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Efficient Photosensitization by a Chlorin−Polyoxometalate Supramolecular Complex

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Supporting Information

ABSTRACT: The 4:1 supramolecular complexed ionic salt between pyridinium chlorin and polyanionic $[\alpha\text{-}SiMo_{12}O_{40}]^{4-}$ exhibits significantly enhanced photodynamic activity against A549 cell lines because of increased singlet oxygen photogeneration through high cellular penetration and localization of the chlorin molecules on the ionic salt into the cancer cell. Confocal laser scanning microscopy images clearly represent a higher uptake and photodynamic effect of this supramolecular complex corresponding to the lower IC_{50} value compared to the free chlorin.

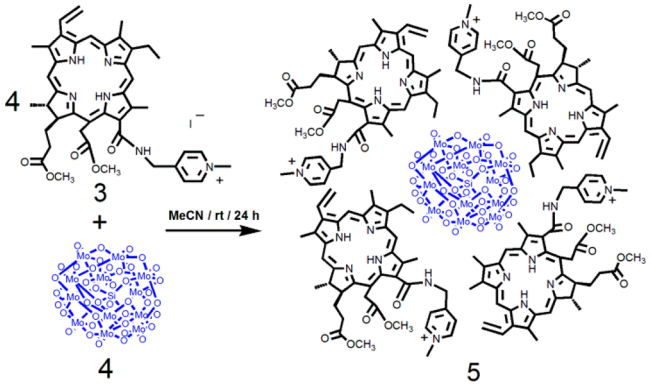
Photodynamic therapy (PDT) is a promising noninvasive cancer treatment based on the activation of a photosensitizer (PS) upon light irradiation for the generation of reactive oxygen species (e.g., singlet oxygen, $^1O_2$) to destroy the tumors.[1] The development of desirable PSs as well as delivery systems of PSs has attracted much attention for highly selective targeting of tumor sites.[2] Cationic chlorins or porphyrins have advantages compared to neutral (nonionic) chlorins or porphyrins for higher uptake and retention for tumor targeting, which has potential applications such as DNA binding and photocleavage[3] and PSs for PDT.[4]

Polyoxometalates (POMs), well-defined early-transition-metal oxygen anion clusters, have attracted extensive interest because of their intriguing properties including catalytic, electronic, optical, and magnetic functionalities.[5] In addition, POMs have interesting medicinal properties such as antitumor and antiviral activities.[6] However, there are very rare reports for medicinal applications using POM and organic molecules. This is the first example of an inorganic−organic hybrid molecular assembly using POM and chlorin (the PS) molecules that can enhance the photodynamic activity against the cell lines. Previously, we developed long-wavelength cationic chlorins (purpurinimide derivatives with a pyridinium or quinolinium unit) via a stereoselective aldol-like condensation method as a potential PS, resulting in relatively high phototoxicity against HeLa cell lines.[7]

In this work, we synthesized new cationic chlorin derivative **3** containing pyridinium (Scheme S1 in the Supporting Information, SI). Methyl pheophorbide-a (**1**) was obtained from extraction of chlorophyll-a paste (*excrementum bombycis*) in acidic methanol followed by column chromatography separation in 5% yield.[8] Combining **1** and an excess amount of 4-(aminomethyl)pyridine in chloroform, followed by column chromatography, afforded neutral chlorin **2** as a dark-green solid in 61% yield. Methylation of **2** with methyl iodide afforded cationic chlorin **3** as a dark-green solid in quantitative yield without further purification.

In order to introduce POM, we selected the well-known Keggin-structure-type POM **4**,[9] $[\alpha\text{-}SiMo_{12}O_{40}]^{4-}$, which can be soluble in common organic solvents such as acetonitrile and dimethyl sulfoxide (DMSO) after cation exchange as an organic salt form (e.g., tetrabutylammonium).[10] The preparation of the chlorin−POM complex **5** from the cationic chlorin **3** and the Keggin-type POM **4** is straightforward, as shown in Scheme 1. The chlorin−POM complex **5** was separated as a precipitate at the reaction condition.

Scheme 1. Synthesis of the Chlorin−POM Complex 5



The structures of **2**−**5** were characterized by combination analysis of ^1H NMR, UV/vis, and Fourier transform infrared (FTIR) spectroscopies, atomic analysis, and high-resolution fast atom bombardment mass spectrometry (MS; Figures S1−S13 in the SI). ^1H NMR spectra of **2** and **3** present two doublet signals at 8.51 and 7.22 ppm and at 7.41 and 6.46 ppm (in $CDCl_3$) corresponding to the protons on the pyridine units, respectively (Figures S1 and S4 in the SI). Also, the ^1H NMR spectrum of **3** presents methyl proton signals at 3.65 ppm for successful methylation from **2**. The ^1H NMR spectrum of **5** (Figure S8 in the SI) reveals disappeared proton signals of a tetrabutylammonium salt from starting POM, indicating complete cation

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exchange with the pyridinium chlorin **3**, resulting in a 4:1 ratio (chlorin−POM), as shown in Scheme 1. UV/vis spectra of **3**−**5** in DMSO are shown in Figure S9 in the SI. The FTIR spectrum of **5** presents specific signals for the POM, such as Si═O at 946 cm^{-1}, Mo═O at 899 cm^{-1}, and Mo−O−Mo at 791 cm^{-1} (Figure S10 in the SI). The scanning electron microscopy (SEM) image of complex **5** (Figure S11 in the SI) shows ca. 70−110 nm size range after solid separation as a precipitate. Elemental analysis (in the SI) and a ^1H NMR titration experiment (Figure S12 in the SI) confirm formation of the chlorin−POM complex **5** having a 4:1 ratio (chlorin−POM). In addition, thermogravimetric analysis (TGA; Figure S13 in the SI) of the chlorin−POM complex **5**, which evidences direct measurement of the weight loss percentages of both organic and inorganic contents as 62% of four organic pyridinium (4^+) chlorin molecules and 38% of one inorganic POM (4^-) molecule.

Photocytotoxicity and dark cytotoxicity of chlorin **3**, POM **4**, and their complex **5** were investigated against A549 cells using a halogen lamp equipped with a band-pass filter (640−710 nm) as a light source (total light dose 2 J cm^{-2}; irradiation time 15 min; Figure 1). A549 cells (10×10^4 cells/well) were incubated with

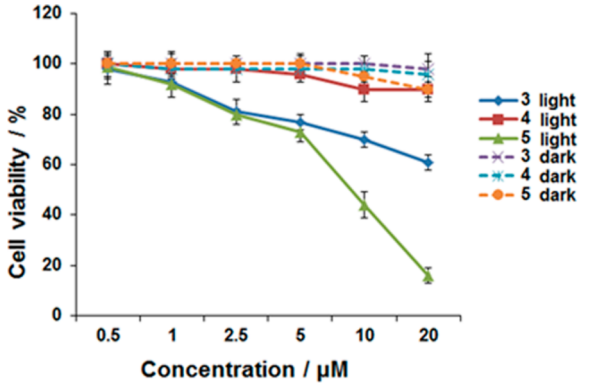

Figure 1. Cell viability of photocytotoxicity and dark cytotoxicity of chlorin **3**, POM **4**, and their complex **5** against A549 cells at a concentration range of 0.5−20 μM and a light dose of 2 J cm^{-1}. The percentage of cell viability was determined by a MTT assay at 24 h incubation time before (dark) and after (light) photoirradiation. Error bars represent the standard deviation of three replicate experiments.

PSs for 24 h and photoirradiated for a photocytotoxicity test. At 24 and 48 h incubation times after photoirradiation, the cell viability (%) was estimated based on the mitochondrial activity of NADH (reduced form of nicotinamide adenine dinucleotide) dehydrogenase using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (summarized in Tables S1 and S2 in the SI). Figure 1 exhibits the cell viability results of photocytotoxicity and dark cytotoxicity of chlorin **3**, POM **4**, and their complex **5** at a concentration range of 0.5−20 μM at 24 h incubation time after photoirradiation (also see Figures S14−S17 in the SI).

In all of the compounds, upon photoirradiation, the cell viability decreased, consistent with increasing incubation time after photoirradiation. All of the compounds showed low dark cytotoxicity up to 20 μM. POM **4** displayed low phototoxicity (79% at 48 h and 90% at 24 h incubation times at 20 μM), and cationic chlorin **3** gave good phototoxicity (59% at 48 h and 61% at 24 h incubation times at 20 μM). As we expected, the phototoxicity efficiency was significantly improved in the chlorin−POM complex **5** (14% at 48 h and 16% at 24 h incubation times at 20 μM). This supramolecular complex demonstrated a significant decrease in the IC_{50} value to 6.61 μM at 48 h and 8.60 μM at 24 h incubation times compared to free chlorin **3** (IC_{50} is more than 20 μM in this studied condition). It is noted that this result confirms that the chlorin−POM complex can maximize its own photodynamic activity of the PS (in this case **3**) alone, which significantly corresponds to the excellent delivery of **3** through the complex system **5** into the tumor cells.

This increased phototoxicity result of the chlorin−POM complex **5** is consistent with the increased number of cationic chlorins on complex **5** by stable and strong electrostatic interactions between the POM (having 4− charge) and the four cationic pyridinium chlorins. In the complex system, the POM has an important role as a delivery vector of the (cationic) chlorins (as polar attractors).[6]

POM **4** has no fluorescence; however, it quenches fluorescence of chlorin **3** in a DMSO solution because of the electron-transfer interactions between the POM and chlorin molecules (Figure S18 in the SI), which is shown as a Stern−Volmer plot (Figure S19 in the SI). This fluorescence quenching by POM **4** may inhibit $^1O_2$ generation of chlorin by electron transfer,[11] however, which was not so much affected to decrease the photodynamic activity in complex **5**. Figure 2 evidences the

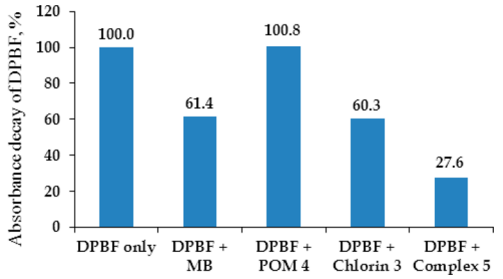

Figure 2. Absorbance decay (%) of DPBF (50 mM in DMSO) at 418 nm after photoirradiation (total light dose 2 J cm^{-2}; irradiation time 15 min) in the absence (control) and presence of 1 mM methylene blue (MB), chlorin **3**, POM **4**, and the chlorin−POM complex **5**.

relative differences of $^1O_2$ photogeneration between chlorin **3**, POM **4**, and their complex **5** using 1,3-diphenylisobenzofuran (DPBF) as a selective $^1O_2$ acceptor.[12] Complex **5** allows significantly increased $^1O_2$ photogeneration compared to chlorin **3** only. Consequently, this result proves that the increased photodynamic activity was attributed the fact that the chlorin−POM complex induced higher $^1O_2$ photogeneration through high cellular penetration and localization of the chlorin molecules into the cells compared to free chlorin **3** via an endocytosis approach.[13]

Cellular penetration and localization of chlorin **3** and the chlorin−POM complex **5** for in vitro antitumor activity were confirmed by confocal laser scanning microscopy (CLSM; Figure 3).[2,14] Fluorescence of the chlorin molecules (red color) from both **3** and **5** was clearly shown inside the cells, indicating successful cellular uptake of the pyridinium chlorin molecules in the cytoplasm. CLSM images show that **5** allows higher uptake than **3** because of the excellent delivery system of the chlorin−POM complex **5**, resulting in lower cell viability in **5** compared to **3** (Figure 1).

Before irradiation (Figure 3a−c,g−i), morphologies between **5** and **3** are almost the same; however, after irradiation, complex **5** (Figure 3d−f) presents a different shape of morphology than that

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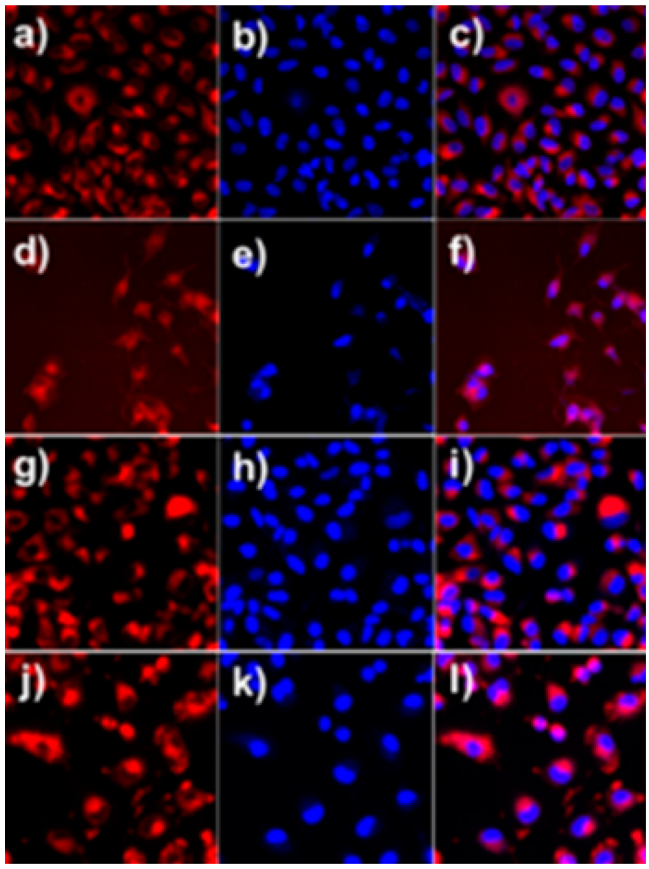

Figure 3. CLSM images of A549 cells: (a–c) complex **5** before irradiation; (d–f) complex **5** after irradiation of 640–710 nm for 15 min at 2 J cm^{-1}; (g–i) chlorin **3** before irradiation; (j–l) chlorin **3** after irradiation. (a, d, g, and j) Chlorins (red) in **3** or **5**. (b, e, h, and k) Nuclear dye DAPI (blue). (c, f, i, and l) Merged.

of chlorin **3** (Figure 3j–l) because of the preparation of apoptotic bodies by higher photodynamic activity compared to **3**.[6,15] Overlapped images (Figure 3c,f,i,l) of cytoplasms (red color) and nuclei (blue color) stained with 4′,6-diamidino-2-phenylindole (DAPI) are clearly present, indicating that the cationic chlorins can penetrate the cytoplasm (not the nuclei).

For medicinal applications using POM, it is a significant consideration that many POMs have low stability in water at the physiological pH and decompose into a mixture of inorganic products.[16] A stability test was performed by inductively coupled plasma (ICP) measurement (elemental analysis change)[17] of POM **4** (NH_4^+ form) and complex **5** (dissolved in DMSO/water) for 0–48 h at 37 °C in phosphate-buffered saline (pH 7.4) aqueous solutions (Table S3 in the SI). The Mo atom in **5** showed ca. 11% decomposition at 24 h and was retained at 48 h in the test condition. Otherwise, the Si atom in **5** showed no decomposition until 48 h. Also, the Mo atom in **4** showed ca. 2% at 24 h and 11% at 48 h decomposition. Also, the Si atom in **4** showed ca. 7% decomposition at 48 h. There are many efforts to increase the stability and activity of the POMs, especially using polymeric nanoparticles and liposomes.[18]

This supramolecular complex may reveal that a lot of cationic chlorin molecules could be bound with a polyanionic POM molecule, which depends on the number of charges of chlorin and POM molecules. However, for further applications using this complex system, one needs to keep a suitable hydrolytic stability of this complex. This observation could be useful both in the development of PDT using POMs and cationic chlorins as well as for the pursuit of a new design and synthesis of potential PSs and PS delivery vectors.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and cell viability data and Figures S1–S19 and Tables S1–S3 showing ^{1}H NMR, UV/vis, and fluorescence spectra and SEM, TGA, MS, and ICP data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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