs than the C11Pc, despite the shorter carbon chain which was expected to quench the fluorescence. In addition, the C3Pc nanoparticle conjugates exhibited an enhancement in the production of singlet oxygen ($^1O_2$). The metal-enhanced $^1O_2$ production led to a remarkable photodynamic efficacy for the treatment of human breast cancer cells.

Introduction

Photodynamic therapy (PDT) of cancer is a minimally invasive treatment that involves the activation of a photosensitiser with visible or near-infrared light, leading to the production of reactive oxygen species, including singlet oxygen ($^1O_2$), and thus cell death.[1-2] Optimal photosensitisers are typically hydrophobic in character, enabling interactions with the lipid bilayers of the cell membrane.[3-4] However, the hydrophobicity of photosensitisers hinders the delivery and transport through the body.[3-5] To avoid such issues, metal nanoparticles have been used as delivery systems for PDT since they can be tailored to provide water solubility while the photosensitiser retains its hydrophobic properties.[3-6] A further advantage of combining metal nanoparticles with photosensitisers is the reports of enhanced $^1O_2$ production as compared to the free photosensitiser.[7-8]

Metal-enhanced fluorescence (MEF) has been particularly studied as a means of enhancing the fluorescence of fluorophores placed on the surface of metal nanoparticles, including photosensitisers.[9-10] MEF depends on several factors, including the size and shape of the nanoparticles, the distance between the core of the nanoparticle and the fluorophore and the orientation of their respective molecular dipoles.[11-14] It has been reported that it is possible to enhance the fluorescence of fluorophores placed *ca.* 5-30 nm away from the metal core, while shorter distances could lead to fluorescence quenching.[10] The enhancement in the fluorescence has been reported to be due to the non-radiative energy transfer between the fluorophore and the surface plasmon (SP) of the metal nanoparticles.[15] The SP of the metal nanoparticles is then able to re-radiate the transmitted energy of the coupling fluorophore, leading to an increase in fluorescence.[15] Of importance for PDT, a relationship between MEF, metal-enhanced phosphorescence (MEP) and metal-enhanced $^1O_2$ production (ME$^1O_2$) has been reported.[16-18] Even though MEF, MEP and ME$^1O_2$ are competitive processes, these phenomena have been reported to occur within the same system.[17-18] Geddes and co-workers have provided two explanations by which MEF and MEP can occur simultaneously.[17-18] Firstly, there is an increased net absorption by the system, leading to an increase

in both singlet and triplet yields. Secondly, reverse intersystem crossing could be possible, by which the excited triplet state transmits its energy to the singlet excited state.[17-18]

The enhancement in $^1O_2$ production as a result of MEF has been found to be distance-dependent in the same manner as MEF.[16-20] Huang et al. conjugated the photosensitiser chlorin e6 to gold nanorods and showed MEF, which correlated with a high $^1O_2$ production and strong PDT effect in vitro as compared to the free chlorin e6.[21] The use of MEF to reactivate the quenched fluorescence of fluorophores on the surface of gold nanoparticles upon aggregation has also been reported for PDT.[22] However, to the best of our knowledge, studies on the use of gold nanoparticles (AuNPs) for distance-dependent, metal-enhanced PDT have not been reported. Herein we describe, for the first time, the use of AuNPs in combination with zinc phthalocyanines (ZnPcs) for a metal enhanced photodynamic effect for treating breast cancer cells. AuNPs were functionalised with a mixed monolayer of a hydrophobic ZnPc and polyethylene glycol (PEG). Two ZnPcs, consisting of a dimeric structure composed of two macrocycles linked to one another by a disulfide bond, were explored. The difference between the two ZnPcs relies on the length of the carbon chain that connects the macrocycle to the sulfur atom and hence the surface of the AuNPs. One ZnPc is connected via three carbon atoms (C3Pc), whereas the other is connected via eleven (C11Pc) (Fig 1).[23] In solution, the C11Pc photosensitiser exhibited significantly greater fluorescence than the C3Pc. Intriguingly, despite the shorter anchor chain and the expectant quenching of the fluorescence of the C3Pc photosensitiser, the C3Pc was found to be more fluorescent when attached to the surface of the AuNPs than the C11Pc with the longer chain. Additionally, the C3Pc-PEG-AuNP conjugates were observed to produce an increase in $^1O_2$, as a consequence of ME$^1O_2$. The enhancement of $^1O_2$ production of the C3Pc-AuNP conjugates elicited a remarkable photodynamic efficacy for the treatment of SK-BR-3 human breast cancer cells.

Experimental

Materials

All reagents were of analytical grade, used as received and purchased from Sigma-Aldrich (UK) unless otherwise stated. Tetrahydrofuran (THF), toluene, sterile deionised water (H_2O) and sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) were purchased from Fisher Scientific (UK). Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), sodium chloride (NaCl), phosphate buffered saline (PBS) tablets, foetal bovine serum (FBS), 75 cm^2 Nunc Easy tissue culture flasks with porous caps, Nunc 6-well multidishes, Nunc Nunclon™ Δ Surface 96-well white-bottom and clear-bottom microplates and 18 mm diameter glass coverslips were purchased from Thermo Fisher Scientific (UK). Costar™ sterile disposable reagent reservoirs, sterile centrifuge tubes and sterile disposable serological pipettes were purchased from Corning B.V. Life Sciences (The Netherlands). Trypsin 0.25 % with ethylenediaminetetraacetic acid (EDTA), McCOY's 5A phenol red-free medium containing L-glutamine, McCOY's 5A medium containing phenol red and L-glutamine (200 mM) were all purchased from Invitrogen (UK). CellTiter-Blue® cell viability assay was purchased from Promega (UK). Millex GP syringe driven filter units (0.22 μm) were purchased from Millipore Corporation (USA). Sodium hydrogen carbonate ($NaHCO_3$) was purchased from BDH Laboratory Supplies Poole (UK). Vivaspin™ 500 (100 kDa MWCO; PES membrane) centrifuge columns were purchased from Sartorius Stedim Biotech (UK). The α-thio-ω-carboxy polyethylene glycol (3,000 Da; PEG) was purchased from Iris Biotech GmbH (Germany). Staurosporine free base (> 99 %) was purchased from LC Laboratories (USA). Holey carbon film 300 mesh copper grids were obtained from Agar Scientific (UK). A JDS Uniphase 633 nm Helium-Neon (HeNe) laser (10 mW) was purchased from Edmund Optics (USA). SK-

BR-3 human breast adenocarcinoma cells were purchased from LGC Standards and kindly provided by Prof Dylan R. Edwards (Norwich Medical School, University of East Anglia, UK).

Methods

UV-vis spectra of the samples were recorded using a Hitachi U-3010 spectrophotometer at room temperature. Quartz cuvettes with a 1 cm path length were used. Fluorescence excitation and emission spectra of the samples were obtained using a Hitachi F-4500 fluorescence spectrometer in quartz cuvettes with a 1 cm path length. For the plate assays, absorbance, fluorescence and luminescence measurements were performed using a CLARIOstar® (BMG Labtech) microplate reader at room temperature.

Transmission electron microscopy (TEM) images were obtained using a JEOL JEM-2010 Electron Microscope operating at 200 kV. The samples were deposited on holey carbon film 300 mesh copper grids.

Centrifugation of Eppendorf tubes (1.5 mL) and Vivaspin™ 500 columns was performed using a Beckman Coulter Allegra™ X-22R centrifuge. Centrifugation of biological samples in 15-50 mL centrifuge tubes was performed using an Eppendorf 5810R centrifuge.

Confocal microscopy was performed using a Carl Zeiss LSM 510 META confocal laser scanning microscope. The images were acquired with a plan-apochromat 63x/1.4 Oil DIC objective and processed using ImageJ/Fiji software.

Buffer solutions and imaging medium

Phosphate buffered saline (PBS) was prepared in 10 mM phosphate buffer (PB) containing NaCl (150 mM) and calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$; 100 μM). The 10 mM PB was prepared using $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}2H_2O$ stock solutions (200 mM). The pH of the PBS was adjusted to 7.4 using aqueous solutions of sodium hydroxide (NaOH; 5 M) and hydrochloric acid (HCl; 0.6 M). Phosphate buffered saline for the biological experiments (PBS-B) was prepared by dissolving 10 PBS tablets in H_2O (1 L). The solution was sterilised by autoclaving at 110 °C for 10 min. The final PBS-B solution contained Na_2HPO_4 (8 mM), potassium phosphate monobasic (KH_2PO_4; 1 mM), NaCl (160 mM), potassium chloride (KCl; 3 mM) and a pH of 7.3. MES buffer was prepared in water containing 2-(N-morpholino)ethanesulfonic acid (MES; 50 mM) and Tween-20 (0.05 %). The pH of the MES buffer was adjusted to 5.5 using aqueous solutions of NaOH (5 M) and HCl (0.6 M). Imaging medium based on Hank's balanced salt solution (HBSS) for the biological experiments was prepared in water containing NaCl (120 mM), KCl (5 mM), $CaCl_2{\cdot}2H_2O$ (2 mM), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$; 1 mM), NaH_2PO_4 (1 mM), NaHCO3 (1 mM), 4-(2-hydroxyethyl)piperazine-4-ethanesulfonic acid (HEPES; 25 mM), D-glucose (11 mM) and bovine serum albumin (BSA; 1 mg/mL). The pH of the imaging medium was adjusted to 7.4 using an aqueous solution of NaOH (1 M). Prior to use, all buffer solutions and imaging media were sterilised by filtration through Millex GP syringe driven filter units (0.22 μm).

Synthesis and characterisation of the C11Pc/C3Pc-PEG-AuNPs

The synthesis of the two zinc phthalocyanines (Pc) used in this work, composed of either a three carbon-atom chain (C3Pc) or an eleven carbon-atom chain (C11Pc) connecting two macrocycles via two sulfur atoms (Fig 1), has been reported previously.[24-25]

Fig. 1. Structure of the phthalocyanines (a) C3Pc and (b) C11Pc.

Gold nanoparticles (*ca.* 4 nm; AuNPs) were synthesised by sodium borohydride ($NaBH_4$) reduction and functionalised with a mixed monolayer of PEG (3,073 Da) together with either C11Pc or C3Pc; using a previously described method but with modifications.[8] C11Pc (2.43 mg; 0.95 µmol) or C3Pc (2.22 mg; 0.95 µmol) were dissolved in tetrahydrofuran (THF; 1 mL) and left to stir for 5 min at room temperature. Then, PEG (7.5 mg; 2.5 µmol) was dissolved in THF (2 mL), added to the phthalocyanine solution and stirred at room temperature for 5 min. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$; 1.2 mg; 3.0 µmol) was dissolved in THF (1.2 mL), added to the Pc-PEG mixtures and stirred at room temperature for a further 5 min. Finally, a solution of $NaBH_4$ (1.5 mg; 39.65 µmol) was prepared in distilled water (1.2 mL) and added to the Pc-PEG-$HAuCl_4$ mixture rapidly and under vigorous stirring. The reaction was stirred overnight (*ca.* 17 h) at room temperature in the dark at moderate speed.

The resulting solution was a mixture of free Pc, AuNPs functionalised with Pc only, AuNPs functionalised with PEG only and AuNPs functionalised with both Pc and PEG. To purify the mixture, excess THF (5.4 mL) was added rapidly and under vigorous stirring, followed by centrifugation in Eppendorf tubes (1.5 mL) at 1,300 rpm for 2 min at 4 °C. The supernatant was collected and the brown pellet containing AuNPs functionalised only with PEG was discarded. The solvent mixture in the supernatant was rotary evaporated under reduced pressure at 70 °C. At this point, MES buffer (5 mL; 50 mM MES; 0.05 % Tween-20; pH 5.5) was added and the mixture was solubilised using an ultrasonic bath. The solution was centrifuged in Eppendorf tubes (1.5 mL) at 14,000 rpm for 30 min at 4 °C. The pellet containing free Pc and AuNPs functionalised only with Pc, and thus not soluble in an aqueous solution, was discarded. The supernatant containing the Pc-PEG-AuNPs was collected, filtered through a Millex GP syringe driven filter unit (0.22 µm) and stored at 4 °C. The Pc functionalised AuNPs were obtained with a yield of *ca.* 2 %.

The synthesised Pc-PEG-AuNPs were characterised using UV-vis and fluorescence spectroscopies and TEM. The UV-vis extinction spectra were recorded between 300-800 nm. The fluorescence emission spectra were recorded using an excitation wavelength of 633 nm, between 653-850 nm. The fluorescence excitation spectrum was recorded, using an emission wavelength of 780 nm, between 500-750 nm.

The size and morphology of the nanoparticles was characterised by TEM. A droplet of the AuNPs (20 µL) in MES buffer was placed onto a holey carbon film 300 mesh copper grid and left for 2 min to allow the AuNPs to sediment onto the grid. Excess MES buffer was then removed by gently tapping the side of the grid with filter paper. The grid was left to dry completely before imaging.

Estimation of the fluorescence quantum yields

The fluorescence quantum yield for the free Pcs and the respective Pc-PEG-AuNPs were obtained using UV-vis and fluorescence spectroscopies. Zinc 2,9,16,23-tetra-tert-butyl-29H,31H-phthalocyanine

(ZnPc-ref) was used as the reference, as it is known to have a fluorescence quantum yield of 0.33 in toluene.[26] Free C3Pc (0.52 mg) was dissolved in THF (2 mL) to give an initial concentration of 111 µM. C3Pc-PEG-AuNPs dissolved in MES buffer at an initial C3Pc concentration of 3.6 µM were used. Free C11Pc (2.27 mg) was dissolved in THF (2 mL) to give an initial concentration of 443 µM. C11Pc-PEG-AuNPs dissolved in MES buffer at an initial C11Pc concentration of 1.3 µM were used. The reference standard ZnPc-ref (0.66 mg) was dissolved in toluene (2 mL) to give an initial concentration of 411 µM. All samples and reference sample were further diluted to give Pc concentrations ranging from 0-1.2 µM. Five dilutions were analysed for each sample. The UV-vis absorption spectra of all samples were recorded and the absorbance intensity at 640 nm was noted. Then, the fluorescence emission spectra of the samples, using an excitation wavelength of 640 nm, were recorded between 660-850 nm. The fluorescence quantum yields were obtained using a calibration curve of integrated fluorescence intensity (*i.e.,* area under the curve) vs absorbance at the excitation wavelength, 640 nm. The calibration curve produced a straight line with gradient "m" and intercept "0". The gradient was proportional to the fluorescence quantum yield of the sample. By comparing the gradient of the unknown samples to the gradient of the reference standard, the fluorescence quantum yields were estimated.

Measurement of singlet oxygen production

Production of singlet oxygen was measured using the singlet oxygen probe 9,10-anthracenediyl-bis (methylene) dimalonic acid (ABMA). ABMA (0.63 mg) was dissolved in methanol (MeOH; 3 mL) to give an initial concentration of 0.512 mM. The Pc-PEG-AuNPs (511 µL) in MES buffer were placed in a 1.5 mL quartz cuvette together with ABMA (1 µL; 1 µM) and a magnetic stirrer bar. Oxygen was bubbled through the solution and the quartz cuvette was stoppered. The fluorescence emission spectrum of the initial solution was recorded using an excitation wavelength of 380 nm between 390-600 nm. The sample was then irradiated at 633 nm using a 10 mW HeNe laser for 40 min with continuous stirring. The laser was placed 50 cm away from the cuvette. The fluorescence emission spectrum of the sample was recorded every 5 min.

In vitro cell work

SK-BR-3 human breast adenocarcinoma cells were routinely cultured in McCOY's 5A phenol red-free medium containing L-glutamine and supplemented with foetal bovine serum (FBS; 10 %); and grown in an incubator at 37 ˚C under a 5 % CO_2 atmosphere.

SK-BR-3 cells were subcultured (1:4) every 5 days, when they reached near confluence. The culture medium was discarded and the cells were washed with PBS-B (5 mL). The cells were dislodged from the flasks by addition of trypsin 0.25 % (1x) EDTA (5 mL) and incubated for 5 min at 37 °C under a 5 % CO_2 atmosphere. Trypsin was deactivated by addition of the McCOY's 5A culture medium (5 mL) and removed by centrifugation at 800 relative centrifugal force (rcf) for 5 min at 21 °C. The pellets containing the cells were then resuspended in McCOY's 5A medium. The cells were counted with a Neubauer hemocytometer.

Confocal microscopy. For imaging experiments, SK-BR-3 cells were cultured in McCOY's 5A medium containing phenol red and supplemented with L-glutamine (1 %) and FBS (10 %). SK-BR-3 cells were

seeded onto 18 mm diameter glass coverslips inside Nunc 6-well multidishes at a concentration of 2×10^4 cells/mL (3 mL/well). The cells were incubated for *ca.* 48 h at 37 °C under a 5 % CO2 atmosphere.

The culture medium was removed and the cells were washed once with PBS-B (1 mL). The nanoparticle samples, C11Pc-PEG-AuNPs and C3Pc-PEG-AuNPs (0.23 μM Pc) in FBS-free McCOY's 5A medium supplemented with L-glutamine (1 %), were then added (1 mL/well). Control cells not loaded with any AuNP samples were treated with FBS-free McCOY's 5A medium supplemented with L-glutamine (1 %). The cells were incubated with the samples and controls for 3 h at 37 °C under a 5 % CO_2 atmosphere.

Following incubation with the samples, the cells were washed with PBS-B (1 mL) three times. They were then resuspended in complete McCOY's 5A medium (2 mL) supplemented with L-glutamine (1 %) and FBS (10 %). At this point, the cells were irradiated at 633 nm using a 10 mW HeNe laser fitted with a biconvex diverging lens for 6 min per coverslip. The multidishes containing the non-irradiated coverslips were left covered in aluminium foil in the dark. Following irradiation, the cells were further incubated for 19 h at 37 °C under a 5 % CO_2 atmosphere.

For imaging in the confocal microscope, the 18 mm coverslips were placed in a Ludin chamber, which was securely tightened. The cells in the coverslips were washed with imaging medium (*ca.* 1 mL) three times and finally resuspended in imaging medium (*ca.* 1 mL). The Ludin chamber was fitted on a heating stage at 37 °C in the confocal microscope. A 633 nm HeNe laser was used to excite the Pc on the AuNPs and the fluorescence emission was collected in the red channel with a 650 nm long pass filter. Differential interference contrast (DIC) images were collected simultaneously using a 488 nm argon-ion laser. To test for dead cells, the dead cell marker propidium iodide was used. Propidium iodide (5 μL; 1 mg/mL in PBS) was mixed with imaging medium (1 mL). The solution was directly added to the coverslips, which had been previously placed in the Ludin chamber and mounted on the heating stage. The propidium iodide solution was incubated with the cells at 37 °C for 5 min in the dark. A 543 nm HeNe laser was used to excite the propidium iodide and the fluorescence emission was collected in the pink channel with a band pass filter between 560-615 nm.

Celltiter-Blue® cell viability assays. SK-BR-3 cells were seeded onto two white-bottom Nunc Nunclon™ Δ Surface 96- well microplates at a concentration of 20×10^4 cells/mL (100 μL/well). The cells were incubated for *ca.* 48 h at 37 °C under a 5 % CO_2 atmosphere.

The culture medium was removed using a micropipette and the cells were washed once with PBS-B (100 μL). The nanoparticle samples, C11Pc-PEG-AuNPs and C3Pc-PEG-AuNPs at various Pc concentrations in FBS-free McCOY's 5A medium containing L-glutamine were then added (50 μL/well). Additionally, a solution of staurosporine (1 mM in DMSO) dispersed in FBS-free McCOY's 5A medium containing L-glutamine (50 μL; 20 μM) was also used as a positive control for cytotoxicity.3,27 Control cells without any AuNPs loaded were treated with FBS-free McCOY's 5A medium containing L-glutamine. The cells were incubated with the samples and controls for 3 h at 37 °C under a 5 % CO_2 atmosphere.

Following incubation with the AuNPs, the samples were removed and the cells were washed with PBS-B (100 μL) three times. The cells were then resuspended in complete McCOY's 5A medium (100 μL) containing L-glutamine and supplemented with FBS (10 %). At this point, one of the plates was irradiated at 633 nm using a 10 mW HeNe laser fitted with a biconvex diverging lens for 6 min per well. The laser was located 50 cm above the 96-well plate, giving an irradiance of 29 mW/cm^2 and a total light dose of 10.5 J/cm2. The plate not being irradiated was kept covered in aluminium foil in the dark.

The cells were further incubated for *ca.* 48 h at 37 °C under a 5 % CO_2 atmosphere prior to measuring cell viability.

Following incubation post-PDT treatment, CellTiter-Blue® reagent (20 µL) was added to each well and incubated for 4 h at 37 °C under a 5 % CO_2 atmosphere. Fluorescence emission was then measured at 594 nm following excitation at 561 nm. Background fluorescence was corrected by subtracting the fluorescence emission from McCOY's 5A medium alone incubated with CellTiter-Blue®. Cell viability was calculated as a percentage of non-treated, non-irradiated cells. All samples were analysed in triplicate. Statistical significance between means was determined using a two-tailed Student's t-test and P values < 0.05 were considered significant.[28]

Results and discussion

Gold nanoparticles were simultaneously synthesised and functionalised with PEG and either C3Pc or C11Pc by sodium borohydride reduction. During nanoparticle synthesis, the disulfide bond leads to self-assembly of the photosensitiser onto the AuNP surface. The self-assembly causes the reduction of the disulfide bond, leading to the functionalization of the gold core with monomeric structures, consisting of only one macrocycle. Considering the distance between the sulfur atom and the Pc, the Pc macrocycle of the C3Pc is expected to be closer to the gold core than the Pc of C11Pc. Schematic representations of the synthesised AuNPs, with either C3Pc (C3Pc-PEG-AuNPs) or C11Pc (C11Pc-PEG-AuNPs), are shown in Fig 2.

Fig. 2. Schematic representation of the synthesised gold nanoparticles containing a mixed monolayer of PEG and either (a) C3Pc or (b) C11Pc.

The C3Pc-PEG-AuNPs and C11Pc-PEG-AuNPs were characterised by recording the UV-vis extinction spectra (Fig 3a), which confirmed that either ZnPcs are present on the surface of the AuNPs in the active monomeric form. Additionally, the nanoparticles were stable in aqueous solutions, highlighting the functionalisation of the gold surface with PEG. The mean diameter of the nanoparticles was found to be 3.83 ± 0.99 nm for the C3Pc-PEG-AuNPs and 3.41 ± 1.16 nm for the C11Pc-PEG-AuNPs, as analysed by TEM (Fig S1). An estimation of the ratio of Pc per AuNP was calculated, obtaining values of *ca.* 99 C3Pc per AuNP and *ca.* 115 C11Pc per AuNP (Fig S2, Table S1).

Fig. 3. (a) UV-vis extinction spectra of the synthesised (i) C11Pc-PEG-AuNPs and (ii) C3Pc-PEG-AuNPs. (b) Normalised fluorescence emission intensity of (i) free C11Pc and (ii) free C3Pc in THF (λ_{ex}=633 nm; λ_{em}=660-850 nm). (c) Normalised fluorescence emission intensity of (i) C11Pc-PEG-AuNPs and (ii) C3Pc-PEG-AuNPs in MES buffer. (d) Photobleaching of ABMA in the presence of (i) C11Pc-PEG-AuNPs or (ii) C3Pc-PEG-AuNPs (1 µM Pc) as a function of time; and no photobleaching observed for (iii) control PEG-AuNPs. Error bars represent the SD (n = 3) within a 95 % confidence interval.

The fluorescence emission of the AuNPs was analysed and compared to the fluorescence emission obtained from the free Pc, to assess any potential MEF arising from the presence of the gold metal. With consideration of the respective solubility, the free Pcs were dissolved in THF, whereas the

functionalised AuNPs were dissolved in MES buffer. In solution, the fluorescence emission intensity of free C3Pc is three times lower than that of C11Pc (Fig 3b). However, AuNPs functionalised with the C3Pc exhibited higher fluorescence than those functionalised with C11Pc (Fig 3c). These, somewhat surprising, results were further confirmed by the fluorescence quantum yields of each sample. The fluorescence quantum yield for the free C11Pc (4.8 %) was greater than the value of the free C3Pc (2.6 %) (Table S2). When the Pcs were assembled to the AuNPs, there is a quenching of the fluorescence for both the C11Pc and C3Pc derivatives. However, the fluorescence quantum yield for the C11Pc-PEG- AuNPs (0.2 %) was found to be lower than that of C3Pc-PEG-AuNPs (0.42 %) (Fig S3, Table S2). It is important to note that the values of the fluorescence quantum yield for the Pc-PEG-AuNPs were obtained based on the assumption that the refractive index of the MES buffer, in which they were dissolved, was that of water. However, the presence of MES leads to an increase in the refractive index, concomitant with MES concentration in the solution. Therefore, the "true" values of the fluorescence quantum yields of the Pc-PEG-AuNPs are expected to be higher than those reported here. The fluorescence intensity behaviour can be rationalised by considering the interaction between the fluorophores and the AuNPs. Lakowicz's radiating plasmon model suggests that small spherical nanoparticles (< 40 nm) will quench the fluorescence of the fluorophores since such nanoparticles primarily absorb light.[11] This explains the quenching effect seen for both C11Pc and C3Pc when they are attached to the AuNPs. The second result, by which the fluorescence from C3Pc is not quenched as much as that of C11Pc on the surface of the AuNPs, can be rationalised by the distance-dependence and the orientation-dependence parameter between the fluorophores and the AuNPs. The distance-dependent parameter states that close distances between the AuNPs and the fluorophores (≤ 5 nm) will lead to fluorescence quenching.[29-30] However, medium distances (*ca.* 5-30 nm) will lead to fluorescence enhancement.[10,29,30] The orientation adopted by the fluorophores and their molecular dipoles relative to the AuNPs is also important. In a parallel orientation, quenching is allowed because the dipole in the metal cancels the dipole in the fluorophore. The cancellation of the dipole further slows the radiative decay and favours the quenching of the fluorescence.[12] On the contrary, a perpendicular orientation can induce maximum fluorescence enhancement, due to cooperation of the dipole in the metal and the dipole in the fluorophore, which enhances the radiative decay and allows the enhancement of the fluorescence.[12] The orientation of the fluorophore depends on the angle at which the anchor chain connecting the fluorophore to the metal is positioned with respect to the metal core.[31] In the present study, the fluorescence emission intensity of the C3Pc was found to be greater than that of the C11Pc on the surface of the AuNPs, suggesting that the C11Pc lies more parallel relative to the gold core as compared to the C3Pc, as speculated by Battistini et al. in a similar study performed with pyrenes connected to AuNPs by either 4 or 11 carbon atoms.[31] These authors speculated that intermolecular interactions were the driving force for the final orientation of the chains and hence the macrocycles. In the present study, it is possible that the C11Pc chain will adopt a more parallel configuration relative to the AuNPs in order to maximise interactions between the chains. On the other hand, the C3Pc chain adopts a more perpendicular orientation due to the short chain, in which chain-chain interactions are minimal. What is evident from the spectra shown in b and c of Fig 3 is the contrasting and quite unpredicted reversal of emission intensities from C11Pc and C3Pc when free and bound on the gold nanoparticles. Such a phenomenon would arise from the differing molecular packings and/or orientations of the Pc units of the two compounds when bound to the nanoparticle surface.

Given the higher fluorescence for C3Pc on the surface of the AuNPs and with consideration that MEF is related to ME$^1O_2$ production,[19-21] it was anticipated that the C3Pc-PEG-AuNPs would enhance the production of $^1O_2$ as compared to the C11Pc-PEG-AuNPs. The $^1O_2$ probe 9,10-anthracenediyl-bis(methylene) dimalonic acid (ABMA) selectively reacts with $^1O_2$, leading to the formation of a non-

fluorescent 9,10-endoperoxide product.[32] For the experiment, the AuNP conjugates were mixed with ABMA and irradiated with a 633 nm HeNe laser for 40 mins. The fluorescence emission of ABMA was monitored every 5 mins. To confirm that the production of $^1O_2$ was due to the presence of the Pc on the AuNPs, AuNPs functionalised with PEG only were used as a control and they were shown to produce a negligible amount of $^1O_2$. The results for both C11Pc-PEG-AuNPs and C3Pc-PEG-AuNPs (Fig 3d) confirm that $^1O_2$ is produced as there is a decrease in ABMA fluorescence over time. Further, the results suggest that the photobleaching of ABMA is greater in the presence of C3Pc-PEG-AuNPs as compared to C11Pc-PEG-AuNPs. The use of ABMA to detect the production of $^1O_2$ is a qualitative measurement and it can be subject to high errors, as seen for the C3Pc-PEG-AuNPs sample. However, it can be clearly seen that C3Pc-PEG-AuNPs induce more photobleaching of ABMA than C11Pc-PEG-AuNPs after 40 mins. The higher photobleaching of ABMA in the presence of C3Pc-PEG-AuNPs over time is a strong indication that the C3Pc-PEG-AuNPs produce more $^1O_2$ than the C11Pc-PEG-AuNPs.

To confirm whether the higher production of $^1O_2$ by the C3Pc-PEG-AuNPs would lead to a greater photodynamic efficacy *in vitro*, intracellular studies using SK-BR-3 cells were performed. Visual inspection of the nanoparticle internalisation by the SK-BR-3 cells together with inspection of the cell death was performed using confocal microscopy. The confocal microscopy studies confirm the superiority of C3Pc-PEG-AuNPs to induce cell death following PDT. SK-BR-3 cells appear to internalise both the C11Pc-PEG-AuNPs and the C3Pc-PEG-AuNPs in a similar manner, as shown in Fig 4a and 4b by the red fluorescence of the respective Pc.

Fig. 4. Confocal fluorescence microscopy images of SK-BR-3 cells incubated with either C11Pc-PEG-AuNPs (a, c) or C3Pc-PEG-AuNPs (b, d) (0.23 μM Pc) for 3 h before (a-b) or after (c-d) irradiation with a 633 nm HeNe laser for 6 mins. Images are the composite of differential interference contrast (DIC), fluorescence from C11Pc or C3Pc collected in the red channel (λ_{ex} = 633 nm; above 650 nm) and fluorescence from propidium iodide collected in the pink channel (λ_{ex} = 543 nm; 560-615 nm). Scale bars 5 μm.

Following light irradiation, cell death was visualised using propidium iodide (PI), a dead cell marker that binds the DNA in the nuclei of those cells where the membrane has been damaged, shown in pink.[33] Additionally, the dramatic change in cell morphology before and after treatment with PDT is a further indication of PDT damage to the cells. While the cell damage post-PDT in SK-BR-3 cells treated with C11Pc-PEG-AuNPs is minimal (Fig 4c), the majority of the cells incubated with C3Pc-PEG-AuNPs have been killed (Fig 4d).

Celltiter-Blue® cell viability assays were performed for the quantification of the cell death induced post-PDT, as shown in Fig 5. The Celltiter-Blue® assay contains the non-fluorescent active ingredient resazurin, which is reduced in the presence of metabolically active cells to the fluorescent resorufin.[34] The fluorescence of resorufin can be measured at 594 nm, in order to quantify cell viability.

Fig 5. CellTiter-Blue® cell viability assay for SK-BR-3 cells incubated with (a) C11Pc-PEG-AuNPs or (b) C3Pc-PEG-AuNPs. Cells were either irradiated with a 633 nm HeNe laser (a) orange, b) yellow) or non-irradiated (a) dark cyan, b) green). Staurosporine (+ve St) was used as a positive control for cytotoxicity via apoptosis. Error bars represent the SD (n = 3) within a 95 % confidence interval. Statistically significant difference between C11Pc-PEG-AuNPs and C3Pc-PEG-AuNPs is indicated by * at $P < 0.008$

and ** at $P < 0.0001$, obtained using a two-tailed Student's t-test, where $P < 0.05$ is considered statistically significant.

SK-BR-3 cells were incubated with either C11Pc-PEG-AuNPs or C3Pc-PEG-AuNPs for 3 h. Following incubation, the cells were washed to remove all the non-internalised AuNPs and subjected to PDT by irradiation with a 633 nm HeNe laser located 50 cm above the cells for 6 mins, giving an irradiance of 29 mW/cm^2 and a total light dose of 10.5 J/cm^2. As it can be seen in Fig 5, there is no, or limited, dark toxicity for either of the AuNP conjugates, as cell viability remains high with minimal cell death following incubation with the AuNPs.

Furthermore, cells subjected to PDT following incubation with the C11Pc-PEG-AuNPs remain largely undamaged, as minimal cell death was observed. On the contrary, cells subjected to PDT after incubation with the C3Pc-PEG-AuNPs show increasing levels of cell death as the concentration of C3Pc is increased, reaching up to 85 % cell death when 0.23 μM of C3Pc is used. Staurosporine (+ve St) is a positive control for cytotoxicity via apoptosis.[27] The results in Fig 5 indicate that the C3Pc-PEG-AuNPs are more effective at inducing cytotoxicity following light irradiation, as confirmed by the P values that show a statistically significant difference between the C11Pc-PEG-AuNPs and the C3Pc-PEG-AuNPs. These results, in agreement with Fig 4, highlight that the surface enhanced production of $^1O_2$ by the C3Pc-PEG-AuNPs provides exceptional PDT efficacy.

Conclusions

In summary, we have shown the different behaviour of two ZnPc photosensitisers, C3Pc and C11Pc, assembled on the surface of AuNPs. In solution, free C11Pc presents higher fluorescence emission intensity than C3Pc. On the surface of the AuNPs, the fluorescence emission intensities from C11Pc-PEG-AuNPs and C3Pc-PEG-AuNPs are reversed. The photosensitiser with the shorter anchor chain, C3Pc, exhibits significantly greater fluorescence than the photosensitiser with the longer chain, C11Pc. It is thought that the differing molecular packings, distances and/or orientations of the Pc macrocycles of C3Pc and C11Pc when bound to the AuNPs are the main parameters affecting the fluorescence. The higher fluorescence of C3Pc on the surface of the AuNPs provides an ideal photosensitiser for metal-enhanced fluorescence (MEF) and thus significantly improved PDT. It was demonstrated that the enhanced fluorescence of C3Pc led to an enhancement of $^1O_2$ production. The spectroscopic results were extended by in vitro experiments, which clearly showed that PDT treatment of SK-BR-3 breast cancer cells was more efficient when C3Pc-PEG-AuNPs were used (85 % cell death) as compared to C11Pc-PEG-AuNPs (negligible cell death). The results here presented show the exciting potential of metal-enhanced $^1O_2$ production to increase efficacy of cell death via AuNP-mediated PDT of cancer.

Conflicts of interests

The authors declare that there are no conflicts of interests.

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