ABSTRACT: Photodynamic therapy (PDT) is an interesting and promising approach to tackle a broad spectrum of cancer types. With the combination of a photosensitizer, light and oxygen, PDT achieves a unique selectivity by the production of localized reactive oxygen species (ROS) inside cells, which leads to their destruction. In addition, the luminescence properties of photosensitizers can be exploited to develop imaging tools. Unfortunately, the cancer selectivity and homogeneity of most photosensitizers are frequently limiting the performances of PDT and cancer detection/characterization by luminescence imaging. Consequently, our study aims to use cellulose nanocrystals to transport and deliver radiolabeled photo-responsive metalla-assemblies to create a new generation of theranostic agents for PDT and imaging applications. The synthesis, structural characterization, cytotoxicity evaluation, and in vivo biodistribution imaging of the compounds are presented. The best candidates show excellent biological activity and selectivity towards ovarian carcinoma cell line (A2780), cisplatin resistant ovarian carcinoma cell line (A2780cis) versus normal human embryonic kidney cells (HEK293T), as well as efficient imaging properties, suggesting a potential use as multimodal theranostic agents.

KEYWORDS: photodynamic therapy; imaging; porphyrin; cellulose nanocrystal; metalla-assembly; theranostic agent.

INTRODUCTION

Cancer is estimated to be the second leading cause of death globally according to the World Health Organization, with a 9.6 million death count in 2018 [1]. To reduce the significant social, economic, psychological, and physical impact caused by cancer worldwide, efforts in early diagnosis, screening, treatment, and palliative care are needed.

An attractive cancer therapy that holds great promise for treating cancer is photodynamic therapy (PDT). This is mainly due to its precise controllability, minimally invasive nature, and high spatiotemporal accuracy [2]. However, even with these specificities, PDT still needs improvements, in particular concerning selectivity and targeting [3].

Nanocarriers stand at the forefront of nanomedicine research when it comes to drug targeting and delivery [4,5]. Given that, solid tumors suffer from leaky vasculature and poor lymphatic drainage due to slacked junctions between endothelial cells, it is therefore possible to use this against them through a passive accumulation of nanoparticles (50–200 nm) [6,7].

In order to take advantage of the enhanced permeability and retention (EPR) effect, nanoparticles must be able to stay intact in the bloodstream for a prolonged blood circulation time [8,9], as well as being small, constituted of natural compounds and possessing a neutral and hydrophilic surface [10]. Among nanoparticles, those with PEG [11], with polysaccharide coating [12], or cellulose nanocrystals (CNCs) have retained our attention. These needle-like nanomaterials are natural, and possess several attractive characteristics such as renewability, low cost, and biodegradability [13]. They
also present the great advantage of being non-toxic [14]. In addition, cellulose-derived materials have certain physico-chemical properties that provide them with synthetic advantages. For example, they can be easily functionalized, having a large number of hydroxyl group present at the surface [15]. Porphyrins have been previously attached to nanoparticles to modulate their antimicrobial, anticancer and catalytic properties as well as making them more water soluble [16–20]. Recently, Imlimthnan et al. reported the use of modified CNCs as radiolabeled molecular imaging probes for in vivo medical applications [21].

However, most porphyrin derivatives are planar aromatic compounds that tend to aggregate in biological media, hampering their photophysical behavior [22]. A possible solution to limit aggregations between PS is their incorporation into supramolecular structures. The benefit of using supramolecular metal-based assemblies to avoid aggregation has been recently reviewed by Sun and Stang [23]. We have prepared porphyrin-based metallas- assemblies to avoid aggregation and to increase water solubility of PS [24]. For example, a series of arene ruthenium metalla-cubes built from tetrapyridylporphyrin panels (Fig. 1) have showed excellent PDT activity on cancer cells [25–27], as well as on fibroblast-like synoviocytes [28].

**Fig. 1.** Two porphyrin-based arene ruthenium metalla-cubes previously used as PDT agents [25].

These octacationic complexes are water soluble and show no aggregation in biological media, probably due to steric and electrostatic repulsion. Moreover, they possess an excellent photoactivity, with a tremendous phototoxicity index [29,30], thus showing great potential as a new generation of PDT agents.

It is also widely recognized that accurate cancer detection is imperative towards fruitful treatments. Only by identifying the area the tumor is situated at, recognizing the kind of cancer, its limits and whether the disease is restricted or has metastasized to different organs, it is possible to truly give the best treatment for the patient and consequently obtain the most ideal result. It is therefore obvious that when choosing a specific cancer treatment methodology, diagnostic agents assume a critical role [31,32]. Knowing this, imaging is crucial in a successful treatment, and interestingly, photosensitizers can be used not only to treat (PDT), but also to visualized cancer [2]. An elegant strategy to add imaging properties to photosensitizers is by the attachment of radioactive isotopes. Indeed, insertion of a radionuclide at the core of a porphyrin-based PS is relatively easy to achieve and has showed great potential [2, 33–35], the most common isotopes used in this manner being copper-64 [36], gallium-68 [37], technetium-99m [38], and indium-111 [39].

Herein we present an extension to our versatile system, adding two more functionalities to arene ruthenium porphyrin-based metallas- assemblies. The insertion of technetium-99m in the core of the pyridylporphyrin panels aims to incorporate imaging properties, while the covalent grafting of the arene ruthenium metalla-assemblies to cellulose nanocrystals to exploit the EPR effect. The influence on the PDT efficiency upon introduction of these new characteristics has been evaluated on cancerous (A2780 and A2780cis) and non-cancerous (HEK293T) cell lines, showing that both additions have a positive impact on the *in vitro* activity. In addition, preliminary *in vivo* imaging experiments show the biodistribution of the compounds, confirming their imaging properties.

**RESULTS AND DISCUSSION**

Supramolecular arene ruthenium metalla-assemblies are built from two main components, dinuclear arene ruthenium metalla-clips, and multidentate N-donor connectors (panels) [40]. These components can be functionalized to modify the physico-chemical properties of metalla-assemblies [2,41]. Therefore, to introduce imaging and grafting opportunities, functionalized tris-pyridyl-porphyrin panels were used (Fig. 2).

**Fig. 2.** Molecular structures of the functionalized tris-pyridyl porphyrin derivatives P1 and P2.

In these panels, the replacement of a pyridyl group by an hydroxyphenyl or aminophenyl group provides a fixation point for the insertion of cellulose nanocrystals,
while keeping two photosensitizers within the supramolecular structure. The porphyrin derivatives (5-(4-hydroxyphenyl)-10,15,20-tris(4-pyridyl)porphyrin) (P1) and (5-(4-aminoaryl)-10,15,20-tris(4-pyridyl)porphyrin) (P2) were prepared according to or by simple modifications of published methods [42,43].

From the tri-pyridyl porphyrin derivatives P1 and P2, the synthesis of the metalla-assemblies is straightforward. As illustrated in Scheme 1, the first step is to react the dinuclear arene ruthenium clip [Ru(η6-p-cymene)2(2,5-bis(2-hydroxyethylamino)-1,4-benzoquinone)Cl2] with silver triflate, followed by the addition of the corresponding panel, respectively P1 and P2. Then, the hexacationic metalla-assemblies M1 and M2 are isolated in good yield as their triflate salts (see Exp. part). The molecular structure of these two metalla-assemblies was confirmed by a combination of NMR spectroscopy and mass spectrometry.

Scheme 1. Synthesis of metalla-assemblies M1 and M2.

Upon formation of the metalla-assemblies M1 and M2, the ¹H NMR signals (Fig. S1 and S2, supplementary information) assigned to the metalla-clip can still be observed, while new signals associated with the porphyrin panels appear (10.0-7.5 ppm, aromatic signals of H-β pyrrolic proton and meso aryl groups). These observations are consistent with the formation of the metalla-assemblies.

The p-cymene moiety shows peaks around δ ~1.3-2.5 ppm for the protons of the isopropyl and methyl groups (alkyl protons), and signals in the 6.0-5.3 ppm region corresponding to aromatic protons. Around 9.1-8.0 ppm, more aromatic signals can be found corresponding to the pyridyl and β-pyrrolic protons of the porphyrin moieties. In addition, at ~3.8-3.5 ppm, the benzoquinone aryl protons are observed. In complexes M1 and M2, additional signals at ~4.73 and 5.98 ppm can be found for the protons of the hydroxyl and primary amine protons of the hydroxypheyl and aminophenyl substituents at the meso positions of both porphyrins, while peaks of the porphyrinic NH protons were observed at ~2.9 ppm. Finally, at δ ~ 4.1 ppm, a peak corresponding to the benzoquinone hydroxyl protons is observed.

Mass spectrometry (Fig. S3 and S4, supplementary information) provides further evidence for the formation of the metalla-assemblies. A series of peaks with different charge states from 3+ to 6+ were observed for both M1 and M2, because of the loss of the triflate (OTf−) counterions.

UV-Vis absorption and fluorescence spectra were measured in DMSO for metalla-assemblies M1 and M2. Their absorption spectra (Fig. S5, supplementary information) showed the typical Soret band (412 and 415 nm for M1 and M2, respectively) and Q-bands (region of 450-600 nm) of porphyrin-based compounds, which are attributed to the absorption transition between the ground state and the first excited singlet state (S0–S1).

Then, the two metalla-assemblies were combined with cellulose nanocrystals (CNCs). Prior to the grafting, CNCs were obtained by hydrolysis of cellulose with sulfuric acid solution (64% w/w) followed by several washings, centrifugation, and dialysis against distilled water until neutrality [17]. The purpose of this acid hydrolysis consists of breaking cellulose chains within the amorphous domain, thus getting free nanocrystals, which can then be selectively oxidized in primary alcohols with TEMPO. The obtained CNCs suspension can be easily linked to other moieties through the newly added carboxylic acid groups, thus forming with M1 and M2, the corresponding metalla-assembly grafted to CNCs, MC1 and MC2 respectively (Fig. 3).

Fig. 3. Molecular structures of MC1 and MC2.
The FTIR spectra of CNCs precursor, MC1 and MC2, (Fig. S6) in the wavenumber range of 3700 - 2900 cm⁻¹ showed similar bands of cellulose around 3360 cm⁻¹ (O-H stretching), 2890 cm⁻¹ (C-H stretching), 1430 cm⁻¹ (CH₂ symmetrical bending), and 1057 cm⁻¹ (ether C-O-C) [44]. Spectrum of oxidized CNCs also displayed a signal at 1720 cm⁻¹ due to the carbonyl stretching of the carboxylic acid function [16]. Compared to the CNCs precursor, the FTIR spectra of MC1 shows a strong peak at 1739 cm⁻¹, which suggests the presence of an ester bond, presumably between the hydroxyl group of the porphyrin in metalla-assembly M1 or the hydroxyl groups in the alkyl chain of the metalla-assembly with the cellulose nanocrystals [45]. On the other hand, a peak around 1644 cm⁻¹ for MC2 suggests the presence of an amide bond between the carboxylic function of CNCs and the amine group of the porphyrin [44].

Simultaneously, the ATR FT-IR spectrum of both CNCs-linked metalla-cages showed that the intensities of the C–H vibration band of methyl groups (2820–2976 cm⁻¹) are strengthened. The N–H bending vibration peak of porphyrin units appeared at 1658 cm⁻¹. All these indicate the successful combination of CNCs with the metalla-assemblies.

i. Radiochemistry

After the syntheses and characterizations, radiolabeling experiments were performed to determine if these supramolecular assemblies can be used as multimodal imaging agents.

First, we have determined the importance of reaction time, stoichiometry, and temperature on the formation of the radiolabeled products. Moreover, for comparison, the incorporation of technetium-99m in P1 and M1 was also performed. Interestingly, time and temperature have a significant impact on the nature of the compound, and the best conditions for the radiolabeling of these compounds were at 60 °C for a minimum of 45 minutes; any temperature or reaction time inferior to these gave a poor radiolabeling completion and/or a mixture of compounds. After having established these conditions, labeling with technetium-99m was achieved (Fig. 4) with an incorporation of over 90% for both [⁹⁹ᵐTc]MC1' and [⁹⁹ᵐTc]MC2' CNCs grafted metalla-assemblies. The incorporation of more than 90% of technetium-99m in the core of the porphyrin units was confirmed by performing,

under the same conditions, the radiolabeling of P1 and M1. These two compounds show an incorporation of 93 and 98% respectively, which are similar to those found for the CNCs-grafted compounds.

Fig. 4. Proposed molecular structures of [⁹⁹ᵐTc]MC1' and [⁹⁹ᵐTc]MC2'.

The stability of the [⁹⁹ᵐTc]-derivatives was also evaluated. Fig. 5 shows the successful radiolabeling of these compounds, and the stability at 37 °C of [⁹⁹ᵐTc]MC1' in different mediums at different timepoints to better replicate physiological conditions, from 15 minutes to up to 24 h. The different media for the radiochemical purity studies were chosen with the intent of mimicking the main components of the bloodstream. In addition, since EDTA and DTPA form stable complexes with most transition metals such as Cu, Tc, and Zn, the behavior of these compounds in DTPA and EDTA solutions was also studied.

Finally, it was also important to verify the stability of these compounds in HEPES and NH₄AcO buffer solutions, because they are the main buffers used for radiolabeling studies. From these experiments, it was possible to determine that there is no significant difference for at least 24 h when it comes to the radiochemical purity under most of the media tested, with some differences occurring mainly in the cases of EDTA and plasma, right after 15 min. However, after this timepoint they seem to stay stable for the rest of the study. This could be due to the fact that EDTA is a strong chelator of technetium-99m, and that plasma also has components such as magnesium ions that can compete with the technetium for the coordination with the center of the porphyrin units.
ii. In Vitro Studies

Following the stability study, the cytotoxicity of the metalla-assemblies, along with their corresponding porphyrins and cellulose grafted compounds (Scheme 1), were evaluated on two different human ovarian carcinoma cancer cell lines, A2780 and A2780cis, as well as on the human liver cell line HEK293T. The results (Table 1) of their metabolic activity inhibition against all cell lines (IC_{50}) are presented in the form of dose-response curves in Figures 6 and 7.

Regarding P1 and its corresponding metalla-assembly M1, although the porphyrin by itself showed IC_{50} values after irradiation between 1 μM and 3 μM (Table 1, first line), when it is further functionalized (Table 1, second line) it shows higher cytotoxicity with IC_{50} values up to only 0.5 μM. It must be noted that the IC_{50} values in the dark for all the compounds were at least 10x lower than those after irradiation. When it comes to P2 (Table 1, fourth line), and its corresponding metalla-assembly M2 (Table 1, fifth line), the same results can be observed with lower IC_{50} values under both conditions.

In the dark, the metalla-assemblies (M1 and M2) are more cytotoxic than the porphyrins (P1 and P2). Upon irradiation, however, an interesting result is observed: P2 appears to be a better PDT agent than the corresponding metalla-assembly M2 (Table 1, fourth and fifth lines). In addition, we can notice better IC_{50} values for the porphyrin when compared to its hydroxyphenyl analogue (Table 1, first and fourth lines), but higher IC_{50} values when both metalla-assemblies are being compared, M1 and M2 respectively (Table 1, second and fifth lines). This suggests that the difference in activity of the two porphyrins can be most likely linked to their phenol and amino groups, and that when we further functionalized these porphyrins by conjugation with metalla-clips, it seems that for one this modification increases its activity and for the other, it decreases it. When analyzing the in vitro results, at first sight it could be thought that the amino group of P2 is increasing toxicity as opposed to the hydroxyl group of P1, however, when M1 and M2 are compared, the opposite is observed.
Fig. 6. Metabolic activity of P1, P2, M1, and M2 against two different ovarian cancer cell lines (A2780 and A2780cis) with and without irradiation. Error bar stands for standard error (±SE), calculated from 3 repeated measurements.

Fig. 7. Metabolic activity of P1, P2, M1, and M2 against a normal liver cell line (HEK293T) with and without irradiation. Error bar stands for standard error (±SE), calculated from 3 repeated measurements.
Regarding MC1 and MC2 (Table 1, third and sixth lines), by linking cellulose nanocrystals to these metalla- assemblies, it was intended not only to increase the selectivity towards cancer cells (which cannot be tested in vitro, given that this selectivity is expected to come from the EPR effect) but also hydrophilicity, thus determining the effect on the PDT activity that this further modulation will bring. We can observe just by looking at the results that the IC$_{50}$ decreases significantly when the metalla-assembly is grafted to CNCs. By analyzing the results, we can also determine that they follow the expected behavior, which is a lower IC$_{50}$ when compared to their predecessors. This might be due to the improved solubility that is introduced. From these results, it is possible to conclude that new PDT agents that not only show enhanced chemical and structure stability but also increased hydrophilicity and consequent higher cytotoxicity were developed.

It has been previously reported that one of the factors contributing to the greater efficiency of PDT is the accumulation of PS in tumor cells [46]. Therefore, this increase in the cytotoxicity of the more amphiphilic derivatives will, theoretically, be related to their increased uptake by cells. Furthermore, when the photodynamic activity of these compounds against the A2780 and A2780cis cell lines is compared, it can be concluded that there is no significant difference between the two. This is important because the A2780cis cell line is resistant to Cisplatin®, one of the most used metal-based chemotherapeutic drugs to treat cancers, including ovarian cancer. Consequently, drugs that can inhibit its growth just as much as they would to normal A2780 cell line are in need. These compounds seem to not discriminate between the two lines, especially when irradiation is applied, which is a very important and exciting result because they could potentially be used to treat cancers resistant to common oncogenic drugs such as Cisplatin®. Additionally, given that these compounds are built from arene ruthenium units, a classical p-cymene ruthenium PTA (PTA = 1,3,5-triaza-7-phosphaadamantane) chemotherapeutic RAPTA-C [47], was also tested and compared. When analyzed, the IC$_{50}$ of RAPTA-C and of these metalla-assemblies show a significant difference: RAPTA-C has IC$_{50}$ values always higher than 200 µM, independently of the cell line, and no expected difference when irradiated, as expected since it doesn’t have a photosensitive agent in its structure. Another interesting remark is that further functionalization of the initial porphyrins P1 and P2 are translated into not only lower IC$_{50}$ values when irradiation is applied, but also without irradiation, which means that the photodynamic efficiency is partially lost in the final MC1 and MC2 derivatives.

Finally, from all tested compounds, M1 showed the best overall results in vitro, suggesting that MC1 could work better in vivo, when other factors such as the EPR effect can be considered as well. All the studied compounds revealed dose-dependent anti-proliferative effects. In addition, derivatives with increased hydrophilicity, obtained by cellulose linkage or by coordination with ruthenium metalla-clips, showed the best results. Furthermore, no expected difference when irradiated, as expected since it doesn’t have a photosensitive agent in its structure. Another interesting remark is that further functionalization of the initial porphyrins P1 and P2 are translated into not only lower IC$_{50}$ values when irradiation is applied, but also without irradiation, which means that the photodynamic efficiency is partially lost in the final MC1 and MC2 derivatives.

To evaluate their cellular uptake, the $^{99m}$Tc-labeled versions of these compounds were used in in vivo imaging animal models. An administered dose of 3 µg/ml was used, which is lower than the IC$_{50}$ without irradiation

**Table 1.** IC$_{50}$ values ± standard deviation (in µM) of both porphyrins, corresponding metalla-assemblies, and metalla-assemblies linked to the CNCs against two ovarian carcinoma cell lines (A2780 and A2780cis) and one hepatic healthy cell line (HEK293T).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>A2780cis</th>
<th>A2780</th>
<th>HEK293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without irradiation</td>
<td>Irradiated</td>
<td>Without irradiation</td>
<td>Irradiated</td>
</tr>
<tr>
<td>P1</td>
<td>61.01 ± 2.67</td>
<td>1.41 ± 0.08</td>
<td>52.30 ± 4.50</td>
</tr>
<tr>
<td>M1</td>
<td>6.79 ± 0.70</td>
<td>0.33 ± 0.05</td>
<td>4.52 ± 0.28</td>
</tr>
<tr>
<td>MC1</td>
<td>0.96 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>1.65 ± 0.27</td>
</tr>
<tr>
<td>P2</td>
<td>32.03 ± 2.41</td>
<td>0.41 ± 0.01</td>
<td>28.81 ± 3.92</td>
</tr>
<tr>
<td>M2</td>
<td>7.11 ± 0.58</td>
<td>0.61 ± 0.09</td>
<td>2.43 ± 0.21</td>
</tr>
<tr>
<td>MC2</td>
<td>0.89 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>Cisplatin®</td>
<td>26.01 ± 3.01</td>
<td>-</td>
<td>2.02 ± 0.92</td>
</tr>
<tr>
<td>RAPTA-C</td>
<td>&gt;200</td>
<td>-</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>
(> 8 µg/ml), but higher than the IC<sub>50</sub> with irradiation (< 1 µg/ml). Therefore, more imaging agent can be injected, without harming cells, since no radiation is used for this procedure. As expected by our previous metabolic activity assays, the internalization assay denoted a variation of cell uptake from 6 to 13% depending on cell lines. Indeed, a higher uptake in the A2780 cancer cells is observed over the normal liver cells HEK293T over time (Fig. 8). In addition, by increasing the size of the photosensitizer and upon grafting to CNCs, a faster and stronger uptake is observed (Fig. 8).

Fig. 8. Graph presenting the estimated cell uptake values expressed in % for [<sup>99m</sup>Tc]MC1' in both A2780, A2780cis, and HEK293T cell lines (left); and graph presenting the estimated cell uptake values expressed in % for [<sup>99m</sup>Tc]P1', for [<sup>99m</sup>Tc]M1', and [<sup>99m</sup>Tc]MC1' in the A2780 cell line (right). Error bar stands for standard error (±SE), calculated from 3 repeated measurements.

### iii. In Vivo Imaging Studies

For the in vivo imaging studies, the radiolabelled compounds [<sup>99m</sup>Tc]M1' and [<sup>99m</sup>Tc]MC1' were selected, since they showed the most promising results in vitro, therefore showing the best characteristics for PDT and in vivo applications. Although [<sup>99m</sup>Tc]MC1' was expected to be the ideal PS as a multimodal agent, due to the added CNC moiety which is supposed to help with targeting and biodistribution, [<sup>99m</sup>Tc]M1' was also used in order to compare what this addition brings in vivo.

The protocol consisted of a 100 µL retro-orbital injection of either [<sup>99m</sup>Tc]M1' or [<sup>99m</sup>Tc]MC1' with a radioactivity of approximately 100 µCi in SCID mice, with their consequent imaging at different timepoints. It should be noted that preliminary results using different concentrations of [<sup>99m</sup>Tc]MC1', more specifically 1500 nM and 750 nM, were tested before achieving the ideal concentration of 375 nM. For the highest concentration (1500 nM), some mortality was observed (33 %), and both compounds seemed to accumulate at the lungs instead of the liver and intestines (Fig. 9), which was deemed dangerous, and therefore the concentration was lowered to 750 nM. At this concentration, the biodistribution was similar to the one found later for 375 nM, but with some extra accumulation at the injection site (Fig. 10). This is thought to be the cause for the observed local photoreaction, which damaged the animal’s eye (point of injection). Due to this, it was decided to lower the dose once again with the aim of trying to achieve the same imaging results with a lower concentration of compound to protect the animal while still giving good images of their biodistribution, and a concentration of 375 nM was selected. This concentration was considered safe through the analysis of the images for [<sup>99m</sup>Tc]MC1, which showed the expected accumulation at both the liver and the injection site for this compound, but with no evidence of photoreaction to occur (no damage to the eyes of the mice were observed as well as no mortality).
Fig. 9. Indicative static 20 – 50 min scintigraphy/x-ray images of a normal Swiss Albino mouse intratracheal administered with [\(^{99m}\)Tc]MC1' (1500 nM) at 1, 2, 4 and 24 hours p.i. The gradual alteration in colour indicates a lower to a higher number of recorded counts.

Through the analysis of the images acquired for [\(^{99m}\)Tc]M1' (Fig. 11) and [\(^{99m}\)Tc]MC1' (Fig. 12) at 375 nM, it was clear that the biodistribution for both compounds from 1 to 24 h post-injection (p.i.) was very similar. Although with some accumulation in a portion of the intestines, in the first hour they seem to accumulate preferentially in the liver. This is the most prominent, extending all the way to the 24 h mark, and it can suggest that these compounds, if administered in a dose that has an effect without irradiation, could have some inherent hepatotoxicity.

Fig. 10. Indicative static 20 – 50 min scintigraphy/x-ray images of a normal Swiss Albino mouse intratracheal administered with [\(^{99m}\)Tc]MC1' (750 nM) at 1, 2, 4 and 24 hours p.i. The gradual alteration in colour indicates a lower to a higher number of recorded counts.

The accumulation of [\(^{99m}\)Tc]MC1' in the injection site at 375 nM, but not for [\(^{99m}\)Tc]M1' at the same concentration, is most probably due to its larger size, which might increase its retention at the injection site. Another interesting result is observed when a further look is taken into the clearance of the compounds (Fig. 13). All compounds show similar clearance yields, depending more on the concentration of the injected solution rather than if the compound is bound to the cellulose nanocrystals or not. This is better evidenced when we compare the 3 different concentrations of [\(^{99m}\)Tc]MC1' (375, 750 and 1500 nM) and notice that the higher the concentration administered, the longer it takes for the compound to be cleared from the mice. At 375 nM, for example, we have a clearance at 24 h of around 50%, while for 1500 nM, at the same timepoint, the clearance is less than half, being about 20%. Lastly, it seems that the grafting of CNC mostly exerts its influence at the clearance level at earlier timepoints, and becomes less impactful the longer it stays in the body.
Fig. 11. Indicative static 20 – 50 min scintigraphy/x-ray images of a normal Swiss Albino mouse intratracheal administered with \[^{99m}Tc\] M1’ (375 nM) at 1, 2, 4 and 24 hours p.i. The gradual alteration in colour indicates a lower to a higher number of recorded counts.

Fig. 12. Indicative static 20 – 50 min scintigraphy/x-ray images of a normal Swiss Albino mouse intratracheal administered with \[^{99m}Tc\]MC1’ (375 nM) at 1, 2, 4 and 24 hours p.i. The gradual alteration in colour indicates a lower to a higher number of recorded counts.

Fig. 13. Clearance yields for \[^{99m}Tc\]MC1’ at different concentrations: 375, 750 and 1500 nM (left); and for \[^{99m}Tc\]M1’, and \[^{99m}Tc\]MC1’ at 375 nM (right). Error bar stands for standard error (±SE), calculated from three repeated measurements. Error bar stands for standard error (±SE), calculated from three repeated measurements.
EXPERIMENTAL

Materials and Analytical methods

All non-synthesized reagents were commercially available (Sigma-Aldrich, Brunschwig, Basel, Switzerland) and used as received. NMR spectra were recorded with a Bruker Advance Neo Ascent 600MHz spectrometer (600.13 MHz for $^1$H NMR spectra). UV–vis absorption spectra were recorded with a PerkinElmer UV–vis spectrophotometer (Waltham, MA, USA). Infrared spectra were recorded with a Thermoscientific Nicolet iS5 spectrometer. Electrospray ionization mass spectrometry (ESI-MS) were performed in positive or negative mode with a LCQ Finnigan mass spectrometer. Microanalyses were carried out by the Mikroelementaranalytisches Laboratorium, ETH Zürich (Zürich, Switzerland). Dynamic light scattering (DLS) was used for the measurement of average hydrodynamic diameters (Malvern Zetasizer Nano-ZS, Malvern Instruments, UK). Each suspension (0.025 wt%) in pure water was analyzed in triplicate at 20 °C with a scattering angle of 173°. Pure water was used as a reference-dispersing medium. The ζ-potential data were collected through electrophoretic light scattering at 20 °C, 150 V, in triplicate for each suspension (0.25 wt%) using the same instrument and at neutral pH. The instrument was calibrated with a Malvern -68 mV standard before each analysis cycle.

Technetium-99m in the form of $[^{99m}Tc]NaTcO_4$ was collected by elution of a $[^{99m}Mo]Mo/[^{99m}Tc]Tc$ TEKCIS generator (Auckland, New Zealand) calibrated at 6 GBq, and all radiochromatography was performed with a miniGITA Dual radio TLC system by Elysis Raytest (Straubenhardt, Germany).

A2780 and A2780cis cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and HEK293T cells were obtained from the American Type Culture Collection (ATCC, CRL-1619). All cell lines were maintained according to the suppliers’ recommendations at 37 °C in a humidified atmosphere with 95% air and 5% CO₂ in a HeraCell 150 incubator. Dulbecco’s Modified Eagle’s culture Medium (DMEM, Sigma D-5648) supplemented with 10% fetal bovine serum (Sigma F7524), 250 μM sodium pyruvate (Gibco 11360), and 1% antibiotic (100 U/ml penicillin and 10 μg/ml streptomycin; Sigma A5955) was used for the HEK293T cell line; while for the A2780 and A2780cis cell lines, Roswell Park Memorial Institute 1640 Medium (RPMI, Sigma R6504) culture medium supplemented with 10% fetal bovine serum, 1mM sodium pyruvate and 1% antibiotic was used.

The in vivo studies were performed at the NCSR Demokritos (Aghia-Paraskevi, Attica Prefecture, Greece), using female SICD mice (15-25 g) purchased from the Breeding Facilities of the NCSR Demokritos (Permit Number: EL 25 BIO 019, EL 25 BIO 020). The protocol and all the animal procedures were approved by the General Directorate of Veterinary Services (Athens, Attica Prefecture, Greece) and by the Bioethical Committee of the Institution (Permit number: EL 25 BIO 022, EL 25 BIO 042) on the basis of the European Directive 2010/63/EU on the protection of animals used for experimental purposes. The imaging studies were performed on a dedicated benchtop mouse-sized gamma camera ($\gamma$-eye™ by BioEmTech).

i. Synthesis

General procedure for the synthesis of the metallaclip C1, $[\text{Ru}(\eta^6-p\text{-cymene})\{2,5\text{-bis(2-hydroxyethylamino)}\text{-1,4-benzoquinone})\text{Cl}]$:

A mixture of 2,5-bis(2-hydroxyethylamino)cyclohexa-2,5-diene-1,4-dione (52.0 mg, 0.23 mmol), CH₃COONa (38.4 mg, 0.46 mmol), and [Ru(p-cymene)Cl₂] (145.0 mg, 0.23 mmol), were dissolved in ethanol (25 mL) and stirred at reflux for 24 h. The reaction was allowed to cool to room temperature, the volume was reduced to half, and the mixture was stored in the refrigerator overnight. The precipitate was filtered off and washed with cold H₂O to isolate the desired product as a violet solid (71% yield).

$^1$H NMR (600 MHz, DMSO-d₆); δ (ppm) 7.30 (s, 2H, CH₃-benzoquinone), 5.87 (d, 2H, CH₃-cymene), 5.64 (d, 2H, CH₃-cymene), 5.58 (d, 2H, CH₃-cymene), 5.39 (d, 2H, CH₃-cymene), 4.09 (s, 2H, OH), 3.76 (m, 4H, CH₂), 3.54 (m, 4H, CH₂), 2.51 (s, 6H, CH₃), 2.20 (br, 2H, CH(CH₃)₂), 1.29 (m, 12H, CH(CH₃)₂). ESI-MS (positive mode): [C₂H₅Cl₂N₂O₂-Cl⁻]: Calcd 765.08 (m/z), Found 765.12 (m/z). Elem anal. Calcd for C₂H₅Cl₂N₂O₂-Ru₂ (764.08): C, 47.18; H, 5.54; N, 7.34. Found: C, 47.21; H, 5.61; N, 7.37.

General procedure for the synthesis of the metalla-assemblies M1 and M2

In a round bottom flask, 3 eq. of C1 (37.5 mg) were suspended in MeOH (20 mL), and AgCF₃SO₃ (6 eq., 24 mg) was added. The mixture was stirred at RT for 3 h. At this point, P1 (20 mg) or P2 (20 mg) were added (2 eq.), and the mixture heated to 60 °C. The reaction was stirred for 24 h. Then, the solution was filtered to remove silver chloride and allowed to cool to room temperature. The solvent was removed under reduced pressure, and the crude product was dried overnight. CH₃Cl (1 mL) was added to dissolve the solid, and diethyl ether was added to induce precipitation. The solid was filtered off and dried under vacuum (71% and 66% yield for M1 and M2, respectively).
M1: $^{1}$H-NMR: (600 MHz, DMSO-d$_{6}$): δ (ppm) 8.78 (s, 16H, CH$_{ar}$-porphyrin), 8.29 (d, 12H, CH$_{ar}$-pyridine), 8.25 (d, 4H, CH$_{ar}$-phenyl), 8.21 (d, 12H, CH$_{ar}$-pyridine), 7.11 (d, 4H, CH$_{ar}$-phenyl), 5.82 (m, 12H, CH$_{ar}$-cyrene), 5.71 (s, 6H, CH$_{ar}$-benzoquinone) 5.39 (m, 12H, CH$_{ar}$-cyrene), 4.73 (s, 2H, OH$_{phenyl}$), 4.10 (s, 6H, OH), 3.76 (m, 12H, CH$_{2}$), 3.53 (m, 12H, CH$_{3}$), 2.53 (s, 18H, CH$_{2}$), 2.20 (m, 6H, CH(CH$_{3}$)$_{2}$), 1.29 (m, 36H, CH(CH$_{3}$)$_{2}$), -2.90 (s, 4H, NH). ESI-MS (positive mode): [M1-3CF$_{3}$SO$_{3}$]$^{+}$: Calcd 1267.2 m/z. Found 1267.2 m/z; [M1-4CF$_{3}$SO$_{3}$]$^{+}$: Calcd 913.1 m/z. Found 913.1 m/z; [M1-5CF$_{3}$SO$_{3}$]$^{+}$: Calcd 700.7 m/z. Found 700.7 m/z. 

Elem. anal. Caled for C$_{78}$H$_{172}$F$_{18}$N$_{20}$O$_{32}$Ru$_{6}$(4248.49): C, 50.35; H, 4.13; N, 76.60. Found: C, 51.01; H, 4.19; N, 67.67. UV-vis (C$_{2}$H$_{6}$OS): $\lambda_{max}$ nm (log ε) 412(5.59), 514(4.18), 550(3.74), 588(3.68), 649(3.52).

M2: $^{1}$H-NMR: (600 MHz, DMSO-d$_{6}$): δ (ppm) 9.05 (s, 16H, CH$_{ar}$-porphyrin), 8.87 (br, 12H, CH$_{ar}$-pyridine), 8.18 (d, 12H, CH$_{ar}$-pyridine), 7.61 (d, 4H, CH$_{ar}$-phenyl), 5.98 (br, 4H, NH$_{2}$), 5.81 (m, 12H, CH$_{ar}$-pyridine), 5.70 (s, 6H, CH$_{ar}$-benzoquinone) 5.39 (m, 12H, CH$_{ar}$-cyrene), 4.12 (s, 6H, OH), 3.76 (m, 12H, CH$_{2}$), 3.52 (m, 12H, CH$_{2}$), 2.53 (s, 18H, CH$_{3}$), 2.20 (m, 6H, CH(CH$_{3}$)$_{2}$), 1.30 (m, 36H, CH(CH$_{3}$)$_{2}$), -2.90 (s, 4H, NH). ESI-MS (positive mode): [M2-3CF$_{3}$SO$_{3}$]$^{+}$: Calcd 1265.5 m/z. Found: 1265.5 m/z; [M2-4CF$_{3}$SO$_{3}$]$^{+}$: Calcd 912.6 m/z. Found: 912.6 m/z; [M2-5CF$_{3}$SO$_{3}$]$^{+}$: Calcd 700.3 m/z. Found: 700.3 m/z. 

Elem. anal. Caled for C$_{77}$H$_{171}$F$_{18}$N$_{20}$O$_{32}$Ru$_{6}$(4246.52): C, 50.37 H, 4.18; N, 7.26. Found: C, 50.41; H, 4.41; N, 7.60. UV-vis (C$_{2}$H$_{6}$OS): $\lambda_{max}$ nm (log ε) 415(5.72), 513(4.23), 549(3.86), 587(3.78), 648(3.57).

General procedure for the synthesis of metalla-assemblies linked to CNCS MC1 and MC2

First, CNCs were prepared and characterized according to the literature [17,48]. Then, the oxidation of the primary hydroxyl groups of the cellulose nanocrystals to COOH functions was performed, also following a well-established protocol [16]. For the grafting of M1 and M2, two 50 ml solutions of oxidized CNCs (1 eq.) in deionized water were activated with NHS (2 eq.) and ethylene dichloride (2 eq.) each for 2 h at room temperature. Each metalla-assembly (M1 or M2; 1 eq.) was then dissolved in 20 mL of methanol and added dropwise to the respective oxidized CNCs solution. The mixture was left to react for 24 h. After stirring for 1 day at room temperature, the CNC-metalla-assemblies (MC1 and MC2) were recovered by ultracentrifugation at 13000 rpm, after several washes with deionized water. The final solutions were collected and both z-average size and also zeta potential were determined. For compound MC1, for example, these values were 184.2 nm (Fig. S7, supplementary information), and -38.3 ± 4.5 mV (Fig. S8, supplementary information), respectively.

ii. Radiochemistry

$[^{99m}$Tc]$\text{Pertechnetate}$ was first eluted from a $[^{99m}$Mo]$\text{Mo}$/[^{99m}$Tc]$\text{Tc}$ generator. After elution, a 74 MBq aliquot was then reduced with 0.5 ml of a solution of 0.1 mg SnCl$_{2}$ in 0.05 N HCl. After shaking vigorously for 1 min and allowing to stand at room temperature for 15 min, the pH of the reduced technetium-99m solution was adjusted to 7.4 with a NaOH 1M solution. A 1 mL aliquot of P1, M1 or MC1, previously dissolved in DMSO and diluted to a concentration of 10 mg/ml, is then added to the neutralized $[^{99m}$Tc]$\text{Tc}$SnCl$_{2}$ mixture and incubated at 45 °C for 30-60 min depending on the porphyrin/metallic cage, and consequently labeled with ~55.5 MBq of technetium-99m by the chemical method described above. The incorporation efficiency is assessed by ascending paper radiochromatography with Whatman 3MM paper developed in acetone or saline as the mobile phase. The radiolabeling was successful with all compounds achieving a radiochemical incorporation higher than 93%.

For the radiochemical purity studies performed with the radiolabeled compounds, in order to follow their stability, various media were tested including saline, phosphate-buffered saline (PBS), deionized water, HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)) buffer solution (0.5 M, pH 5.6), ammonium acetate (0.5 M, pH 5.5), Plasma-Lyte 148 (pH 7.4), and saturated EDTA and DTPA solutions, at different time points (15, 30, 60, 120, 240 min, and 24 h). The stability was investigated by incubating the radiolabeled compound in the media (1:10) and was then evaluated through ascending paper radiochromatography with Whatman 3MM paper developed in saline for all $[^{99m}$Tc]$\text{Tc}$SnCl$_{2}$ labeled compounds. From this, it is possible to conclude when the reaction is complete, because the radio-TLC peak for the labelled compounds can be seen at the front of the radio-TLC, while if it is incomplete, traces of reduced technetium-99m stay at the bottom.

iii. In Vitro

The cytotoxicity and therapeutic efficacy of the compounds as photosensitizers were assessed after their synthesis and characterization. A metabolic activity assay was used to determine their activity. Because the goal of this study was to synthesize and characterize porphyrin metalla-assemblies grafted to cellulose nanocrystals, as well as to assess their potential as a photosensitizer agent in cancer PDT, the choice of carcinogenic cell lines was critical. Three human cell lines were used: the A2780 human ovarian cancer cell line and the cisplatin resistant cell line A2780 cis, as well as the HEK293T cell line, originally derived from human embryonic kidney cells,
that we used as an additional control to assess tumor specificity.

**Metabolic activity**

The cytotoxicity of the photosensitizers in all cell lines was tested using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium bromide) colorimetric assay to establish their potential therapeutic impact and to examine the sensitivity of the cell lines to the compounds. 96-Well plates of the A2780, A2780cis, and HEK293T cell lines were prepared for this experiment. Cell culture media was aspirated, and the plates were cleaned with PBS 48 hours after being subjected to the methods outlined in the preceding section. After that, each well was filled with 100 mL of MTT (0.5 mg/ml; Sigma M2128) in PBS, pH 7.4, and each plate was incubated in the dark at 37 °C for at least 3 hours. The contents of each well are homogenized after all formazan crystals have been produced and solubilized with DMSO. Using the Molecular Devices® SpectraMax M5E multi-mode plate reader, the absorbance was measured at 570 nm with a 620 nm reference filter.

The obtained results were evaluated and processed using the OriginPro 9.0 program and were expressed as a percentage of metabolically treated cells against non-treated cells. For each chemical, dose response curves were constructed, and IC₅₀ (half-maximal inhibitory concentration) values were calculated. Dark cytotoxicity tests were carried out as described previously, but without the irradiation phase and with larger sensitizer concentrations (5 to 20 mM). This approach allowed for the creation of dose-response curves and the determination of the sensitizer concentration that suppresses the metabolic activity of the cultures by 50%

**Internalization assays**

Cells were seeded in a 12 well plate (~10⁵ cells/well) overnight at 37°C in a humidified incubator containing 5% CO₂. To the vial with the radiolabeled compound, cell culture medium was added until the 2ml mark, giving a final concentration of 50 µg/ml of compound with 37 to 55.5 MBq. Then, it was added 5.5 -7.5 MBq of this solution to each well (A2780, A2780cis or HEK293T cell lines), which was previously washed with phosphate-buffered saline (PBS) in order to remove any non-attached or dead cells.

The amount of activity added is measured in a dose-calibrator. This process is repeated for the different wells in the 12 well-plates, and after they are left to incubate for different time periods varying from 15 minutes to 4 h. At the end of the desired time point, the wells are washed for at least 3 times with PBS. This step assures that any radioactivity that doesn’t enter the cells or attaches to their membrane is removed, and so the results are accurate and truly represent the uptake process. These washes are collected, and their activity measured. After washing, cold methanol is used 2 or 3 times to lyse the cells and collect any compound that was inside them. Finally, the resulting lysate in each well was collected and the activity measured again in a dose-calibrator as previously described. Each condition is currently being performed in triplicates and the experiments repeated three times as well in order to assure the results are reliable and reproducible.

**Photodynamic Treatment**

Cells were plated and incubated in the incubator overnight for each experiment to allow for cell attachment. Mother solutions at 20 mM in DMSO (Fisher Chemical, 200-664-3) of each photosensitizer were made and diluted to the necessary quantities (from 10 nM to 10 mM). Untreated cell cultures were included as controls on each plate. The light treated cell cultures were always incubated for 48 h at the optimal photosensitizer doses. The cultures were irradiated for 30 minutes with an irradiance of 10 mW/cm², (18 J/cm² light dose) with the setup described in details by Joniova et al. [49]. Irradiations at a wavelength of 652 nm were performed with a diode laser (Ceralas D-50, BIOLITEC, Germany) coupled to a frontal light distributor (FD-1, Medlight SA, Switzerland) for photodynamic therapy.

**iv. In Vivo Imaging Protocol**

Following radiolabeling, the resulting solution was diluted with water to a concentration of 375 nM, with a final volume of 80-100 µL and a final activity of 3.33-3.7 MBq (90-110 µCi): the activity being calculated by measuring in a dose calibrator the syringe before and after the administration. After being previously anesthetized with isoflurane (3.5% for induction), a SCID mouse is injected retro-orbitally, and a scintigraphic imaging profile in normal SCID mice is acquired. The mice were positioned on the animal bed at a <0.5 cm distance from the camera head to allow whole body imaging with maximum spatial resolution and successive 2 min frames were collected for up to 1 h post injection (p.i.). After the first hour, static images were also acquired at 1, 2, 4, and 24 h p.i.

The activity in the animal is also measured for each timepoint to determine the clearance yield of the compound through the difference of the expected activity if none of it was cleared out of the body, with the activity being corrected for the expected decay at each timepoint, and the actual activity measured in the mouse. The animal...
CONCLUSIONS

This study showed very promising results regarding the synthesis and functionalization of multimodal agents for PDT treatment. The combination of cellulose nanocrystals with porphyrin-based metallas-assemblies was intended to ameliorate their delivery, solubility, and metabolism activity, while still keeping the photo-activity of the compounds. Indeed, the synthesized and characterized stable metallas-assemblies grafted to cellulose nanocrystals have demonstrated photochemical properties suitable for PDT and imaging applications.

In addition, they show no tendency to aggregate, possibly because of the steric and electrostatic nature of the CNCs and metallas-assemblies. Secondly, the radiolabeling of these compounds has been successful, and although some adjustments to the protocol might be needed for cellular uptake assays, preliminary experiments show promising results and potential for these molecules to be used not only as therapeutic but also as diagnostic agents. Finally, the in vitro photodynamic effect of these photosensitizers has shown to be promising for therapeutic application having IC\textsubscript{50} values in the nanomolar range in ovarian cancer cells, and preferential cellular uptake from cancer cells in comparison to normal liver cells.

Overall, these results suggest that these compounds possess the main requirements of an ideal PS and are, thereby, potential photosensitizers for PDT application, while the in vitro data confirmed that these compounds possess the characteristics to act as imaging agents.

In the future, we would like to explore other relevant characteristics for their possible use as imaging agents in PDT, by the conjugation of these compounds with chelator agents and the introduction of other radionuclides with different half-times for SPECT in vivo imaging. In addition, we would like to complete the photophysical studies (calculate quantum yields of fluorescence, triplet state formation, and singlet oxygen production), to gain further insights on their photochemical properties.

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Supporting information

\(^1\)H-NMR (Fig. S1 and S2), mass spectrometry analysis (Fig. S3 and S4), UV-Vis (Fig. S5), and FTIR spectra (Fig. S6), as well as the z-average size and zeta potential (Fig. S7 and S8) are given in the supplementary material.

REFERENCES

8. Tsai HC, Tsai CH, Lin SY, Jhang CR, Chiang YS and Hsieu GH. *Biomaterials* 2012; 33: 1827-1837.
Porphyran Metalla-Assemblies Coupled to Cellulose Nanocrystals for PDT and Imaging Applications

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ABSTRACT: Photodynamic therapy (PDT) is an interesting and promising approach to tackle a broad spectrum of cancer types. With the combination of a photosensitizer, light and oxygen, PDT achieves a unique selectivity by the production of localized reactive oxygen species (ROS) inside cells, which leads to their destruction. In addition, the luminescence properties of photosensitizers can be exploited to develop imaging tools. Unfortunately, the cancer selectivity and homogeneity of most photosensitizers are frequently limiting the performances of PDT and cancer detection/characterization by luminescence imaging. Consequently, our study aims to use cellulose nanocrystals to transport and deliver radiolabeled photo-responsive metalla-assemblies to create a new generation of theranostic agents for PDT and imaging applications. The synthesis, structural characterization, cytotoxicity evaluation, and in vivo biodistribution imaging of the compounds are presented. The best candidates show excellent biological activity and selectivity towards ovarian carcinoma cell line (A2780), cisplatin resistant ovarian carcinoma cell line (A2780cis) versus normal human embryonic kidney cells (HEK293T), as well as efficient imaging properties, suggesting a potential use as multimodal theranostic agents.

KEYWORDS: photodynamic therapy; imaging; porphyrin; cellulose nanocrystal; metalla-assembly; theranostic agent.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig_s1}
\caption{\textsuperscript{1}H NMR (600 MHz, DMSO-\textit{d}_6) spectra (between -3 and 11 ppm) of M1.}
\end{figure}
Fig. S2. $^1$H NMR (600 MHz, DMSO-d$_6$) spectra (between -3 and 11 ppm) of M2.

Fig. S3. Mass spectrometry for M1 – ESI (+).
Fig. S4. Mass spectrometry for M2 – ESI (+).

Fig. S5. UV-Vis spectra of M1 (black) and M2 (red) in DMSO. The peaks show the typical Soret band at 412 and 415 nm, respectively, as well as the Q-bands (500-650 nm).
Fig. S6 – IR spectra MC1 (red), MC2 (blue), and free CNC (orange).

Fig. S7 - Dynamic light scattering (DLS) data of MC1 collected for the measurement of average hydrodynamic diameters.

Fig. S8 - ζ-potential data of MC1 collected through electrophoretic light scattering.