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Hyaluronic Acid Grafted Nanoparticles of a Platinum(II)-Silicon(IV) Phthalocyanine Conjugate for Tumor and Mitochondria-Targeted Photodynamic Therapy in Red Light

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Abstract

Herein, we report novel hyaluronic acid formulated nanoparticles containing a platinum(II) conjugated silicon(IV) phthalocyanine (SiPc-Pt-HA) for tumor targeted red light photodynamic therapy and chemotherapy. The SiPc-Pt-HA conjugate showed specific uptake, photo-enhanced cytotoxicity (~1500 fold) and mitochondrial accumulation in breast cancer over normal cells.

Synopsis and Content Figure:

Hyaluronic acid nanoparticles containing a platinum(II)-silicon(IV) phthalocyanine conjugate exhibited selective uptake by cancer cells and potent mitochondria-targeted PDT and chemotherapy.

Photodynamic therapy (PDT), a clinically established modality for treating cancer, is principled on the selective activation of a photosensitizer by light in the presence of tissue oxygen.¹ This spatio-temporal regulation over drug activation, which can be externally

[†]Footnotes relating to the title and/or authors should appear here.

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Conflicts of interest

There are no conflicts to declare.

achieved by light, makes it attractive in comparison to the conventionally used techniques such as surgery, chemotherapy and radiotherapy.² However, PDT comes with its own limitations, such as i) aggregation and poor solubility of photosensitizers in biological media, ii) inefficient singlet oxygen generation within the maximum tissue penetrating and biocompatible spectral window (650–850 nm), iii) oxygen dependence in the tumor microenvironment and iv) non-specific accumulation of photosensitizers causing adverse effects in illuminated healthy cells.³ The first two restraints were bypassed to some extent by an emerging class of photosensitizers, the silicon(IV) phthalocyanines (SiPc), which are characterized by their reduced aqueous aggregation and high singlet oxygen quantum yields when illuminated with tissue-penetrating far-red light.⁴

Combining PDT with chemotherapy offers a potential solution to the ineffectiveness of PDT in hypoxic regions, and also to overcome drug-resistance *via* multiple routes for cytotoxicity.⁵ Recent research has focussed on conjugating cisplatin and its analogues, which are a mainstay of chemotherapeutic drugs,^{6–8} with photosensitizers.^{9–11} These drugs can offer dual mechanisms of cytotoxicity through both light induced reactive oxygen species and formation of lethal Pt-DNA crosslinks.^{9–11} Furthermore, it is known that metal chelation endows photosensitizers with reduced aggregation, improved aqueous solubility and enhanced singlet oxygen generation abilities.^{12,13}

Combining these rationales, we designed and synthesized a dinuclear complex, **SiPc-Pt**, containing a hydrophobic SiPc core which is symmetrically flanked by positively charged monofunctional platinum(II) units on axial positions (Fig. 1a). Though photosensitizer appended platinum(II) complexes exist in literature,¹⁰ none of these molecules have been previously explored as dual-threat tumor-homing anticancer agents. We reasoned that our positively charged complex would be capable of forming nanoassemblies *via* electrostatic interactions with hyaluronic acid (HA), a negatively charged polymer known to enable active cancer cell uptake *via* overexpressed receptors, such as cluster determinant 44 (CD44) and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (Fig. 1b).¹⁴ Herein, we describe the synthesis of these nanoparticles, **SiPc-Pt-HA**, which display selective uptake and excellent PDT activity in CD44 overexpressing breast cancer cells.

We first prepared the silicon phthalocyanine precursor, **SiPc-1** by reacting SiPcCl₂ with nicotinic acid (Scheme S1a, ESI†).¹⁵ The platinum(II) complex, **SiPc-Pt** (Fig. 1a) was isolated in a two-step reaction using cisplatin and **SiPc-1** (Scheme S1b, ESI†). The compounds were characterized by NMR and mass spectroscopy (Fig. S1-S4, ESI†). The ¹H NMR spectra of **SiPc-Pt** showed a downfield shift for the protons adjacent to the pyridine N-atom which is characteristic of Pt(II)-pyridine complex formation (Fig. S3a, ESI†). The mass spectra displayed the most abundant peak at m/z of 656.6, having isotopic distribution pattern for Pt and assignable to [M-2NO₃]²⁺ ions (Fig. S4, ESI†).

The hyaluronic acid conjugate **SiPc-Pt-HA** was prepared by stirring **SiPc-Pt** in presence of the negatively charged sodium hyaluronate. Dynamic light scattering (DLS) measurements revealed the formation of nanoparticles of average hydrodynamic diameters of 100 ± 15 nm (Fig. 2a) and a negative surface zeta potential value of -26.5 ± 1.2 mV as expected for HA nanoparticles with high stabilities.¹⁶ Such stable nanoparticle formations were not observed

in controls when only HA or **SiPc-Pt** was dispersed in water. Transmission electron microscopy (TEM) revealed spherical nanoparticles having diameter of 80 ± 20 nm (Fig. 2b). About 45% of the added **SiPc-Pt** was encapsulated in the nanoparticles as calculated by its absorbance. No noticeable change was observed in DLS or absorption intensities monitored over a month for samples kept at room temperature which demonstrated considerable stability of the nanoformulations (Fig. 2a).

SiPc-Pt-HA and **SiPc-Pt** (10 μ M in 10% DMF-PBS, pH 7.4) exhibited a strong absorption band around 700 nm with a high extinction coefficient of $20 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Fig. 2c, Table S1, ESI[†]). The nanoconjugation resulted in strong quenching of emission intensity at ~ 705 nm of **SiPc-Pt** ($\phi_f = 0.20$) (Fig. 2d, Table S1, ESI[†]).¹⁶ **SiPc-Pt** also was more water soluble and less prone to aggregation as evidenced by dilution experiments (Fig. S5, ESI[†]). We attribute this to the electrostatic repulsion of the positively charged platinum(II) units which decreases the π -stacking tendencies of the hydrophobic SiPc core. Both **SiPc-Pt-HA** and **SiPc-Pt** showed enhanced singlet oxygen quantum yields ($\phi = 0.24$) than **SiPc-1** ($\phi = 0.15$) as evident from the rates of decay of DPBF, 1,3-diphenylisobenzofuran (Fig. 2e, f, Fig. S6, Table S1, ESI[†]).

We then investigated the interaction of **SiPc-Pt-HA** and **SiPc-Pt** with DNA using an ethidium bromide (EtBr) competition assay. We observed that Pt-DNA interactions were blocked in **SiPc-Pt-HA** as evident from no change in EtBr emission intensity, likely due to the negative surface charges on the hyaluronic acid coated nanoparticles (Fig. 2g). However, as expected, **SiPc-Pt** lowered the emission intensity of DNA-intercalated-ethidium bromide indicating Pt-DNA electrostatic and covalent adduct formation (Fig. 2g). The ICP-MS quantification of Pt incorporated in ctDNA yielded 1.5 ppm and 0.64 ppm of Pt for **SiPc-Pt** and cisplatin-treated ctDNA respectively (Fig. 2h).

Recent literature reports suggest red-light induced degradation of Si-O bonds in SiPcs.¹⁷ This encouraged us to study the photochemical behaviour of these silicon phthalocyanine conjugates. DLS and absorbance measurements of **SiPc-Pt-HA** solutions exposed to red light demonstrated its enhanced photostability (Fig. 2a, Fig. 7c, ESI[†]). In contrast, **SiPc-Pt** (10 μ M in 10% DMF-PBS, pH = 7.4) displayed a gradual decay in absorption intensity at 700 nm and formation of a blue precipitate only on exposure to red light indicating photo-triggered degradation of the complex (Fig. S7a, d, ESI[†]). The rate of photodegradation increased slightly in hypoxic conditions as compared to that in aerated solutions (Fig. S7b, c, ESI[†]). This enhancement in the rate of photolysis in absence of oxygen is possibly due to the increased lifetime of both the triplet state and the intermediate radical anion species.¹⁷ Mass spectral analysis of photolysed **SiPc-Pt** solutions showed m/z peaks at 574.5 and 386.8 which are assignable to SiPc(OH)₂ and a platinum(II)-pyridine complex, indicating breaking of the axial Si-O bonds in red light (Fig. S8, ESI[†]).

We speculated that **SiPc-Pt-HA** should result in rapid uptake selectively in HA receptor-overexpressing cancer cells like MDA-MB-231 (human breast carcinoma) but not by HEK293T (human kidney cells) which are devoid of these particular receptors.¹⁴ The significantly higher cellular uptake of **SiPc-Pt-HA** in MDA-MB-231 cells as compared to HEK293T cells as evaluated from flow cytometric analysis validated our hypothesis (Fig.

3a, Fig. S9, ESI[†]). To further verify the CD-44 receptor mediated uptake (Fig. 1), we pre-treated MDA-MB-231 cells with free hyaluronic acid. The saturation of the receptors blocked the facile entry of **SiPc-Pt-HA** as supported by the lower emission intensity values (Fig. 3a). In contrast, the hyaluronic acid free complex **SiPc-Pt** showed non-specific uptake in both MDA-MB-231 and HEK293T cells (Fig. 3a).

Encouraged by the selective uptake in MDA-MB-231 cells, we further attempted to understand the sub-cellular targets of **SiPc-Pt-HA** (Fig. 3b, S10, ESI[†]). Confocal microscopic images of MDA-MB-231 cells revealed mitochondrial co-localization of **SiPc-Pt-HA** as evident from the yellow colour of the merged images in the first row of Fig. 3b. It was also noted that the red emission of the complex showed zero overlap with the blue emission of the nuclear stain (DAPI) indicating negligible co-localization of **SiPc-Pt-HA** in the nucleus. The HA free conjugate, **SiPc-Pt** displayed a similar mitochondrial accumulation profile supporting enzymatic degradation of hyaluronic acid nanoparticles and release of the **SiPc-Pt** in MDA-MB-231 cells (second row of Fig. 3b). This is important as the vicinity of **SiPc-Pt** to this vital organelle will allow the complex to exert light triggered mitochondrial damage by short-lived singlet oxygen. Also, mitochondria targeted PDT is known to overcome hypoxia and result in increased cytotoxicity.¹⁸ Furthermore, mtDNA lacks the nuclear excision repair (NER) apparatus which promotes cell death by preventing recovery of distorted Pt-DNA structures.¹⁹ Similar experiments were carried out in normal HEK293T cells to further confirm the selective uptake of **SiPc-Pt-HA** nanoparticles only in cancer cells (Fig. S11, ESI[†]).

Based on these promising properties, we then measured the cytotoxicity of **SiPc-Pt-HA** in cells by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Table 1, Fig. 4, Fig. S12, ESI[†]). To our satisfaction, the **SiPc-Pt-HA** nanoparticles caused potent enhancement of cytotoxicity (1500-fold) in red light to CD44 receptor overexpressing MDA-MB-231 ($IC_{50}^{RL} = 0.53 \mu M$) while remaining non-toxic in the HA-receptor negative HEK293T ($IC_{50}^{RL} > 50 \mu M$). No toxicity was observed in absence of light with a short drug-light interval of 4 h, indicative of a tumor-targeted strong PDT effect (Fig. 4). Cells treated with **SiPc-Pt-HA** for longer incubation times did weak dark toxicity ($IC_{50} = 70\text{--}150 \mu M$), likely due to interactions of the monofunctional platinum(II) with DNA (Table 1, Fig. S12, ESI[†]). As expected, unformulated **SiPc-Pt** showed non-specific PDT effects in red light resulting in similar cytotoxic behaviour in both MDA-MB-231 and HEK293T cells (Fig. 4c, d, Table 1). On the contrary, platinum lacking **SiPc-1** resulted in poor PDT effects ($IC_{50}^{RL} \sim 13\text{--}22 \mu M$, Fig. S13 ESI[†]) likely due to its poorer singlet oxygen generating efficiency (Fig. 2f), lack of platinum(II), and tendency to aggregate.

We next attempted to elucidate the mechanism of cytotoxicity caused by the nanoparticles in MDA-MB-231 cells. The DCFDA (2,7-dichlorofluorescein diacetate) assays revealed formation of reactive oxygen species only in **SiPc-Pt-HA** treated cells exposed to red light (Fig. S14, ESI[†]). The photo-induced ROS finally led to cell death by a late apoptotic pathway as understood from the annexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay (Fig. S15, ESI[†]).

In summary, we have demonstrated the synthesis and exploitation of HA coated nanoparticles for CD44 receptor-mediated delivery of a platinum(II)-silicon phthalocyanine complex selectively to cancer cells. The **SiPc-Pt-HA** nanoconjugates showed improved aqueous solubility and singlet oxygen quantum yields in red light. They delivered the **SiPc-Pt** molecules only in cancer cells, and this delivery resulted in generation of cytotoxic singlet oxygen and platinum(II).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
 (a) Chemical structure of **SiPc-Pt** (b) Formation of nanoparticles, **SiPc-Pt-HA** and mechanism of cellular internalization and release in (+) CD44 cancer cells.

Fig. 2.

(a) Dynamic light scattering (DLS) measurements showing size distribution and stability of **SiPc-Pt-HA** nanoparticles. (–) L refers to stability of sample kept in dark for 30 days. (+) L is for sample exposed to red light (5 h, 660–680 nm, 5.5 ± 2.5 mW cm⁻²). Inset shows zoomed region. (b) Transmission electron microscopic (TEM) images showing spherical morphology of **SiPc-Pt-HA** nanoparticles. (c) Absorption and (d) emission spectra of **SiPc-Pt** and **SiPc-Pt-HA**. (e) Singlet oxygen generation by **SiPc-Pt-HA** as evident from decrease in absorption intensity of DPBF at 415 nm at 0, 5, 10 and 15 seconds of red light irradiation. (f) Decrease of normalized absorbance of DPBF at 415 nm for **SiPc-Pt-HA**, **SiPc-Pt** and SiPc-1 on red light exposure for different time intervals as shown in the graph. (g) Changes in emission intensity of ctDNA intercalated ethidium bromide (EB, 50 μ M) on treatment with **SiPc-Pt** and **SiPc-Pt-HA**. (h) Pt content (expressed as ppm) in isolated ctDNA treated with **SiPc-Pt** or cisplatin (CP) in dark (–) L or in red light (+) L.

Fig. 3.

(a) Bar diagram showing cellular uptake (mean fluorescence intensity, Ex. 633, Em. 780) as measured by FACS in MDA-MB-231 and HEK293T cells treated with **SiPc-Pt** or **SiPc-Pt-HA** for 4h in presence or absence of hyaluronic acid (HA, 500 μ M) as shown in the table.

(b) Confocal microscopic images showing mitochondrial co-localization of **SiPc-Pt-HA** (1st row) and **SiPc-Pt** (2nd row) in MDA-MB-231 cells. The 1st column shows emission for DAPI (nuclear stain), 2nd column for mitotracker green (MTG) and 3rd column for complex. The last column shows the overlapped images of the first three columns. Scale bar = 10 μ m.

Fig. 4. Dose-dependent cellular viabilities (%) as obtained from MTT assays in (a, c) MDA-MB-231 and (b, d) HEK293T cells treated with (a, b) **SiPc-Pt-HA** or (c, d) **SiPc-Pt** for 4 h and either are exposed to red light (red circles, (+) L, 45 mins, 660–680).

Table 1.IC₅₀ (μM) values as obtained from MTT assay

Complex	Cell lines	(+) Light ^a	(-) Light ^b
SiPc-Pt-HA	MDA-MB-231 HEK293T	0.05 ± 0.01 >50	77 ± 9 120 ± 11
SiPc-Pt	MDA-MB-231 HEK293T	0.09 ± 0.03 0.03 ± 0.01	146 ± 15 61 ± 4
SiPc-1	MDA-MB-231 HEK293T	13.5 ± 2.4 22.0 ± 2.1	>25 >25
Cisplatin	MDA-MB-231 HEK293T	15 ± 2 17 ± 3	17 ± 3 19 ± 1

^a Cells were treated with compounds for 4 h in dark, followed by irradiation (45 mins, 660–680 nm, 5.5 ± 2.5 mW cm⁻²) in phenol red free media. Post incubation = 24 h in dark.

^b Cells were treated with compounds for longer incubation times of 24, 48 or 96 h to assess dark toxicities.