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# Synthesis and aggregation of a porphyrin cored hyperbranched polyglycidol and its application as a macromolecular photosensitizer for photodynamic therapy.

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KEYWORDS Photodynamic therapy, drug delivery, hyperbranched polymers, self-assembly.

## **ABSTRACT**

Macromolecules are potentially useful delivery systems for cancer drugs as their size allows them to utilize the enhanced permeability and retention effect (EPR), which facilitates selective delivery to (and retention within) tumors. In addition, macromolecular delivery systems can prolong circulation times as well as protecting and solubilizing toxic and hydrophobic drug moieties. Overall these properties and abilities can result in an enhanced therapeutic effect. Photodynamic therapy (PDT) combines the use of oxygen and a photosensitizer (PS), that

become toxic upon light-irradiation. We proposed that a PS encapsulated within a water-soluble macromolecule could exploit the EPR effect and safely and selectively deliver the PS to a tumor. In this paper, we describe the synthesis of a porphyrin cored hyperbranched polymer that aggregated into larger micellar structures. DLS and TEM indicated that these aggregated structures had diameters of 45 nm and 20 nm for the solvated and non-solvated species respectively. The porphyrin cored HBP (PC-HBP), along with the non-encapsulated porphyrin (THPP), were screened against EJ bladder carcinoma cells in the dark and light. Both THPP and PC-HBP displayed good toxicity in the light, with LD50 concentrations of 0.5  $\mu\text{M}$  and 1.7  $\mu\text{M}$  respectively. However, in the dark, the non-incorporated porphyrin (THPP) displayed significant toxicity, generating an LD50 of 4  $\mu\text{M}$ . On the other hand, no dark toxicity was observed for the polymer system (PC-HBP) at concentrations of 100  $\mu\text{M}$  or less. As such, incorporation within the large polymer aggregate serves to eliminate dark toxicity, whilst maintaining excellent toxicity when irradiated.

## **INTRODUCTION**

Photodynamic therapy (PDT) is a treatment that exploits the combined action of a photosensitizer (PS), oxygen and a specific light source.<sup>1</sup> Upon irradiation, a PS can activate molecular oxygen, transforming it into a number of therapeutically active oxygen species (singlet oxygen and free radicals) which act to destroy abnormal cells. Although the principle application of PDT has been towards the treatment of cancers,<sup>2</sup> including lung cancer,<sup>3,4</sup> mouth cancer<sup>5</sup> and skin cancer.<sup>6</sup> Other conditions that can be treated with PDT include, actinic keratosis,<sup>7</sup> Bowen's disease (an early form of skin cancer),<sup>8</sup> macular degeneration (an eye

condition leading to vision loss),<sup>9</sup> and Barrett's oesophagus (treats damaged cells in the lining of the oesophagus).<sup>10</sup> More recently, PDT has also shown promise as an antibacterial agent.<sup>11</sup>

There are also examples of PDT systems that involve a synergistic delivery of photosensitizers and antitumor drugs.<sup>12</sup> Although PDT is an established principle, the number of photosensitizers approved for clinical use is limited. This is due to the photosensitizers (PS) either having low water solubility, significant “dark“ toxicity or a lack of specificity. Solubility can be increased and dark toxicity reduced via modification of the PS. However, this has the potential to reduce or destroy the therapeutic efficiency of the PS. Alternatively, the PS can be incorporated within a supramolecular nanocarrier. As well as increasing water solubility and limiting dark toxicity, the use of nanocarriers can result in the preferential accumulation of the PS within tumors due to the *enhanced permeability and retention effect* (EPR effect).<sup>13</sup> For effective EPR, molecules with molecular weights greater than 40 kDa are required (up to 800 kDa).<sup>14</sup> As well as effecting tumor accumulation, another benefit of EPR is an increased half-life within the blood stream, as the sizes involved exceed the threshold limit for renal extraction. Overall, nanocarrier PS can be specifically targeted towards tumors and result in a 10-fold increase in tumor concentration (when compared to smaller PS).<sup>15</sup> Examples of proposed nanocarriers for photodynamic therapy include liposomes,<sup>16,17</sup> polymeric nanoparticles,<sup>18</sup> silica nanoparticles,<sup>19</sup> graphene oxide systems<sup>20</sup> and porphyrin cross-linked polymers.<sup>21</sup> One particularly good exemplifier of a macromolecular PS are the dendrimer functionalized polyion-complexes (PIC).<sup>22</sup> For example, Kataok reported the use of amino terminated dendrimers capable of forming micellar PIC when mixed with PEG-b-poly(Asp). In vitro studies on this PIC showed excellent photodynamic efficacy towards LLC cells, with LD50 values less than 1  $\mu$ M.<sup>23</sup> Although the use of dendrimers was successful, their step-wise synthesis is time consuming, expensive and can be difficult. This has limited their

large-scale production and potential therapeutic exploitation. Hyperbranched polymers (HBP) possess structures and physical properties that are very similar to those displayed by dendrimers.<sup>24</sup> However, their synthesis is quick (often involving a single synthetic step) and inexpensive, which enables them to be made more easily and on a large scale. Our aim was to develop a safe, synthetically simple hyperbranched polymeric photosensitizer, which was non-toxic in the dark, would kill cancer cells when irradiated with light, and target tumors by virtue of their size.

We have previously studied hyperbranched polyglycidols for application as drug delivery systems.<sup>25</sup> These HBPs are water-soluble and although studies have not been exhaustive, they appear to be safe with respect to clinical application.<sup>26</sup> Previous work within our laboratory observed that hyperbranched polyglycidols can aggregate at very low concentrations (critical aggregation concentration/CAC of 0.1 mg/mL).<sup>27</sup> A low aggregation concentration is important with respect to any therapeutic application, as the aggregated PS should be able to maintain its structure as it circulates through the blood stream. Subsequent measurements using Dynamic Light Scattering (DLS) indicated that these self-assembled aggregates had solvated diameters between 40-90 nm, which is similar in size to the dendrimer functionalized PIC.<sup>22</sup> As such, these polymers in their aggregated form make ideal delivery systems for photosensitizers and for application in PDT.

## **EXPERIMENTAL**

**Materials.** All reagents and solvents were obtained from commercial sources (primarily Sigma-Aldrich) and were used without further purification. Dry solvents were obtained from the

University of Sheffield Chemistry Department Grubbs solvent dispensing system. All glassware was cleaned and dried in an oven overnight (100 °C) before use.

**Instrumentation and methods.** UV absorbance was recorded on an Analytic Jena AG Specord s600 uv/vis spectrometer and analyzed using WinASPECT. Infrared spectra were recorded using a Perkin-Elmer UATR Infrared spectrometer. Spectra were analyzed with Spectrum100 software. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded using a Bruker AV1400 MHz machine and chemical shifts referenced to residual solvent signals. Chemical shifts are quoted using ppm and coupling constants quoted in Hertz. The NMR spectra were analyzed using Topspin 3.0 NMR software. Fluorescence Spectroscopy was obtained using a FluoroMax-4 spectrometer. Aqueous Gel Permeation Chromatography (GPC) data was obtained at room temperature using a high molecular weight column (2x aquagel-OH). Calibration was achieved using polyethylene glycol standards (Mn 220-1,500,000 Da) and molecular weights are thus reported relative to these specific standards used. Samples were filtered using Whatman® GD/X syringe filters with a pore size of 0.45 µm before being injected onto the column through a 200 µL sample loop via a Gilson 234 Auto Injector. The samples were prepared and eluted using phosphate buffered saline (PBS) at pH 7.4 as solvent (which was supplied to the columns by a Waters 515 HPLC Pump at 1.00 mL min<sup>-1</sup>). Sample detection was via an Erma ERC-7512 refractive index detector.

**Synthesis.** *Tetrakis (4-hydroxyphenyl)-porphyrin (THPP) 1.* 5, 10, 15, 20-Tetrakis (4-hydroxyphenyl)-porphyrin (THPP) was synthesized according to the Rothmund and Adler-Longo method.<sup>28</sup> In a brief, freshly distilled pyrrole (4.17 mL, 60 mmol) and 4-Hydroxy benzaldehyde (10 g, 60 mmol) were added to refluxing propionic acid (300 mL). The mixture was refluxed for 1 hr, allowed to cool to room temperature and placed for 1 hour at -5 °C. The sample was washed thoroughly with a mixture of cold propionic acid and alcohol (1:1), followed

by cold chloroform. Yield 2.30 g. UV (methanol-nm): 418 ( $\lambda_{\max}$ ), 516, 554, 588, and 649; IR (neat-  $\text{cm}^{-1}$ ): 3249 (OH), 2924 (NH), 2875 (CH), 1609 (C=N), 1463 (C=C),  $^1\text{H NMR}$   $\delta\text{H}$  (DMSO- $\text{d}_6$ ): 9.99 (s, 4H,  $\text{OH}$ ), 8.85 (s, 8H,  $\beta$ -Pyrrole), 7.97 (d, , J=9.0 Hz, 8H phenylic m- $\text{CH}$ ), 7.18 (d, J=9.0 Hz, 8H, phenylic o- $\text{CH}$ ), -2.92 (s, 2H,  $\text{NH}$ -pyrrole).  $^{13}\text{C NMR}$  (DMSO- $\text{d}_6$ ): 169.1, 157.3, 136.8, 129.2, 114.7, 117.1, 110.9. ESI-MS: 679;  $\text{MH}^+ = 679 \text{ gmol}^{-1}$ .

*Tetrakis (4-hydroxyphenyl)-porphyrin-spacer (THPP-spacer) 3.* 5,10,15,20-Tetrakis(4-hydroxyphenyl)-21H,23H-porphyrin (177 mg, 0.261 mmol),  $\text{K}_2\text{CO}_3$  (453 mg, 3.13 mmol), and 18-crown-6 (10 mg) were added to 30 mL of dimethylformamide (DMF). 1,3-Bromopropanol (291 mg, 2.09 mmol,) was added and the reaction was vigorously stirred under reflux overnight followed by TLC. The DMF was evaporated and the reaction mixture was dissolved in 200 mL of ethyl acetate and extracted three times with water (100 mL). The solvents were evaporated to yield (80%) a purple solid. UV (methanol-nm): 418 ( $\lambda_{\max}$ ), 515, 554, 588, and 649 IR (neat-  $\text{cm}^{-1}$ ): 3249 (OH), 2924 (NH), 2870 (C-H), 1609 (C=N), 1463 (C=C),  $^1\text{H NMR}$  (DMSO- $\text{d}_6$ ):  $\delta$  8.85 (s, 8H,  $\beta$ -Pyrrole), 7.97 (d, J=9.0 Hz, 8H, phenylic m- $\text{CH}$ ), 7.18 (d, J=9.0 Hz, 8H, phenylic o- $\text{CH}$ ), 4.58 (s, 4H, phenylic m- $\text{OH}$ ), 4.20 (t, J=7.0 Hz, 8H, -O- $\text{CH}_2$ - $\text{CH}_2$ -), 3.63 (t, J=7.0 Hz, 8H, - $\text{CH}_2$ -OH), 2.47 (m, 8H, - $\text{CH}_2$ - $\text{CH}_2$ - $\text{CH}_2$ -), -3.01 (s, 2H,  $\text{NH}$ ).  $^{13}\text{C NMR}$  (DMSO- $\text{d}_6$ ):  $\delta$  158.4, 135.4, 135.4, 134.6, 119.5, 112.0, 56.9, 54.0, 31.6. ESI-MS: 911:  $\text{M}^+ = 911 \text{ gmol}^{-1}$ .

*Porphyrin cored hyperbranched polyglycerol (PC-HBP) 4.* The THPP-spacer **3** (0.1 g, 0.11 mmol) was added to diethylene glycol dimethyl ether (10 mL) in a round bottomed flask fitted with a condenser. The solution was stirred at  $90^\circ\text{C}$  until the porphyrin **3** had dissolved. Potassium methoxide (0.031 g 0.43 mmol) was added and the reaction left for a further 90 minutes. Glycidol (1.63 g 0.43 mmol) was added drop-wise via a syringe pump over 4 hours and

the reaction left for a further 5 hours at 90 °C. The mixture was allowed to cool to room temperature. The solvent was decanted off and a minimum amount of methanol was added to cause precipitation of the by-products. The solid material was then removed by filtration, the filtrate poured into acetone, and the mixture stirred for 1 hour. The solvent was then decanted off. The material that remained was washed several times with acetone, which was decanted off after each wash. This was repeated until the washings were colorless. After removal of all solvent and drying under vacuum, the porphyrin cored hyperbranched polyglycerol was obtained as an extremely viscous red solid in a yield of 75% by mass. UV (methanol-nm): 424 ( $\lambda_{\text{max}}$ ), 516, 556, 593 and 645; IR (neat-  $\text{cm}^{-1}$ ): 3350 (OH), 2875 (CH), 1340 (O-C-O), 1070 (C-O).  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta$  8.88 (br-s, 8H,  $\beta$ -Pyrrole), 8.05 (br-d, phenylic m- $\text{CH}$ ), 7.25 (br-d, phenylic o- $\text{CH}$ ), 4.08-3.20 (br-m, H, remaining  $\text{CH}$ , and  $\text{CH}_2$ ).  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ ): 158, 150.7, 146.3, 131.2, 78.7, 77.0, 72.1, 70.3, 66.4, 62.3, 60.4, 42.2; GPC; Mn 32500, Mw 69200.

**Critical Aggregation Concentration (CAC) by pyrene emission.** CACs of HPG polymers were determined by fluorescence measurements using pyrene as a probe. 100  $\mu\text{L}$  of an aqueous pyrene solution ( $6 \times 10^{-7}$  M) was added to a series of polymer solutions (4 mL) with concentrations ranging from 0.001 mg/mL to 1.0 mg/mL. Fluorescence spectra were recorded using an excitation wavelength of 334nm, and emissions at 372 nm and 384 nm were monitored. The CACs were estimated by plotting the intensity ratio  $I_{384}/I_{372}$  vs polymer concentration.

**Cell culture.** EJ (bladder carcinoma) cells were purchased from American Type Culture Collection–LGC partnership (Teddington, UK) and used within 20 passages of purchase. Cells were cultured using Dulbecco’s modified Eagles Medium (DMEM) (Lonza, Cambridge UK) with 10% fetal calf serum (FCS) (Lonza, Cambridge UK) and cultured in an incubator (37 °C, 5



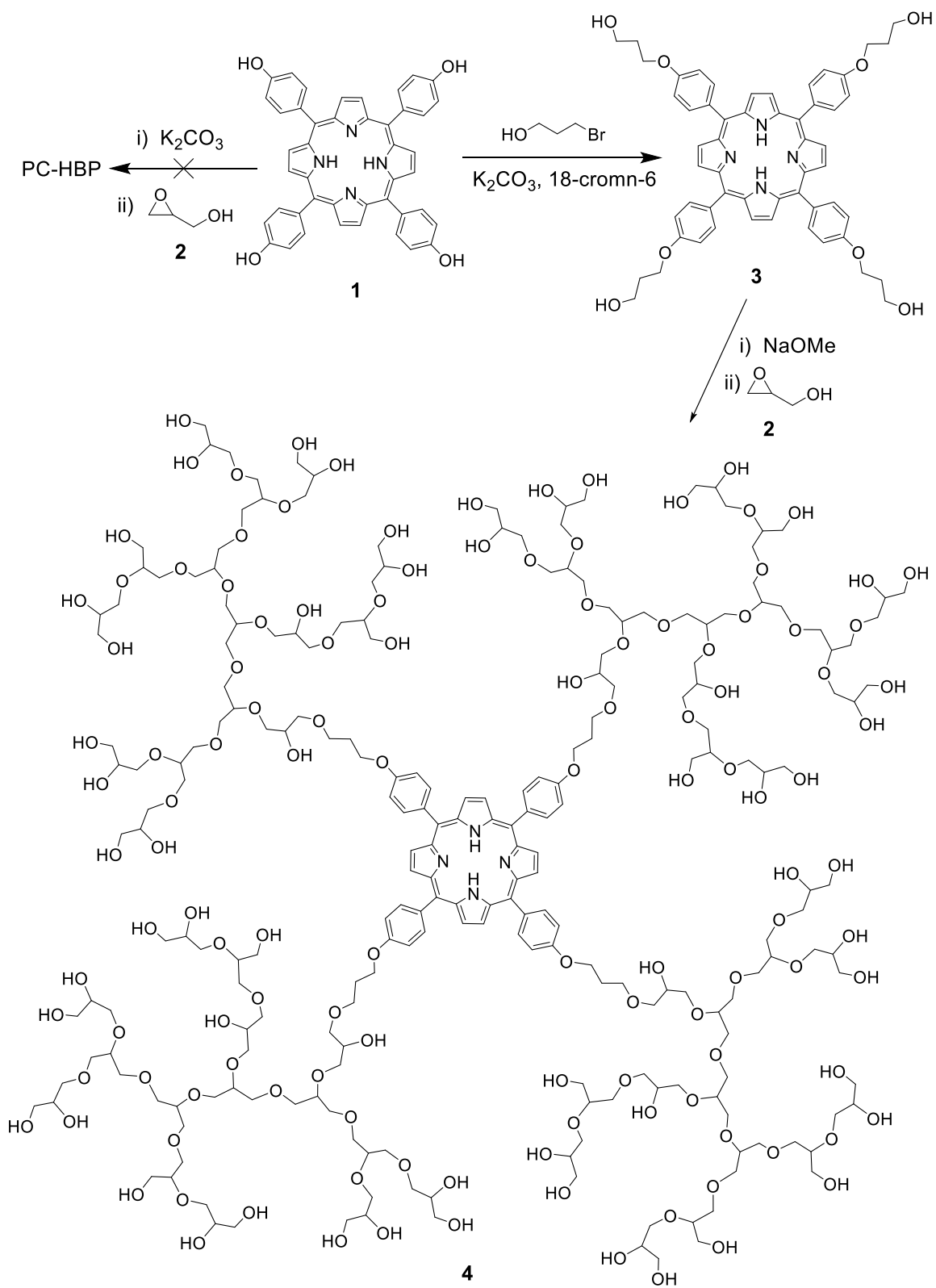
% CO<sub>2</sub>). The cells were passaged when 70-80 % confluence was reached and regularly checked for mycoplasma contamination. Stock solutions of compounds were stored in deionized water, or DMSO, at 10 mM aliquoted in small volumes to avoid freeze/thaw cycles.

**Imaging by confocal microscopy.** EJ (bladder carcinoma) cells were seeded on sterile cover slips (22 x 22 mm) in 6 well dishes at a density of 150,000/well and incubated overnight. Staining solutions of the desired compound were made by dilution into culture media (10 µM) and added to the cells for 24 hours. Once incubation had taken place the cells were washed (PBS) and fixed (4% paraformaldehyde solution in PBS, 4 °C, 20 mins) before being washed again (PBS) and mounted (IMMU-MOUNT, Life Technologies Ltd, Paisley, UK). The slides were imaged by confocal microscopy (Inverted Zeiss LSM 510 NLO microscope) using a 60 × lens, with activation by helium-neon laser ( $\lambda = 633$  nm). Emission was registered in the region 650-710 nm.

**MTT assay - Light and dark toxicity.** 96 well-plates were seeded with EJ cells at 3000/well and incubated overnight. The wells were treated with PC-HBP **4** or THPP **1** and incubated for 24 hours. The media was removed and 40 µL/well PBS was added to each well. For light toxicity experiments the plate was placed in a foil lined box, 6 cm below the light source for 10 minutes at 450/630 nm using an LED array (at 4.20 and 4.85 mW cm<sup>-2</sup> respectively). After storage in the dark, or exposure to light, media was added (DMEM, 10 % FCS, 200 µL/well) and the plates were placed in the incubator. Following 24-hour incubation thiazoyl blue (MTT) solution was added to each well (25 µL, 3 mg ml<sup>-1</sup> in PBS). After a 3-hour incubation the media and MTT solution was removed from each well and DMSO was added (150 µL/well) and crystals dissolved. Optical density of the wells at 540 nm were recorded on a plate reader (Multiskan fc, Thermo Fisher Scientific, Warrington, UK).

## RESULTS AND DISCUSSION

In this work, we proposed to synthesize a hyperbranched polyglycidol with a PS at its core and study its aggregation and its subsequent application as a macromolecular PS. Due to their strong absorption (and emission) at therapeutically useful wavelengths, porphyrins are an established PS for PDT and were selected as our photosensitizer unit. Specifically, the hydrophobic tetra-hydroxyphenylporphyrin (THPP) **1**, was selected as the core for our macromolecular PS. This is a known PS, with good toxicity in light. However, TCPP **1** also has considerable toxicity in the dark. We proposed that its dark toxicity could be reduced, or even prevented, by incorporation within a HBP. Therefore, our initial attempt to synthesize a porphyrin cored HBP started with porphyrin **1**,<sup>29</sup> which was synthesized in good yield from 4-hydroxybenzaldehyde and pyrrole using standard procedures, Scheme 1. Porphyrin **1** possess four phenolic OH groups, each capable of initiating the ring opening polymerization of glycidol **2**. As such, the phenol groups of TCPP **1** were deprotonated (to improve nucleophilicity), and glycidol **2** added by slow monomer addition. After workup, aqueous GPC indicated that a polymer with a modest molecular weight of around 10,000 had been formed, Scheme 1. However, when compared to the polymer backbone, the signals from the porphyrin group were very weak in the <sup>1</sup>H NMR spectrum. This suggested that porphyrin incorporation had been very low. A qualitative assessment of core incorporation was estimated by comparing the Mn values obtained from GPC (a bulk analysis technique) and NMR (a core group analysis technique),<sup>30,31</sup> that indicated that less than 20% of the polymer molecules contained a porphyrin core. The problem was caused by delocalisation of the phenolate ions into the aromatic ring, resulting in poor nucleophilicity and low reactivity towards the glycidol monomer.



**Scheme 1:** Synthesis of the porphyrin cored hyperbranched polymer (PC-HBP) 4.

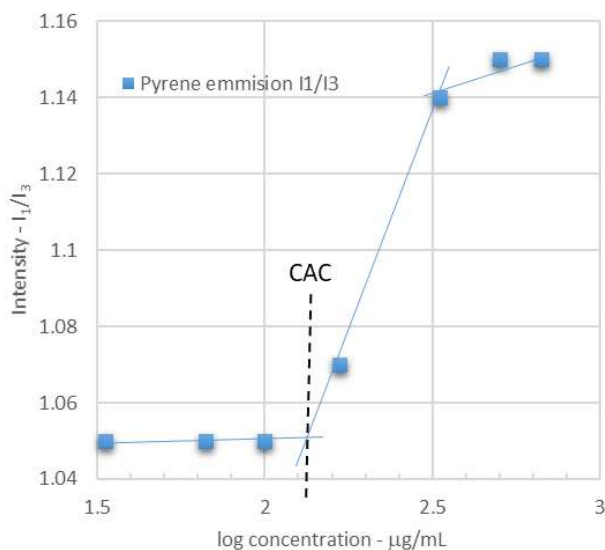
The stability and low reactivity of the phenol units can be overcome by breaking the conjugation between the porphyrin and nucleophilic OH group, which can be achieved by adding a spacer group. This was accomplished by reacting TCPP **1** with a large excess of 3-bromopropanol in the presence of  $K_2CO_3$  and a catalytic amount of 18-crown-6, Scheme 1. After workup and purification, the extended porphyrin **3** was obtained in excellent yield. Subsequent deprotonation using sodium methoxide, followed by slow addition of 50 molar equivalents of glycidol **2**, gave the water-soluble porphyrin cored hyperbranched polymer (PC-HBP) **4** in a good 75 % yield, Scheme 1.

The  $^1H$  NMR spectrum of PC-HBP **4** showed two significant sets of peaks, the first was a group of broad peaks observed in the aromatic region between 7.85–8.40 ppm. These corresponded to the aromatic groups of the porphyrin core. The second set of peaks were larger and appeared as a broad multiplet between 3.25 and 4.20 ppm. This is consistent with the spectra of other hyperbranched polyglycidols and corresponds to the CH,  $CH_2$  and OH groups of the polyglycidol backbone.<sup>32</sup> Comparing the relative intensities of these two regions allowed us to estimate an  $M_n$  value of 32,500 Da.<sup>30</sup> Aqueous GPC indicated that the bulk material had an  $M_n$  of 21,000 Da. Comparing this value to the  $M_n$  obtained via NMR, allowed us to estimate a minimum core incorporation of at least 65%. On first inspection, this may appear somewhat low. However, due to the way in which the two methods calculate  $M_n$ , the actual level of incorporation is much higher. For example, when employing  $^1H$  NMR we assume that every polymer has a porphyrin core, and we compare the intensity of the porphyrin signals with those obtained from the polymer backbone. However, this method cannot differentiate between polymers with or without core and consequently *overestimates*  $M_n$ . On the other hand, GPC *underestimates* the value of  $M_n$  for globular HBPs, due to errors derived from calibration using linear polymers

(which have a larger hydrodynamic radius when compared to globular HBPs with equivalent molecular weights).<sup>33,34</sup> As such we were satisfied that a high proportion of polymers possessed a porphyrin core. Finally, UV analysis of an aqueous solution of HBP 4 confirmed porphyrin incorporation, through the presence of a Soret band at 424 nm and four smaller Q bands at 516, 556, 593 and 645 nm.

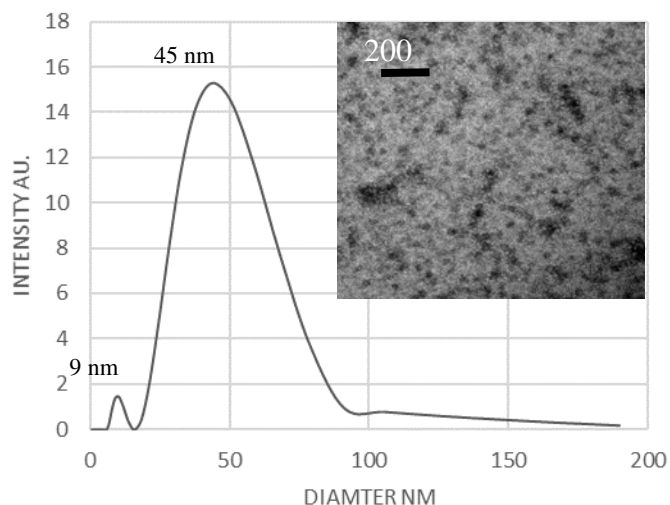
Aggregation of PC-HBP 4 was established through its critical aggregation concentration (CAC). This was determined using a fixed concentration of pyrene and measuring changes in emission intensity with respect to increased polymer concentrations.<sup>35</sup> The plot of polymer concentration vs. changes in pyrene emission is shown in Figure 1. The point at which the pyrene emission changes can be used to estimate the CAC. Our plot shows a break around 0.15 mg/mL and is the concentration at which the aggregate forms, and the pyrene environment and local concentration changes (as pyrene moves from the bulk aqueous phase into the smaller hydrophobic volume of the aggregated polymer). This concentration is typical of the CAC reported for HBPolyglycidols.<sup>36</sup> Although it is not possible to determine a precise molecular weight, and therefore an accurate molar concentration of a disperse polymer, a CAC between  $10^{-8}$  and  $10^{-9}$  M was approximated using the molecular weight determined from GPC. The size of the aggregated PC-HBP 4 was measured using DLS at a concentration above the CAC (at 0.25 mg/mL), and showed two peaks. The first was a very small peak around 9-10 nm and is the non-aggregated PC-HBP 4 (solvated). The second peak, which corresponds to the solvated aggregate of PC-HBP 4, was present at 45 nm. The number of PC-HBPs 4 within the aggregated structures can be crudely estimated by comparing the size of the non-aggregated and aggregated species. The ratio obtained allows us to estimate that at least five of the porphyrin-cored polymers (and therefore five porphyrin PS), combine to form the aggregated structure. Although relatively

small, the aggregate would have a combined molecular weight around 175 KDa. A TEM image of PC-HBP 4 confirmed aggregation and showed spherical structures with diameters around 20-30 nm, Figure 2. This size is large enough to be exploited by the EPR effect, with respect to tumor selective delivery and localization.<sup>13</sup>

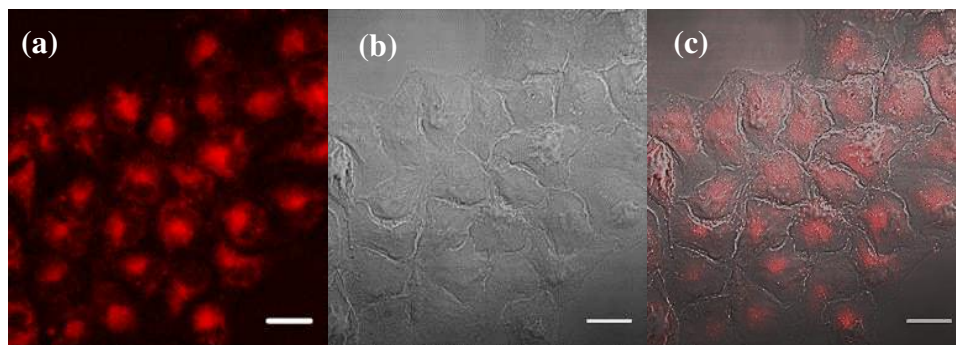


**Figure 1.** Determination of critical aggregation concentrations (CAC) using pyrene encapsulation.

The intracellular localization of PC-HBP 4 into EJ (bladder carcinoma) cells were imaged by confocal microscopy. Figure 3 shows porphyrin fluorescence within the cell, confirming that PC-HBP 4 has been internalized. This is consistent with the intracellular localization observed for other macromolecules, including porphyrin-cored dendrimer PICs.<sup>37</sup>



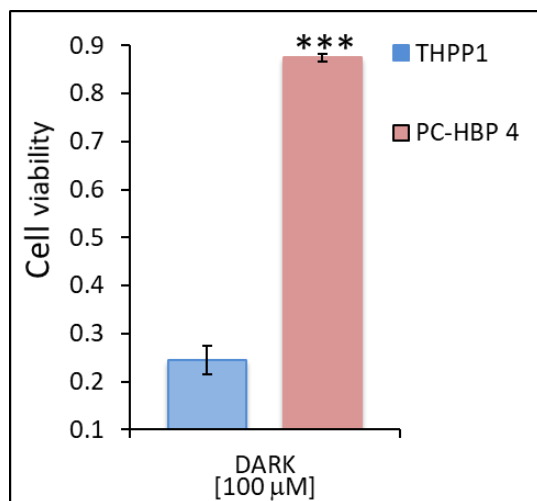
**Figure 2.** DLS and TEM image of the macro-photosensitizer PC-HBP 4.



**Figure 3.** Confocal microscopy images of the EJ cells; (a) red fluorescence from PC-HBP 4 (excited at 420 nm), (b) grayscale image of EJ cells and (c) overlaid images of (a) and (b) showing localization of PC-HBP 4 within the cells. Scale bar indicates 20  $\mu\text{m}$ .

Having established that PC-HBP 4 could be internalized, the in-vitro cytotoxicity of PC-HBP 4 was assessed using EJ (bladder carcinoma) cells and an MTT assay. For comparison, the porphyrin core THPP 1 was also studied. In the case of the polymer PC-HBP 4, concentrations were based on the porphyrin concentration, which was determined using UV and a Beer Lambert

analysis.<sup>38</sup> Toxicity was initially measured by incubating the two compounds with cells for 24 hours in the dark, using 100  $\mu\text{M}$  of each compound. Despite the high concentration, the results indicated a healthy 90-95% cell-viability for PC-HBP 4, confirming very little dark toxicity for the polymeric photosensitizer. However, when using the non-incorporated porphyrin PS (THPP 1), a cell-viability of only 25% was observed. This result was statistically significant such that when comparing the dark toxicity of THPP1 with PC-HBP 4 at 100  $\mu\text{M}$ , a p value of  $1.10302 \times 10^{-5}$  (Student's paired 2 tailed T.Test) was obtained, Figure 4.

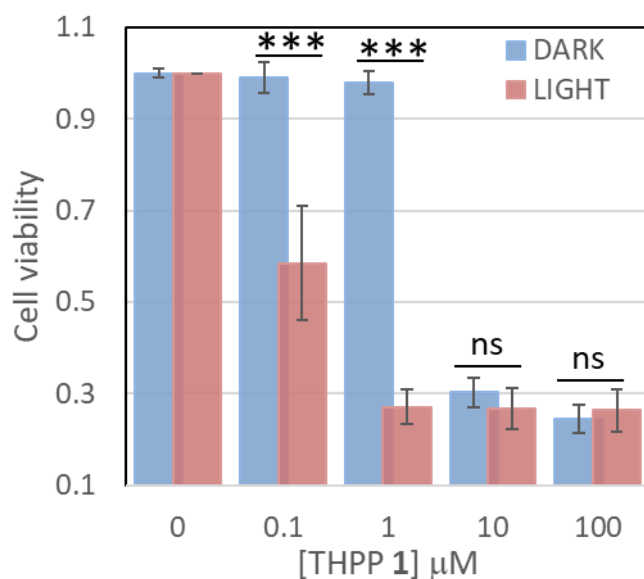


**Figure 4.** Cell viability following exposure to 100  $\mu\text{M}$  THPP1 or PC-THPP4. Mean and SEM of 3 independent repeats are shown. \*\*\* represents  $p < 0.0001$  Student's T.Test.

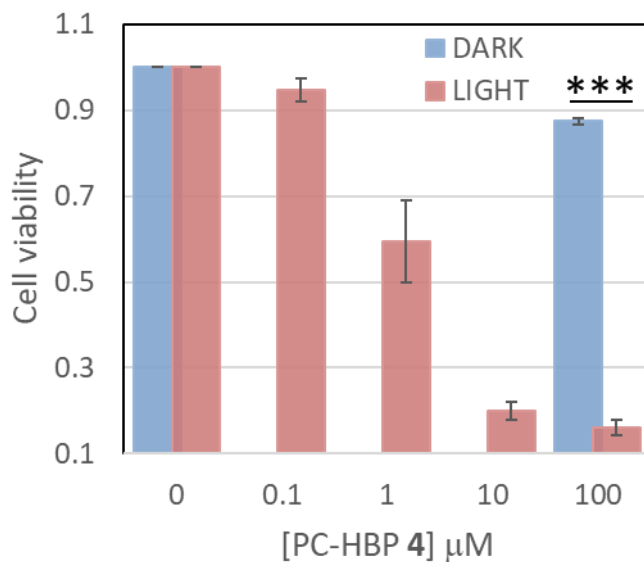
Repeating the dark toxicity experiment at various concentrations of THPP 1 (0.1 – 100  $\mu\text{M}$ ) established an  $\text{LD}_{50(\text{DARK})}$  of 4  $\mu\text{M}$  for the porphyrin core, confirming significant dark toxicity for THPP 1, Figure 5. This is consistent with the study by James et al, who observed an  $\text{LD}_{37}$  of between 1.2 to 3.1  $\mu\text{M}$  for the dark toxicity of THPP 1.<sup>39</sup> The photo toxicity of THPP 1 was



subsequently measured using the same MTT assay, but exposing the cells to low dose light for 10 minutes at 450/630 nm using an LED array (at 4.20 and 4.85 mW cm<sup>-2</sup> respectively). The data is shown in Figure 5 and shows that almost all of the cells were killed at 10 μM, with statistically significant differences ( $p=0.0003$  and  $6.12829\times 10^{-8}$  respectively (Student's paired 2 tailed T-Test)) in toxicity between light and dark conditions only apparent at 0.1 and 1 μM THPP **1** and no difference being seen at 10 μM and 100 μM ( $p=0.22$  and  $0.46$  respectively (Student's paired 2 tailed T-Test)). An LD50(LIGHT) value of 0.5 μM ( $\pm 0.1$  μM), was determined from a plot of cell viability vs concentration, allowing calculation of a photodynamic index (PI) of 8 for THPP **1**, Figure 5. When the photo-toxicity of PC-HBP **4** was measured using the same MTT assay, like THPP **1** almost all of the cells were killed at 10 μM, and photo-toxicities of 1.5 μM and 0.25 μM, were recorded for the LD50 and LD25 values respectively, Figure 6. However in contrast to THPP **1**, PC-HBP **4** did not result in any dark toxicity at 100 μM, Figure 6 and a statistically significant difference in toxicity could be observed between light and dark toxicity at this high dose ( $p=3.95796\times 10^{-7}$ , 2 tailed paired Student's T-Test). At 200 μM, some dark toxicity was observed, with a cell viability of 75% being recorded for PC-HBP **4**, which equates to an LD25 value of 200μM. Given the low dark toxicity of PC-HBP **4** a precise photodynamic index could not be determined using LD50 values, as only 25% of cells were killed at the highest concentration studied (200 μM). However, a photodynamic index of 800 was determined for the polymeric photosensitizer using the LD25 values (0.25 μM and 200 μM for the dark and light toxicities respectively). This significant increase in the photodynamic index as a result in reduced dark toxicity is a major advantage over the simple porphyrin molecule (THPP **1**) with respect to any potential therapeutic application.



**Figure 5.** Cell viability following exposure to increasing doses of THPP 1 in dark or following exposure to light. Mean and SEM of at least 5 technical repeats are shown. \*\*\* represents  $p < 0.0001$  and ns = non-significant in Student's T.Test.



**Figure 6.** Cell viability following exposure to increasing doses of PC-HBP 4 in dark or following exposure to light. Mean and SEM of 3 independent repeats are shown. \*\*\* represents  $p < 0.0001$  in Student's T.Test.

At the outset, we planned to develop a macromolecular photosensitizer that could assemble into a larger aggregate, and take advantage (in principle) of the enhanced permeability and retention effect. This was achieved using a water-soluble porphyrin cored hyperbranched polymer. The polymer was synthesized in two steps and good yield, starting from tetra-(para-hydroxyphenyl) porphyrin. The level of core/PS incorporation was estimated as 65% by comparing the molecular weights determined by core analysis ( $^1\text{H}$  NMR) and bulk analysis (GPC). However, these methods have orthogonal levels of accuracy when measuring the molecular weight of hyperbranched polymers, which result in an overestimation using the bulk method and an underestimation using the core method. As such, the actual level of core incorporation is much higher than the 65% estimated. When added to water, the porphyrin cored hyperbranched polymer self-assembled into larger spherical aggregates with diameters around 20-25 nm, as determined by TEM. Confocal microscopy confirmed that the porphyrin cored hyperbranched polymers could be internalized within EJ bladder carcinoma cells. Subsequent MTT analysis of the non-incorporated porphyrin (4-hydroxyphenyl porphyrin THPP **1**), showed a significant level of dark toxicity with an LD50 of 4  $\mu\text{M}$ . However, when the MTT experiments were repeated using the porphyrin cored hyperbranched polymer, PC-HBP **4**, there was no detectable dark toxicity (at concentrations up to 100  $\mu\text{M}$ ). However, exposing the cells to light for 10 minutes at 450/630 nm (4.20/4.85  $\text{mW cm}^{-2}$  respectively), in the presence of the polymer PC-HBP **4**, indicated  $\mu\text{M}$  toxicity. Studying the activity over a range of concentrations indicated an LD50 of 1.5  $\mu\text{M}$  for the porphyrin-cored polymer. Although an LD50 value could not be determined for the polymer's dark toxicity (toxicity only reached a maximum of 25% at 200  $\mu\text{M}$ ), a photodynamic index of 800 was determined from the LD25 values. These results demonstrate the ability of core functionalized hyperbranched polymers (and their aggregates), to be applied as

effective macromolecular photosensitizer for use in photodynamic therapy. In addition, incorporation of the photosensitizer within the polymeric structure eliminated the dark toxicity. As such, this methodology could provide a useful strategy to overcome any dark toxicity problems associated with otherwise useful photosensitizers. Work is continuing in our laboratories to develop similar systems with more active cores, as well as cores that absorb light at a more clinically useful wavelength.

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