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Selective radiolabelling with ^{68}Ga under mild conditions: a route towards a porphyrin PET/PDT theranostic agent

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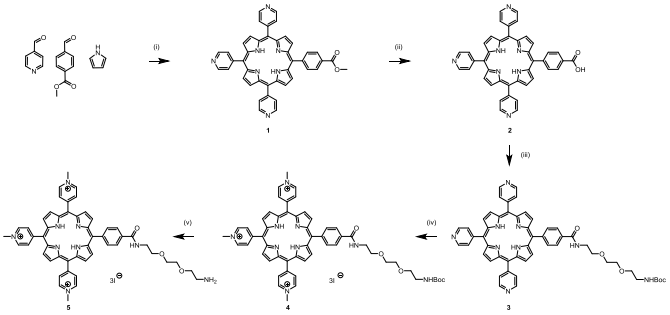
A theranostic conjugate for use as a positron emission tomography (PET) radiotracer and as a photosensitiser for photodynamic therapy (PDT) has been synthesised. A water-soluble porphyrin was coupled with the bifunctional chelate, H_4Dpaa.ga. This conjugate is capable of rapid ^{68}Ga complexation under physiological conditions; with 93% and 80% radiochemical yields achieved, at pH 4.5 and pH 7.4 respectively, in 15 mins at 25 °C. Photocytotoxicity was evaluated on HT-29 cells and showed the conjugate was capable of >50% cell death at 50 μM upon irradiation with light, while causing minimal toxicity in the absence of light (>95% cell survival).

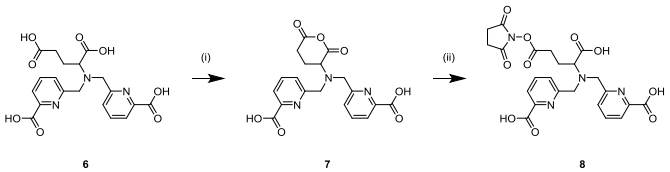
The goal of developing "personalised medicine" has seen a growing interest in the field of theranostics, a term often used for the combination of therapeutic and diagnostic modalities onto a single molecule. Current standardised cancer treatments, which have a "one-size-fits-all" methodology, often lead to significant difference in treatment outcomes.[1] Theranostics, however, allows the optimisation of treatment as it is capable of following the progression of disease before, during, and after treatment.

Photodynamic therapy (PDT) is a minimally invasive treatment that involves the usage of a photosensitiser and molecular oxygen. An ideal photosensitiser will selectively accumulate in the diseased tissues after a given period, and upon irradiation with visible light (400 – 700 nm) the photosensitiser can generate highly toxic reactive oxygen species (ROS) which damage tissues in close proximity to the site of generation. Introducing a diagnostic modality to a PDT agent will allow careful monitoring of the degree of accumulation of photosensitiser, improving treatment outcomes by irradiation when photosensitiser levels are maximal. Porphyrins are chosen here as the photosensitiser because they offer: potent photocytotoxicity, minimal dark toxicity, ease of synthesis and functionalisation, and have selectivity for tumorous tissues.[2–7]



Scheme 1 Synthesis of amine-appended porphyrin **5**. (i) Propionic acid, 170 °C, 1 hr. (ii) EtOH/H_2O, KOH, 40 °C, overnight. (iii) DMF, EDC, HOBt, DMAP, r.t., overnight. (iv) DMF, CH_3I, 40 °C, overnight. (v) DCM, TFA, r.t., 3 hrs.

Positron emission tomography (PET) is a highly sensitive medical imaging technique capable of functional imaging to observe metabolic changes in the human body. A wide variety of PET tracers have been developed,[8] notable amongst these is [^{18}F]-flurodeoxyglucose (FDG). FDG is widely used in cancer imaging.[9] Incorporation of many PET isotopes (^{11}C, ^{13}N, ^{15}O, ^{18}F) requires the formation of covalent bonds in a rapid, specific manner following the production of the radioisotope in a cyclotron. This procedure must be performed rapidly to ensure delivery of a radiotracer with sufficient activity for imaging. This limits the application of PET using these tracers to sites with



Scheme 2 NHS esterification of H_4Dpaa.ga. (i) MeCN, Ac_2O, pyridine, r.t. 30 mins. (ii) DMF, NHS, TEA, r.t. 2 hrs.

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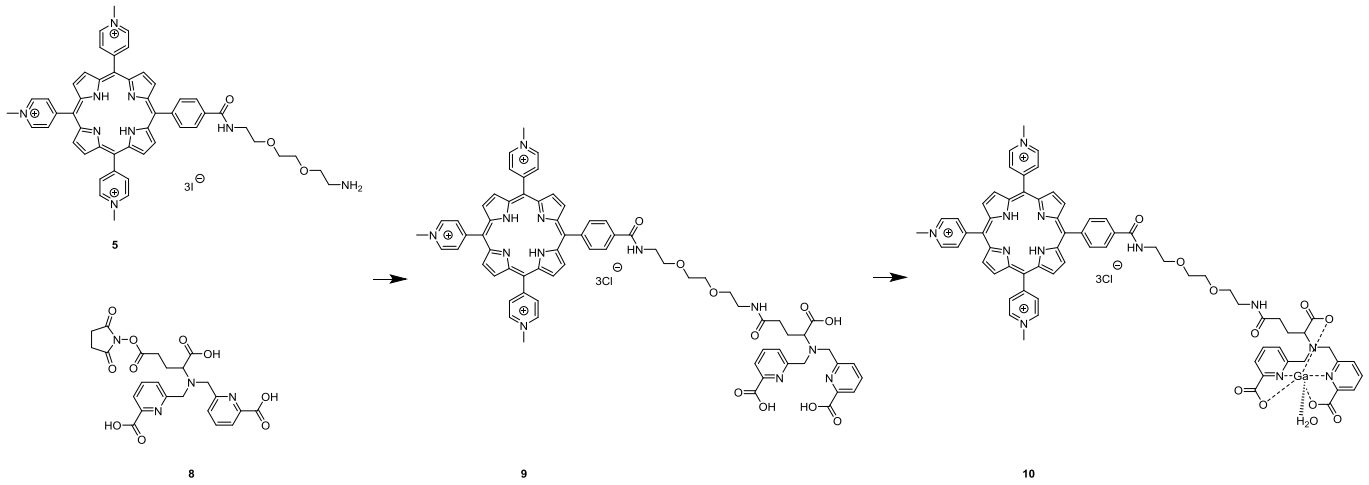

Scheme 3 Peptide coupling and gallium complexation of porphyrin-H_3Dpaa conjugate. (i) DMF, TEA, r.t., overnight. (ii) 0.1 M pH 4.5 acetate buffer, $GaCl_3$, r.t., overnight

ready access to cyclotron produced isotopes and specialised synthesis units.

The use of ^{68}Ga as a PET radioisotope has seen a significant growth in interest,[10–12] culminating in the recent FDA approval of the [^{68}Ga]-DOTATATE in July 2016.[13] This interest is often attributed to development of the ^{68}Ge/^{68}Ga generator, allowing facile on-site generation of the desired radionuclide.[11] ^{68}Ga can be readily incorporated into a radiotracer through the conjugation of a chelate to the targeting unit; this simplifies the synthesis that must be performed following production of the radionuclide.

Porphyrins are known to be able to complex a host of metals.[14] Not surprisingly, there are several reports showing the complexation of ^{68}Ga by porphyrins as theranostic agents.[15–19] However, the complexation of ^{68}Ga within the porphyrin core often requires harsh conditions; with reaction temperatures in excess of 100 °C are required. These conditions would be unsuitable for temperature-sensitive moieties, especially peptide-based targeting motifs and other biomolecules,[12] and some porphyrins have been reported to degrade under these conditions.[17]

Traditional macrocyclic chelators for ^{68}Ga are 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA); however, these chelators also require harsh conditions for complexation which involves heating of over 80 °C (required for DOTA) and acidic conditions (pH 4).[12] Recent developments in the design of chelates for ^{68}Ga have resulted in a number of chelates that are capable of radiolabelling under less acidic conditions.[20–22] Herein we employ the use of a *N,N*-bis[(6-carboxypyridin-2-yl)methyl]glutamic acid (H_4Dpaa.ga, **6**) bifunctional chelator, capable of rapidly chelating ^{68}Ga at ambient temperature under physiological pH (pH 7.4) with excellent radiochemical yield (RCY).[23]

We report here a water-soluble porphyrin-chelate conjugate, **9**, that can be radiolabelled under neutral conditions without heating with good radiochemical yields. The resulting Ga(III) complex **10** displays good phototoxicity in HT-29 cells.

Synthesis of an amine-appended porphyrin, **5**, was achieved in 5 steps (Scheme 1) as previously reported. [24,25]

The porphyrin-chelate conjugate, **9**, was prepared in a 3-step process (Scheme 2) by activation of chelate **6**, *via* an anhydride ring closing, and isolated by precipitation. This precipitate was used immediately to form the NHS ester, **8**, by addition of *N*-hydroxysuccinimide. **8** was used without further purification.

Addition of excess **8** to **5** yielded **9**, (Scheme 3) with quantitative conversion of porphyrin **5** to conjugate **9** observed by TLC and HPLC. Purification was achieved using a simple technique previously reported by our group.[25] The solubility of the porphyrin can be controlled by exchanging the anionic counter-ions. Exchange of the iodide anions with hexafluorophosphate rendered the conjugate insoluble in water. This allowed filtration and removal by washing of excess starting materials and reagents. Subsequent anionic conversion to chloride yielded the water-soluble conjugate **9**.

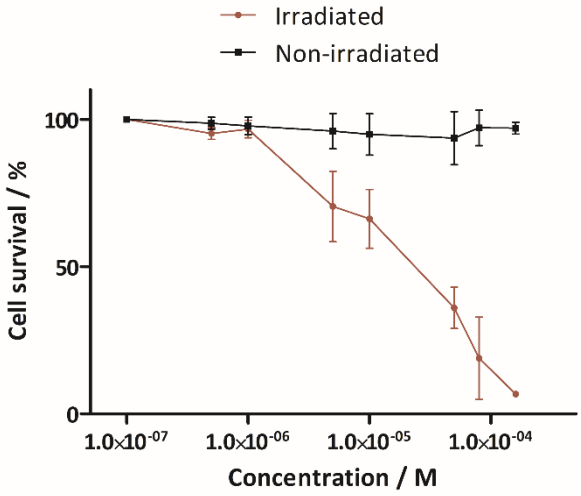

Figure 1 %cell survival of HT-29, irradiated cell and non-irradiated cells (control), determined using MTT assay. Cells were incubated with varying concentration of **10** for 1 hour and irradiated cells received 20 J cm^{-1} white light.

^{1}H NMR spectroscopy indicated the characteristic aromatic signals of a porphyrin, methylated pyridinium protons, and pegylated protons from the linker. The spectrum also indicated picolinic acid protons and protons on the amino acid from H_4Dpaa.ga both in the aromatic and in the alkyl regions. Analytical-HPLC also confirmed reaction (Figure S1 and Figure S2).

Complexation with Ga(III)Cl_3 was carried out under acidic conditions to produce the gallium complex, **10**. Purification of the complex by exchanging the anionic counter-ions was successful in removing excess Ga(III). HPLC analysis confirmed the purity of the resulting complex (Figure S1). The identity of the product was confirmed by high resolution mass spectrometry; indicating the formation of a Ga(III) complex and the stability of the conjugate to the acidic conditions used.

The photocytotoxicity of **9** and **10** was assessed in human adenocarcinoma (HT-29) cells (Figure 1, Figure S11). Cells were incubated with either **9** or **10** at varying concentrations and irradiation was carried using a constant dose of visible light (20 J cm^{-2}; 400 – 700 nm). The results were compared to a non-irradiated control. Although in a clinical setting, red light is more commonly used for PDT, the strength and power of clinical lasers used in PDT are significantly more powerful compared to the quartz tungsten halogen light source used in this study. Hence, to compensate for the lower power, white light was used, covering the whole porphyrin absorbance range, including the strong Soret band at 424 nm (Figure S10).

Under these conditions, >50% cell death was observed at 50 µM, and >90% cell death was observed at a concentration of 160 µM for both **9** and **10** when irradiated. Minimal dark toxicity was observed in the non-irradiated controls with more than 95% cell survival at all concentrations tested (Figure 1). This shows phototoxicity at a similar concentration to a clinically relevant porphyrin PDT agent, Photofrin®, in HT-29 cells.[26]

The radiolabelling efficiency of the conjugate **9** with ^{68}Ga to form [^{68}Ga]-**10** was assessed at two different pHs - pH 4.5 and pH 7.4. At pH 4.5 and 25 °C, **6** and **9** complexed ^{68}Ga with RCY of >99% (Figure S3) and 93% (**Figure 2**) respectively in 15 minutes. At pH 7.4 and 25 °C, **6** and **9** complexed ^{68}Ga with RCY of 96% (Figure S3) and 80% (**Figure 2**) respectively in 15 minutes. Radiolabelling of **5** was also attempted with ^{68}Ga, with conventional heating at 99 °C. At both pH 4.5 and pH 7.4, no radiolabelling was observed (Figure S3). This shows that, even with heating, the porphyrin moiety did not take part in gallium complexation.

The novel agent, [^{68}Ga]-**10**, demonstrates a strategy to produce porphyrin PDT agents that can be selectively and readily radiolabelled under mild conditions with ^{68}Ga for PET. Phototoxicity and toxicity of **9** and **10** was evaluated on human adenocarcinoma (HT-29) cells with and without irradiation with visible light respectively. Neither system showed significant toxicity in the absence of irradiation and both were capable of inducing cell death when irradiated. The conjugate **9** is capable of ^{68}Ga complexation at physiological conditions (15 minutes, pH 7.4, room temperature) to produce [^{68}Ga]-**10** with a RCY of 80%, thus providing a route towards a ^{68}Ga labelled porphyrin PET/PDT theranostic agent.

Table 1 Radiochemical yields of ^{68}Ga labelling reactions.

Ligand	H_4Dpaa.ga **6**[a]	Porphyrin-H_3Dpaa **9**[a]	Porphyrin **5**[b]
pH 4.5[c]	99%	93%	0%
pH 7.4[d]	96%	80%	0%

[a,b][L] = 100 µM ligand. [a]*t* = 15 mins, *T* = 25 °C. [b]*t* = 45 mins, *T* = 99 °C. [c]*I* = 0.1 M acetate buffer. [d]*I* = 0.1 M phosphate buffer.

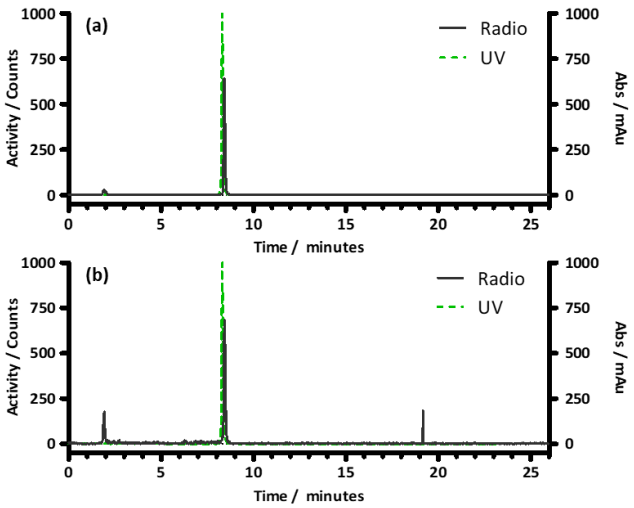

Figure 2 HPLC chromatograms of radiolabelling mixture. (a) pH 4.5, 0.1 M acetate buffer (b) pH 7.4, 0.1 M phosphate buffer. Black solid line indicates radio-HPLC. Green dashed line indicates UV-HPLC of **10**. Radiolabelling conditions: [**9**] = 100 µM, T = 25 °C, t = 15 mins.

Acknowledgements

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Conflicts of interest

There are no conflicts to declare.

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