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The solid-phase conjugation of purpurin-18 with a synthetic targeting peptide

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Abstract—It has been demonstrated that efficient site-specific coupling of the highly active photodynamic therapy sensitiser purpurin-18 to the synthetic targeting peptide G-G-V-K-R-K-K-K-P-G-Y-G can be achieved with greater than 85% purity.

Photodynamic therapy (PDT) is a localised, non-invasive treatment used to combat various types of cancer including oesophageal, bladder and skin cancer. The treatment exploits the interaction of a photosensitising compound, molecular oxygen and visible light of a characteristic wavelength to produce a cytotoxic effect via the production of reactive oxygen species (ROS). One area of importance in PDT is photosensitiser targeting. Photosensitisers can be covalently attached to molecules that will facilitate a targeting effect, such as an antibody, or peptide.

It has previously been shown that photosensitisers can be conjugated (i.e. covalently attached) to a variety of molecules including nuclear targeted insulin conjugates,[1] viral proteins[2] monoclonal antibodies,[3,11] loligomers[4,5] and transferrin.[7] The coupling methods used to date generally provide a non site-specific conjugation, with coupling efficiency often being described as an average number of photosensitisers per appendage. This makes accurate characterisation of the conjugates difficult. It has been demonstrated that the principle of directly attaching a photosensitiser to a synthetic peptide in a one to one ratio (Scheme 1) can be achieved, thus producing an isomer free photosensitiser-peptide conjugate. The method utilises solid phase peptide synthesis chemistry due to the high efficiency and simplicity that it provides.

The photosensitiser of choice was purpurin-18,[8] a chlorophyll derivative that has demonstrated good photodynamic efficacy in vitro.[6] The compound has an absorption peak at 695 nm, which is ideal for PDT as longer wavelength light has improved tissue penetration properties.[10] More importantly, the compound has a single free carboxylic acid moiety for ester activation.

The linear peptide sequence G-G-V-K-R-K-K-K-P-G-Y-G was derived from the T-antigen of the SV40 virus and is a characteristic nuclear localising sequence (NLS).[9] Synthesis of the linear peptide was performed using an automated ABI Applied Biosystems 433A peptide synthesiser. The pre-loaded Wang resin (Fmoc-Gly-Wang resin 0.34 mmol/g loading) had amino acids sequentially added using standard 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) chemistry. The amino acids used were as follows; glycine (*N*-α-Fmoc-glycine), valine (*N*-α-Fmoc-*L*-valine), lysine (*N*-α-Fmoc-*N*-εt.-Boc-*L*-lysine), arginine (*N*-α-Fmoc-N^G-(2,2,5,7,8-pentamethylchroman-6-sulfonyl)-*L*-arginine), proline (*N*-α-Fmoc-*L*-proline) and tyrosine (*N*-α-Fmoc-*L*-tyrosine). All functional groups were protected to prevent unwanted side reactions. For each amino acid addition, 2 molar equivalents were dissolved in dimethylformamide (DMF) along with HBTU (1.9 molar equivalents) and *N*-hydroxybenzotriazole (HOBt, 2 molar equivalents). The diisopropylethylamine in *N*-methylpyrolidine (2 M DIPEA in NMP, 5 molar equivalents) was added to the amino acid solution with gentle agitation before its addition to the resin. The product was a resin bound, fully protected linear peptide with greater than 98% purity as determined by

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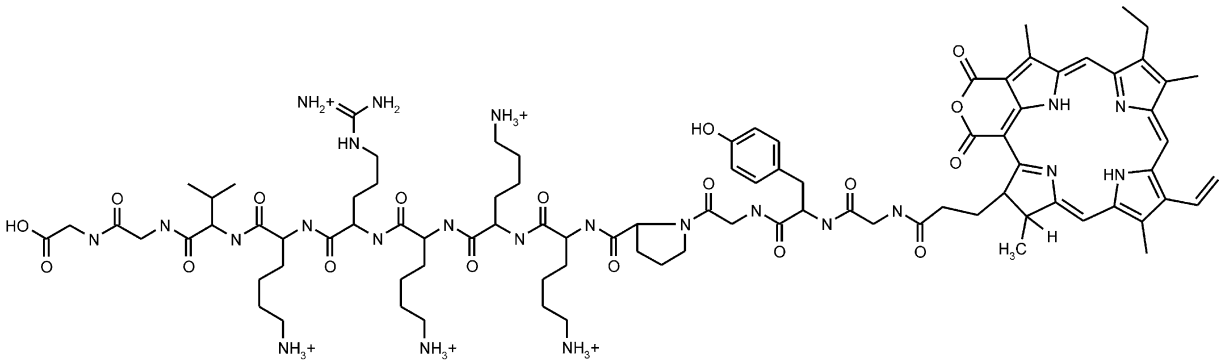

Scheme 1.

reverse phase high performance liquid chromatography (RP-HPLC). The resin was washed twice with 20% piperidine in (DMF) to effect the removal of the N terminal Fmoc protective group yielding a free amine.

During the photosensitiser coupling stage, light exposure was minimised by sealing the reaction vessel in foil to limit the occurrence of unwanted side reactions.

It was found that that conjugation of photosensitisers to the peptide was inefficient following the use of HBTU or 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) coupling procedures. This was due to either low coupling efficacy or the production of unwanted side products (as determined by RP-HPLC or MALDI mass spectrometry).

To circumvent these problems the highly efficient 2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) coupling chemistry was employed.

Purpurin-18 (5 molar equivalents, 14.7 mg) was placed in a glass vial containing 600 μL dry dichloromethane (DCM):DMF:dimethylsulphoxide (DMSO) (1:1:1 (v/v)) and sonicated for 10 min to ensure full dissolution. The coupling reagent HATU (4.9 molar equivalents, 9.7 mg) was then added to the vial and allowed to fully dissolve.

The mixture was then added to the resin (1 molar equivalent 10 mg) which was pre prepared by placing in minimal DCM for 30 min prior to reaction and purged with nitrogen for 2 min before the final addition.

DIPEA (10 molar equivalents, 26 μL) was added to the vial to initiate the reaction, which was left at room temperature for 2.5 h with intermittent agitation.

The reaction mixture was then passed down a sintered glass filtered vial and washed with approximately 15 mL of DMSO followed by 30–40 mL of DCM to remove all waste reagents. The resin was resuspended in DCM, collected in a flask and subjected to gentle rotary

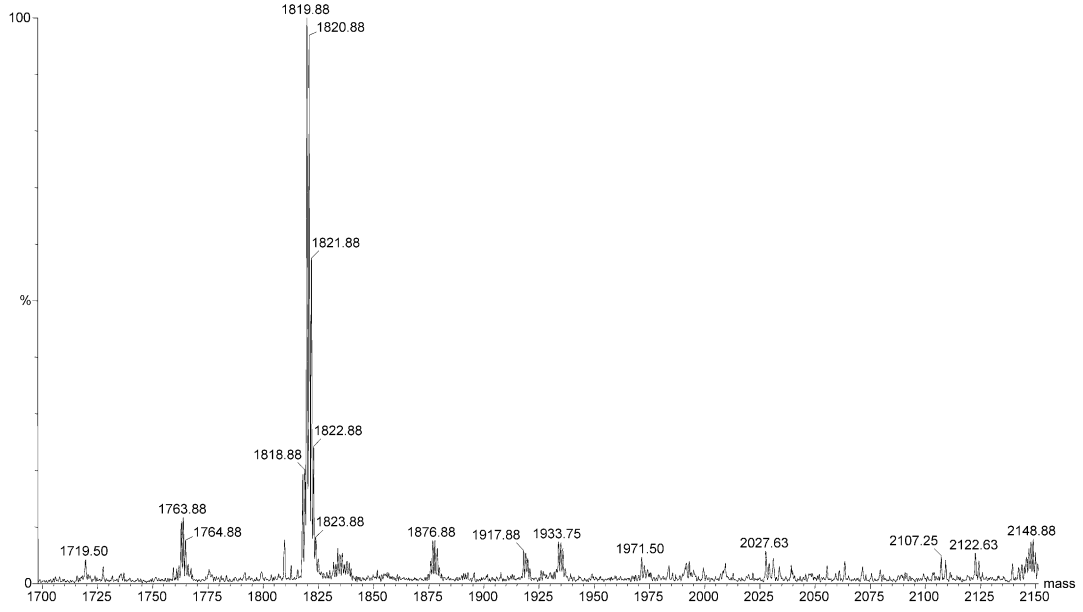

Figure 1.

evaporation until dry. To remove protecting groups and cleave the conjugated peptide from the resin, 2–3 mL of pre-cooled deprotection reagent (95% (v/v) trifluoroacetic acid (TFA), 5% (v/v) water and 5% (w/v) phenol) was added for approximately 3 h with intermittent and gentle agitation.

The mixture was then filtered down a Pasteur pipette containing glass wool, directly into a 50 mL falcon tube containing chilled diethyl ether (35 mL at 4 °C) and placed on ice for 5 min to aid precipitation. The falcon tube was then centrifuged for 5 min at 1000 rpm to give a pellet. The supernatant was removed and the washing procedure repeated a minimum of four times to ensure complete removal of TFA from the sample. The resulting pellet was left to air dry in the fume hood over night before being resuspended in water for analysis.

Analysis by RP-HPLC provided good seperation, with purity of greater than 85%, an improvement over our observations using the HBTU or TBTU coupling procedures. The analysis suggested that the RP-HPLC methodology should also be suitable for preparative purification of the conjugate. Analysis by MALDI mass spectrometry (Fig. 1) determined the main reaction product to have a molecular mass of 1819.88 (expected was 1819.87 for C_{90} H_{129} N_{23} O_{18}) indicative of one molecule of purpurin-18 covalently attached to one NLS peptide molecule. The yield was 28% of the start material. This is based on an assumed loading of the resin (0.34 mmol/g) and one to one coupling. Determination was performed by spectrophotometric quantification of the purpurin-18. It should be noted that yields are generally low when working on such small scales due to physical loss of the material.

In order to measure photodynamic activity, photosensitisers (100 μM) were placed in a solution of tryptophan (100 μM in 60% (v/v) MeOH 40% (v/v) 0.1 M sodium phosphate buffer pH 7.4) and exposed to red light. The reaction was monitored by measuring the decrease in tryptophan fluorescence.

Following the coupling reaction the conjugated purpurin-18 gave a value of 1.14% min^{-1} (± 0.35) compared to the value for free purpurin-18 of 1.08% min^{-1} (± 0.34) ($n = 2$), thus demonstrating no discernible decrease in intrinsic photodynamic activity following conjugation.

In conclusion, it has been determined that the principle of attaching a photosensitiser to a synthetic peptide in a one to one ratio is possible with good purity and acceptable yields, furthermore the procedure causes no discernible decrease in the photodynamic activity of the parent photosensitiser.

Finally, this technique should be amenable to any photosensitiser containing a carboxylic acid moiety for activation.

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