Electronic Supplementary Information

for

Peptide directed phthalocyanine-gold nanoparticles for selective photodynamic therapy of EGFR overexpressing cancers

Zoë Rachael Goddard,[a] Andrew Michael Beekman,[a] Marco M. D. Cominetti,[a] Maria A. O'Connell,[a] Isabelle Chambrier,[b] Michael J. Cook,[b] María J. Marín,[b] David A. Russell[b] and Mark Searcey[a]*

[a]School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

[b]School of Chemistry, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

Email: M.Searcey@uea.ac.uk

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1. Experimental procedures

Instrumentation and chemicals

All reagents were used as received and purchased from Sigma-Aldrich, Fisher Scientific or Fluorochem unless otherwise stated. Fmoc-amino acids and coupling agents were purchased from Novabiochem, Fluorochem or AGTC Bioproducts. Anhydrous solvents were purchased from Sigma Aldrich, used as purchased and assumed to conform to specification. α-Thio-ω-carboxy poly(ethylene glycol (3 kDa HS-PEG-COOH) was purchased from Iris Biotech GmbH. Vivaspin 500 (100 kDa MWCO, PES membrane) centrifuge columns were purchased from Sartorius Stedim Biotech. Sterile 0.2 μm PES syringe filters were purchased from Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) for cell proliferation assays was purchased from Promega. A549 pulmonary adenocarcinoma and HEK293 embryonic kidney cell lines were purchased from ATCC.

Automated peptide synthesis was performed using a Biotage Syro I. MALDI was performed using a Kratos Analytical Axima-CFR MALDI-ToF, using α-cyanno-4-hydroxycinnamic acid (α-CHCA) as the matrix. High resolution ESI was performed using an LTQ Orbitrap XL or a Shimadzu ion-trap ToF fitted with a Shimadzu Prominence/Nexera UHPLC system.

Analytical RP-HPLC was performed using an Agilent 1200 HPLC, fitted with an Agilent eclipse XDB-C18 column (4.6 x 150 mm, 5 μm) and a flow rate of 1 mL/min. Spectra were run with a solvent gradient of 0-100% B over 20 min. Solvent A: H_2O, 0.05% TFA, solvent B: MeOH, 0.05% TFA. Detection wavelengths were 214 nm and 254 nm. Preparative RP-HPLC was performed using an Agilent 1200 HPLC, fitted with an Agilent eclipse XDB-C18 column (21.2 x 150 mm, 5 μm) and a flow rate of 20 mL/min. Spectra were run with a solvent gradient of 0-100% B over 15 min. Solvent A: 95% H_2O, 5% MeOH, 0.05% TFA, solvent B: 95% MeOH, 5% H_2O, 0.05% TFA. Detection wavelengths were 214 nm and 254 nm.

UV-vis were recorded on an Agilent Cary 60 spectrometer at room temperature, using quartz cuvettes with a pathlength of 1 cm. Fluorescence spectra were recorded on a Horiba Jobin Yvon Fluorolog spectrofluorometer at room temperature, using quartz cuvettes with a 1 cm pathlength. For plate assays, a BMG Labtech PolarStar Optima microplate reader was used to record absorbance at 492 nm.

Centrifugation of the nanoparticles was performed in an Eppendorf 5415D centrifuge. Centrifugation of biological samples was performed in a HERMLE Z 326 K Centrifuge. Flow cytometry was performed using a Beckmann Coulter CytoFLEX with a flow rate of 10 μL/min. Fluorescence microscopy was performed using an inverted Leica DMIL fluorescence microscope with an associated Leica DFC420 camera at x10 magnification.

Synthesis of peptide FITC-βAAEYLRK

FITC-βAAEYLRK was synthesised *via* Fmoc-solid phase peptide synthesis using appropriately protected Fmoc-amino acids. Rink amide MBHA resin (100 mg) was swelled for 30 min in N,N-dimethylformamide (DMF). The resin was deprotected with 20% piperidine in DMF for 20 min twice, followed by washing three times with DMF. The coupling of Fmoc-protected amino acids

(4 eq) was completed with N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate/1-hydroxybenzotriazole hydrate (HBTU/HOBt, 4eq) and diisopropylethylamine (DIPEA, 8 eq) and shaken for 45 minutes. Each peptide coupling was repeated, then the resin washed three times with DMF. After each peptide coupling, the growing peptide chain was deprotected with piperidine. Finally, the fluorescein isothiocyanate (FITC) tag was conjugated onto the C-terminus. FITC (1.2 eq) was dissolved in 12:7:5 pyridine:DMF:CH_2Cl_2, added to the resin and shaken overnight in the dark. Excess FITC was washed off the resin by washing six times with DMF. The resin was then washed six times with methanol and dried. Cleavage of the peptide from the resin was completed in 95:2.5:2.5 trifluoroacetic acid:triisopropylsilane:H_2O with shaking for three hours. The resulting solution was evaporated to dryness and washed with cold ether. The resulting peptide was purified by preparative RP-HPLC, then the purity confirmed by analytical reversed phase HPLC, collecting the peptide as a yellow solid (54 mg, 43.6 µmol, 84%). RP-HPLC: t_R = 14.13 min. MALDI-ToF ($[M+H]^+$): $C_{59}H_{75}N_{13}O_{15}S$ calculated 1238.53, found 1238.50. HRMS ESI ($[M+H]^+$): calculated 1238.5301, found 1238.5299.

Synthesis of 5-(3-(3-aminopropyl)thioureido)2-(6-hydroxy-3-oxo-2H-xanthen-9-yl)benzoic acid (FITC-amine)

This synthesis followed the method of Trévisiol *et al*.[1] with modifications. FITC (6.00 mg, 15.41 µmol) was dissolved in methanol (1 mL) alongside 1,3-diaminopropane (2.60 µL, 31.15 µmol) and stirred for 3 h. The solvent was removed under vacuum and the resulting orange solid purified by preparative HPLC, giving the product as an orange powder (6.35 mg, 13.70 µmol, 89%). ^{1}H-NMR (400 MHz, CD_3OD): 8.29 (d, 2H, *J* = 2.1 Hz), 7.86 (dd, 1H, *J* = 2.1, 8.2 Hz), 7.28 (d, 1H, *J* = 8.2 Hz), 7.02 (d, 2H, *J* = 8.9 Hz), 6.93 (d, 2H, *J* = 2.3 Hz), 6.79 (dd, 2H, *J* = 2.3, 8.9 Hz), 3.90 (t, 2H, *J* = 6.6 Hz), 3.08 (t, 2H, *J* = 7.3 Hz), 2.05 (tt, 2H, *J* = 6.6, 7.3 Hz). RP-HPLC t_R = 13.12 min. HRMS ESI ($[M+H]^+$) $C_{24}H_{21}N_3O_5S$ calculated 464.1275, found 464.1284.

Synthesis of FITC-βAAEYLRK-PEG-SH (peptide-PEG) and FITC-PEG

α-Thio-ω-carboxy-poly(ethylene glycol) (HS-PEG-COOH, 7.5 mg, 2.44 µmol) was mixed with 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 1.1 mg, 2.93 µmol, 1.2 eq) and DIPEA (0.8 µL, 4.88 µmol, 2 eq) in DMF (300 µL). FITC-βAAEYLRK (4.53 mg, 3.66 µmol, 1.5 eq) or FITC-amine (1.3 mg, 2.80 µmol, 1.1 eq) in DMF (50 µL) was added to the solution and stirred overnight in the dark. The solution was evaporated to dryness and the crude peptide-PEG carried forward for nanoparticle synthesis.

Synthesis of peptide-C11Pc-PEG-AuNPs and FITC-C11Pc-PEG-AuNPs

This synthesis followed the procedure of Garcia Calavia *et al*.[2] with modifications. The synthesis of 1,1',4,4',8,8',15,15',18,18',22,22'-tetradecakisdecyl-25,25'-(11,11'dithiodiundecyl) diphthalocyanine zinc (C11Pc) has been reported previously.[3,4] C11Pc (2.4 mg, 0.94 µmol) was dissolved in anhydrous THF (1 mL) and stirred in the dark. Peptide-PEG (5.24 mg, 1.22 µmol) or

FITC-PEG (4.29 mg, 1.22 μmol) was dissolved in anhydrous THF (1 mL) and added to the solution alongside HS-PEG-COOH (3.75 mg, 1.22 μmol) in anhydrous THF (1 mL). $HAuCl_4.3H_2O$ (1.2 mg, 3.05 μmol) was dissolved in anhydrous THF (1.2 mL) and added to the solution. Sodium borohydride (1.5 mg, 39.65 μmol) was dissolved in dH_2O (1.2 mL) and added under vigorous stirring. The solution was stirred for *ca*. 17 h in the dark. THF (5.4 mL) was added to the solution, then the solution centrifuged at 1,400 rpm for 2 min. The supernatant was removed and evaporated to dryness. The nanoparticles were resuspended in PBS (2 mL, 10 mM sodium phosphate, 150 mM NaCl, 100 μM $CaCl_2$) then centrifuged at 8,000 rpm for 20 min. The resulting supernatant was filtered through a syringe driven filter (0.2 μm), then purified through Vivaspin 500 columns, centrifuging at 8,000 rpm for 10 min, washing with PBS and repeating the purification a total of two times. The resulting pellet was resuspended in PBS (2 mL, 10 mM sodium phosphate, 150 mM NaCl, 100 μM $CaCl_2$) or RPMI 1640 without phenol red (2 mL) and stored at 4 °C in the dark. The peptide-C11Pc-PEG-AuNPs were characterised by UV-vis between 200-800 nm. The fluorescence excitation spectrum of C11Pc was recorded between 550-750 nm with an emission wavelength of 780 nm. The emission spectrum of C11Pc was recorded between 653-850 nm with an excitation wavelength of 633 nm. The excitation spectrum of FITC was recorded between 400-520 nm with an emission wavelength of 525 nm. The emission spectrum of FITC was recorded between 500-650 nm with an excitation wavelength of 490 nm.

Synthesis of C11Pc-PEG-AuNPs

This synthesis followed the procedure of Garcia Calavia *et al*.[2] C11Pc (2.4 mg, 0.94 μmol) was dissolved in anhydrous THF (1 mL) and stirred in the dark, then PEG (7.5 mg, 2.44 μmol) in anhydrous THF (2 mL) was added to the solution. $HAuCl_4.3H_2O$ (1.2 mg, 3.05 μmol) was dissolved in anhydrous THF (1.2 mL) and added to the solution. Sodium borohydride (1.5 mg, 39.65 μmol) was dissolved in dH_2O (1.2 mL) and added under vigorous stirring. The solution was stirred for *ca*. 17 h in the dark. THF (5.4 mL) was added to the solution, then the solution centrifuged at 1,400 rpm for two minutes. The supernatant was removed and evaporated to dryness. The nanoparticles were resuspended in PBS (2 mL, 10 mM sodium phosphate, 150 mM NaCl, 100 μM $CaCl_2$) or RPMI 1640 without phenol red (2 mL) then centrifuged at 8,000 rpm for 20 min. The resulting supernatant was filtered through a syringe driven filter (0.2 μm) and stored at 4 °C in the dark. The C11Pc-PEG-AuNPs were characterised by UV-vis between 200-800 nm. The fluorescence excitation spectrum was recorded between 550-750 nm with an emission wavelength of 780 nm. The emission spectrum was recorded between 653-850 nm with an excitation wavelength of 633 nm.

General procedure for the synthesis AuNPs without C11Pc

PEG (7.6 mg, 2.47 μmol) and FITC-PEG (8.69 mg, 2.47 μmol), peptide-PEG (10.61 mg, 2.47 μmol) or PEG (7.6 mg, 2.47 μmol) were dissolved in dH_2O (15 mL). $HAuCl_4.3H_2O$ (1.2 mg, 3.05 μmol) was dissolved in anhydrous THF (1.2 mL) and for 5 min. Sodium borohydride (1.5 mg, 39.65 μmol) was dissolved in dH_2O (0.5 mL) and added to the solution under vigorous stirring. The solution was stirred at room temperature for 4 h. The THF was removed from the solution under

vacuum, then the PEG-AuNPs purified by centrifugation in Vivaspin 500 columns at 8,000 rpm for 10 min. The brown pellet was resuspended in PBS (10 mL, 10 mM sodium phosphate, 150 mM NaCl, 100 μM $CaCl_2$), filtered through a syringe driven filter (0.2 μm) and stored at 4 °C. The UV-vis spectra of each type of nanoparticle were recorded between 200-800 nm.

General procedure for the measurement of singlet oxygen production

AuNPs in PBS (1 μM, 511 μL) were added to a quartz cuvette alongside 9,10-anthracenediyl-bis(methylene) dimalonic acid (ABMA, 1 μL, 0.512 mM in MeOH, final concentration 1 μM). Air was bubbled through the solution then the cuvette stoppered and irradiated with a 10 mW HeNe laser at 633nm, with the laser placed 50 cm away. Every 5 min the fluorescence emission spectrum of ABMA was recorded between 390-600 nm with an excitation wavelength of 380 nm. A control of PBS (511 μL, 10 mM sodium phosphate, 150 mM NaCl, 100 μM $CaCl_2$) and ABMA (1 μL, 0.512 mM in MeOH) was also irradiated and the ABMA fluorescence emission spectrum recorded every five minutes.

Flow cytometry for determining receptor presence

A549 or HEK293 cells ($1x10^6$) were centrifuged at 350 g, 4 °C for 5 min and the supernatant removed. Anti-EGFR antibody (5 μL, 1 mg/mL) was diluted in PBS 0.5% BSA (500 μL). The cell pellet was resuspended in this antibody solution (100 μL), then the cells incubated on ice for 1 h. A second tube of cells was incubated with PBS 0.5% BSA (100 μL) on ice for 1 h. PBS 0.5% BSA (1 mL) was added to each tube and then centrifuged at 350 g, 4 °C for 5 min. The supernatant was removed, the pellet resuspended in FITC-goat anti-mouse secondary antibody (1 μL, 2 mg/mL in 100 μL PBS 0.5% BSA) and the cells incubated on ice for 1 h in the dark. PBS 0.5% BSA (1 mL) was added to each tube then centrifuged at 350 g, 4 °C for 5 min. The supernatant was removed and the pellet resuspended in PBS 0.5% BSA (300 μL) and transferred into a flow cytometry tube. Cells were analysed in a Beckman Coulter CytoFLEX flow cytometer. Live cells were gated and cells run at a flow rate of 10 μL/min with 10,000 events recorded.

Fluorescence microscopy for peptide binding

A549 or HEK293 cells were seeded in a 96 well plate at (100 μL, $1x10^5$ cells/mL) and incubated overnight at 37 °C, 5% CO_2. FITC-βAAEYLRK (1 μL, 10 mM) and fluorescein (1 μL, 10 mM) in DMSO were added to separate wells alongside a control well of DMSO (1 μL). These cells were incubated for 1 h at 37 °C, 5% CO_2, then washed 3 times with cold Dulbecco's PBS (DPBS). The cells were imaged using a Leica DMIL fluorescence microscope at x22 magnification.

Assessment of peptide cytotoxicity

A549 or HEK293 cells were seeded in a 96 well plate at (100 μL, $1x10^5$ cells/mL) and incubated overnight at 37 °C, 5% CO_2. FITC-βAAEYLRK (10 mM in DMSO) was diluted in a serial dilution then added to wells in triplicate (1 μL). Cells were incubated for 72 h at 37 °C, 5% CO_2 before the media removed and fresh media added to each well (100 μL). MTS (10 μL) was added to each well, then the cells incubated for 3 h. The absorbance was recorded at 492 nm and corrected for

background absorbance by subtracting the absorbance of media treated with MTS. Cell viability was calculated as a percentage of non-treated cells.

Phototoxicity of AuNPs

A549 or HEK293 cells were seeded in a 96 well plate at (100 µL, $1x10^5$ cells/mL) and incubated overnight at 37 °C, 5% CO_2. The media was removed and AuNPs (50 µL) were added in foetal calf serum (FCS) free RPMI 1640 media and incubated for 3 h. Staurosporine (3 µL, 1 mM in DMSO) was used as a positive control for cytotoxicity and incubated with the cells under the same conditions. The wells were washed three times with DPBS, then complete phenol red free media (100 µL) added to each well. For each experiment, two identical plates were prepared. One plate irradiate with a 633 nm 10 mW HeNe laser, 6 min per well with the laser secured 50 cm above the plate. The other plate was kept in the dark. After irradiation, both plates were incubated for a further 48 h. MTS (10 µL) was added to each well and the plated incubated for 3 h. The absorbance was recorded at 492 nm and corrected for background absorbance by subtracting the absorbance of media treated with MTS. Cell viability was calculated as a percentage of non-treated cells.

2. Analytical HPLC traces

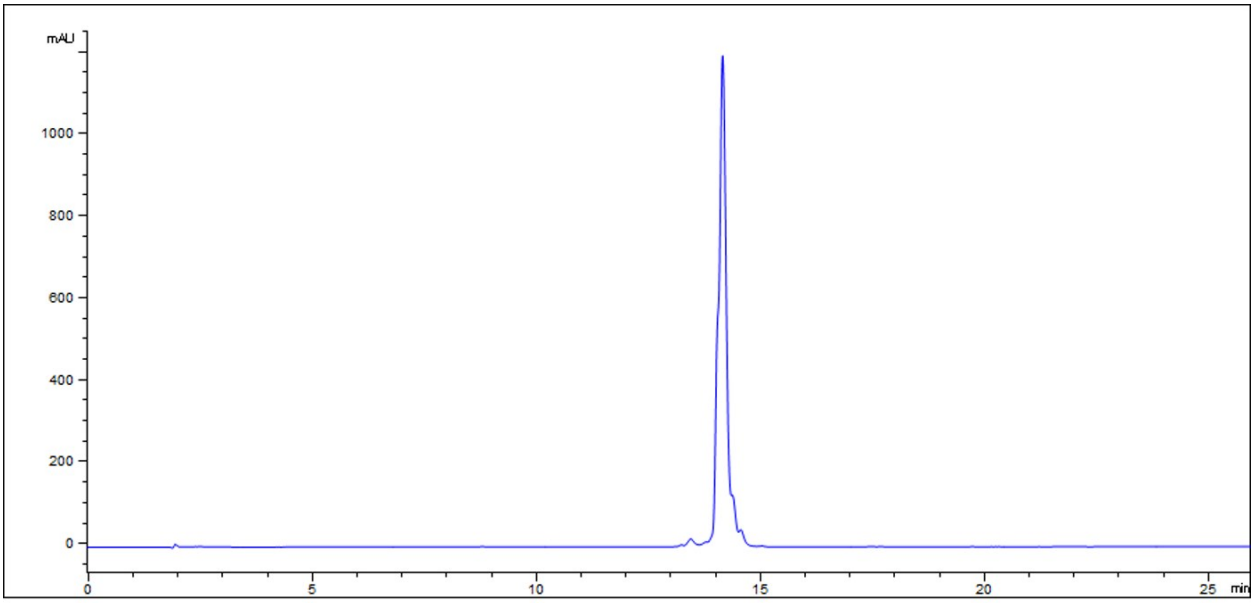

***Figure S1**: HPLC trace of FITC-βAAEYLRK recorded at 254 nm*

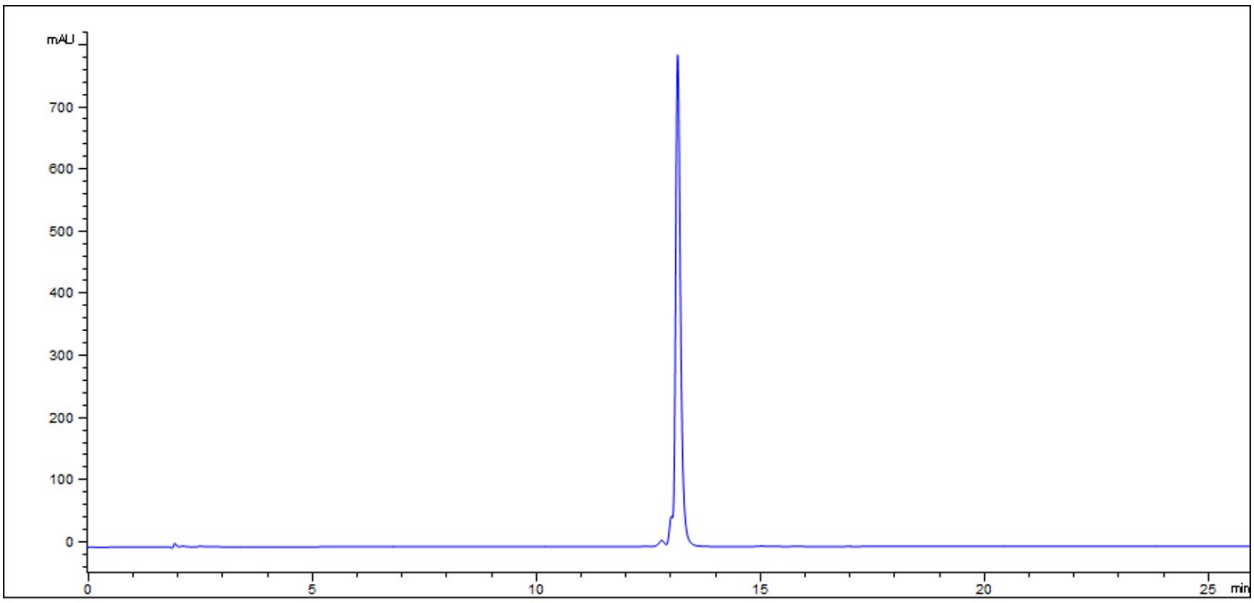

***Figure S2**: HPLC trace of FITC-amine recorded at 254 nm*

3. Flow cytometry for receptor expression

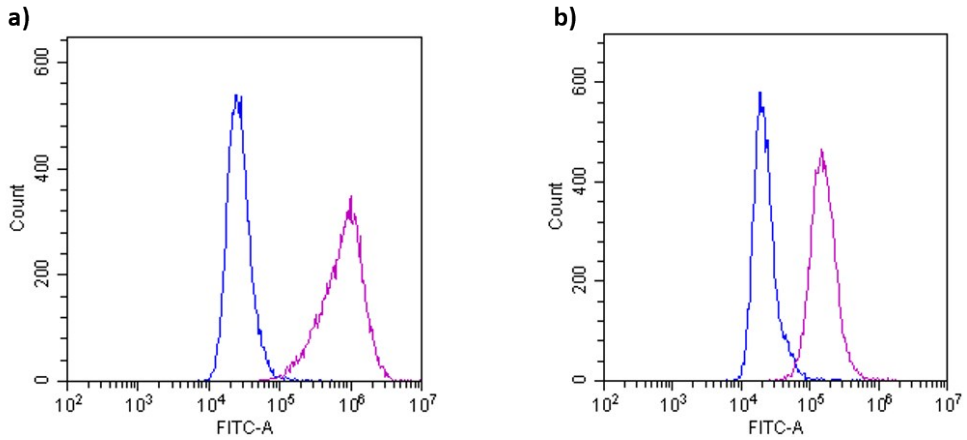

***Figure S3**: Flow cytometry histograms of **a)** A549 and **b)** HEK293 cells incubated with an anti-EGFR Ab then FITC-goat antimouse secondary Ab (purple) or just with the secondary Ab (blue)*

To compare the concentration of the EGFR on the surface of A549 and HEK293 cells, the fold increase of fluorescence upon addition of the primary antibody compared to that of incubation with only the secondary antibody was calculated (table S1). A549 cells displayed a 31-fold increase in fluorescence, compared to a 6.7-fold increase for HEK293s, showing that A549 cells display a higher concentration of EGFR on their surface.

***Table S1**: Mean fluorescence of A549 and HEK293 cells incubated with anti-EGFR antibody, then FITC-goat anti-mouse secondary Ab, or with the secondary Ab alone (control), and the fold increase in fluorescence upon addition of anti-EGFR antibody*

Cell line	Average fluorescence (AU), anti-EGFR treated	Average fluorescence (AU), control	Fold increase in fluorescence
A549	924348.2	29837.9	31.0
HEK293	176635.9	26198.8	6.7

4. Uptake of fluorescein in A549s

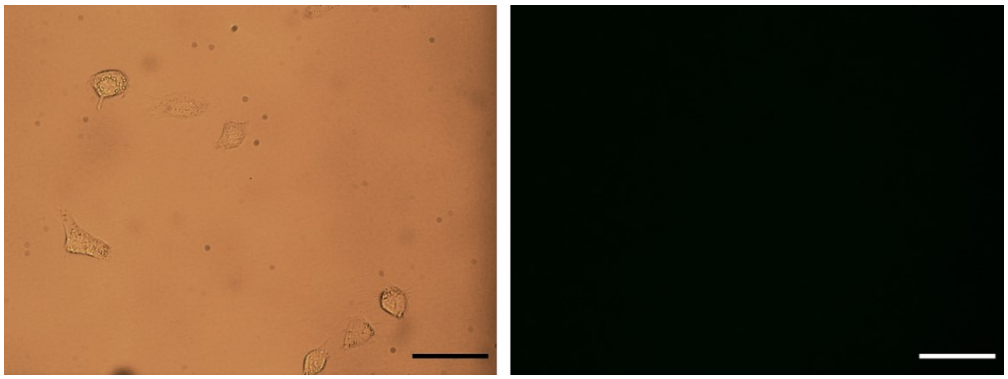

***Figure S4: a)** Bright field and **b)** fluorescence microscopy images of A549 cells incubated with fluorescein. Scale bar = 100 µm*

5. Assessment of peptide cytotoxicity

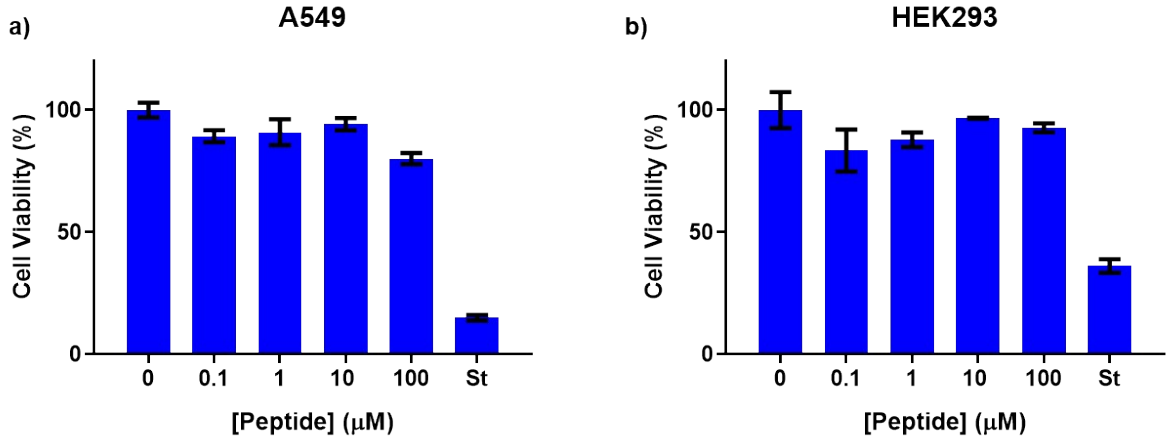

Figure S5*: Cell viability of* ***a)*** *A549 or* ***b)*** *HEK293 cells after 72 h treatment with FITC-βAAEYLRK. St = positive control of staurosporine. Error bars represent SE (n = 3)*

6. Peptide-C11Pc-PEG-AuNPs characterisation

Peptide-C11Pc-PEG-AuNPs were characterised by fluorescence spectroscopy to determine whether the photosensitisers had been quenched by the metal core. The fluorescence of both the C11Pc and FITC was observed. AuNPs quench the fluorescence of fluorophores held close to the core, and therefore the fluorescence of the C11Pc is partially quenched.[2] The FITC is much further away from the core and therefore doesn't experience the same quenching effect. Due to this, the C11Pc shows significantly lower emission than the FITC, even though there is a higher number of C11Pcs on the surface of the AuNPs.

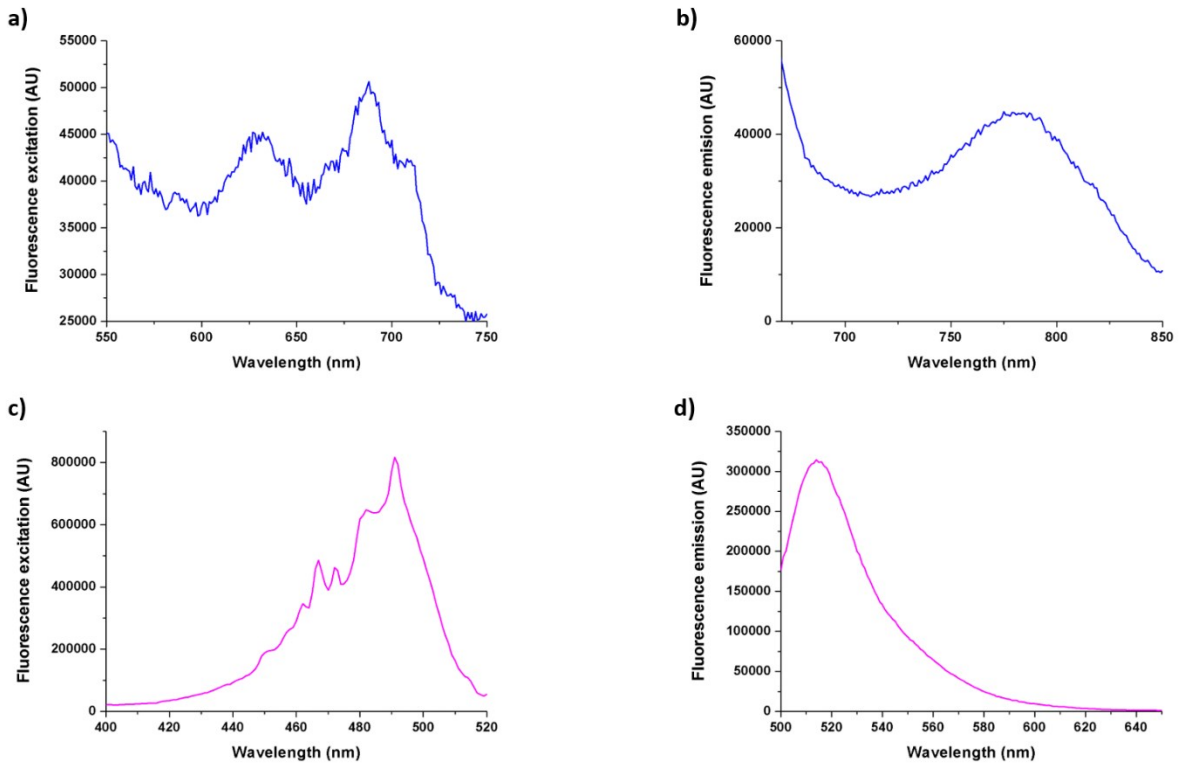

***Figure S6**: Fluorescence excitation spectra of **a)** C11Pc ($\lambda_{em.}$ = 780 nm) and **c)** FITC ($\lambda_{em.}$ = 525 nm) and fluorescence emission spectra of **b)** C11Pc ($\lambda_{ex.}$ = 633 nm) and **d)** FITC ($\lambda_{ex.}$ = 490 nm) ligands on peptide-C11Pc-PEG-AuNPs in PBS*

7. Analysis of ligand concentration on AuNPs

C11Pc concentration was calculated at 696 nm using a molar extinction coefficient of 2.16×10^5 M^{-1} cm^{-1}.

FITC concentration was calculated at 495 nm using a molar extinction coefficient of 7.3×10^4 M^{-1} cm^{-1}.

AuNPs were estimated to have a molar extinction coefficient of 4.78×10^6 M^{-1} cm^{-1} at 400 nm.[5]

8. Singlet oxygen production

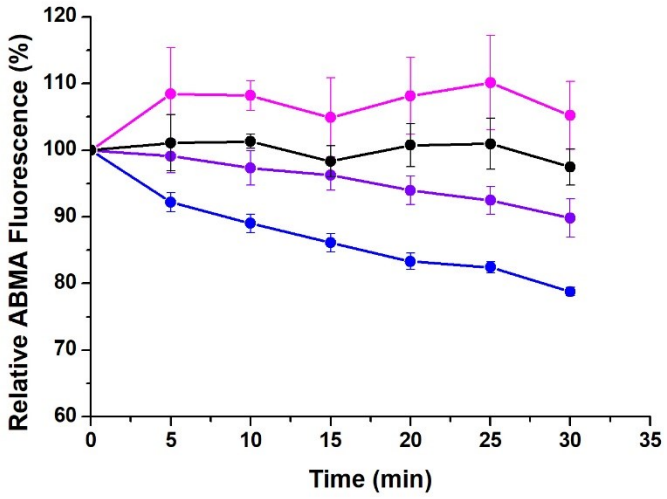

Figure S7: *Singlet oxygen production of PBS (pink), PEG-AuNPs (black), peptide-C11Pc-PEG-AuNPs (purple) and C11Pc-PEG-AuNPs (blue). All AuNPs were tested at 1 µM C11Pc with 1 µM ABMA. Error bars represent SE (n = 3)*

9. A549 phototoxicity

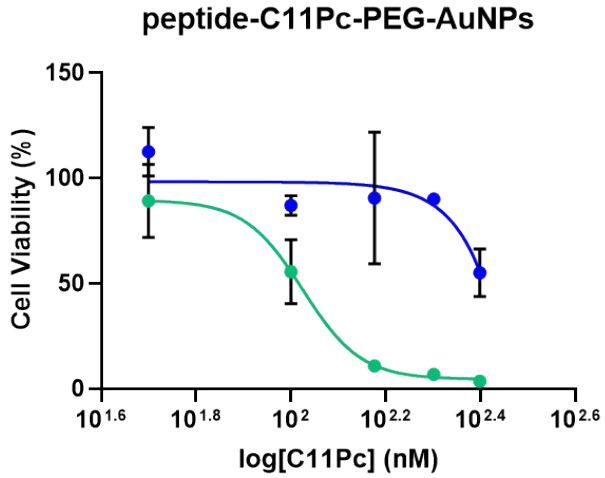

Figure S8: *Cell viability of A549 cells treated with peptide-C11Pc-PEG-AuNPs either with (green) or without (blue) irradiation. Error bars represent SE (n = 3)*

10. HEK293 phototoxicity

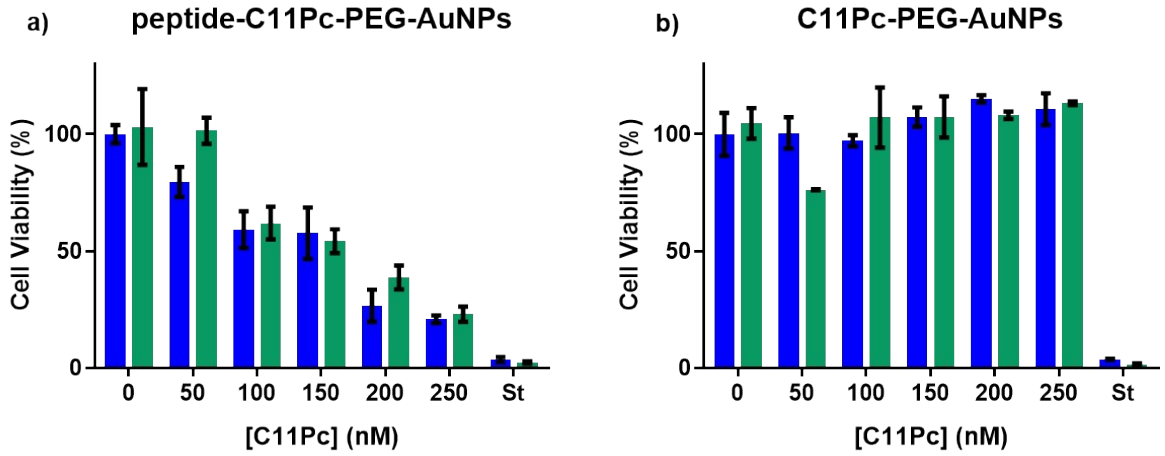

Figure S9: *Cell viability of HEK293 cells treated with* ***a)*** *peptide-C11Pc-PEG-AuNPs or* ***b)*** *C11Pc-PEG-AuNPs either with (green) or without (blue) irradiation. St = positive control of staurosporine. Error bars represent SE (n = 3)*

11. Control AuNPs characterisation

The control AuNPs were all characterized by UV-vis spectroscopy. PEG-AuNPs (fig S5a) displayed the characteristic spectrum of *ca*. 4 nm AuNPs.[6] The presence of FITC and/or C11Pc in the controls was confirmed by characteristic peaks at 495 nm (FITC) or 645/696 nm (C11Pc).

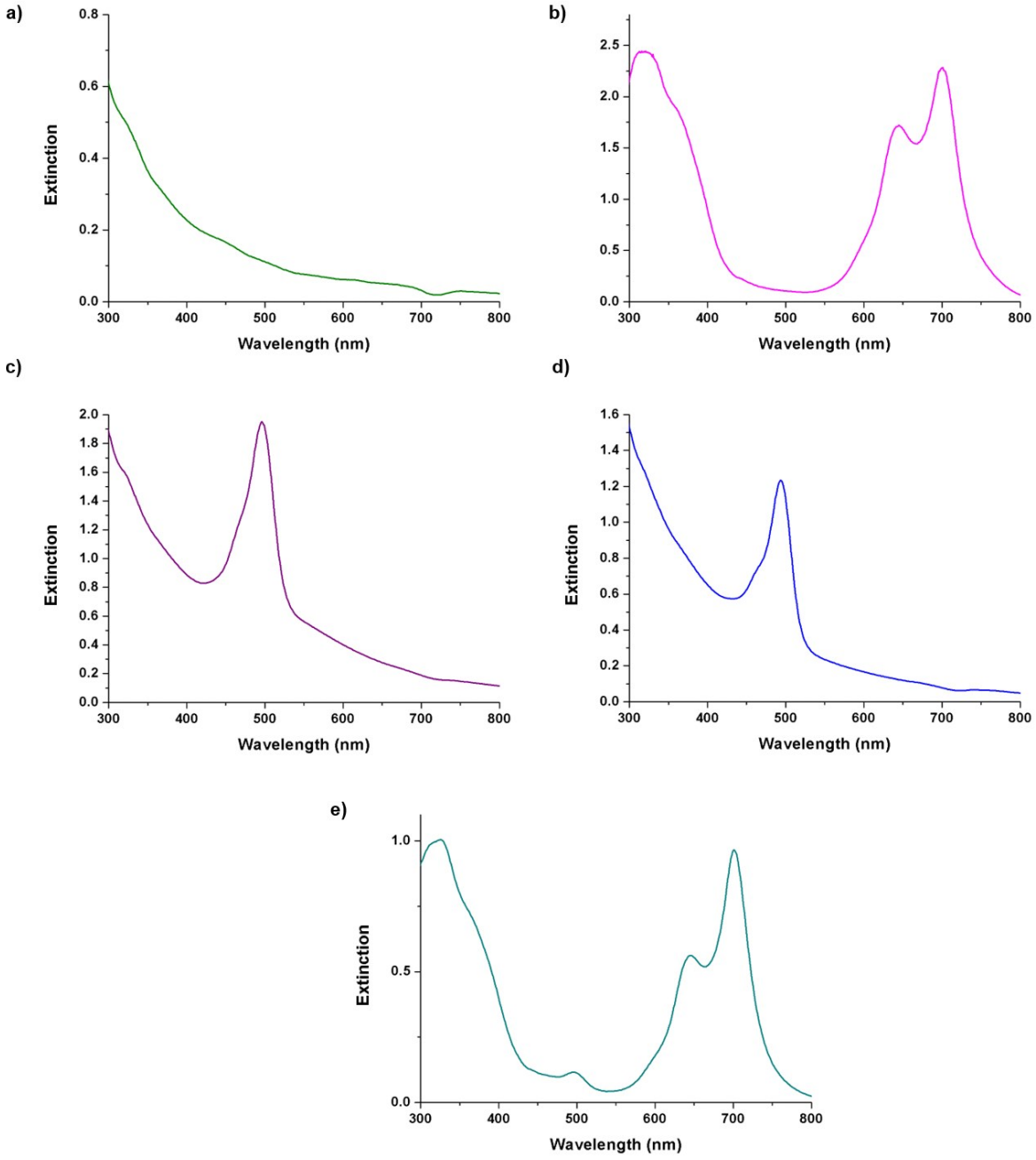

Figure S10: *UV-vis of **a)** PEG-AuNPs, **b)** C11Pc-PEG-AuNPs, **c)** FITC-PEG-AuNPs, **d)** peptide-PEG-AuNPs, **e)** FITC-C11Pc-PEG-AuNPs, recorded in phenol red free RPMI 1640*

12. References

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