Supramolecular Chemistry

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Supramolecular Photosensitizers with Enhanced Antibacterial Efficiency**

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Photosensitizers, a key component in photodynamic therapy (PDT), are compounds that can transfer the energy of light to surrounding oxygen, thereby producing highly reactive oxygen species, for example singlet oxygen ($^1O_2$), to destroy diseased tissues or microorganisms.[1] From a practical application point of view, readily accessible, highly fluorescent photosensitizers with strong absorbance at long wavelengths and high singlet oxygen quantum yields are highly desirable. In particular, porphyrins and their derivatives are popularly employed as one class of important photosensitizers owing to their excellent photophysical properties. They have very intense absorption bands in the visible region and high singlet oxygen quantum yield because of their large π-conjugated aromatic domains.[2] However, the porphyrins easily form aggregates based on hydrophobic π–π interactions in aqueous medium, especially at high local concentrations induced by uptake and accumulation processes inside cells or microorganisms. The aggregation can produce a severe self-quenching effect of the excited state, leading to quenched fluorescence and greatly reducing the ability for $^1O_2$ generation, and therefore lowering the efficiency for phototherapy.[3] To address this issue, it has been common practice to introduce space-demanding hydrophilic substituents to the parent porphyrins, for example, segregate porphyrins into the focal core of hydrophilic dendrimers.[4] In doing so, the quenching effect can be suppressed, which leads to an appreciable improvement of the photocytotoxicity. However, such covalent practice often involves time-consuming tedious chemical synthesis and purification processes, thus raising the costs of preparation. In addition, organic solvents and toxic reagents used in chemical synthesis may be incorporated into photosensitizers and reduce their biocompatibility.

Cucurbit[*n*]uril (CB[*n*]), a family of barrel-shaped macrocyclic hosts, have been developed into an interesting research area, because of their rich host–guest chemistry.[5] The CB[*n*] molecules possess a hydrophilic exterior and hydrophobic cavities. Because of the existence of the hydrophobic cavity, CB[*n*] has been widely used, for example, to encapsulate and solubilize dyes[6] and to enhance weak supramolecular interactions.[7] Generally, compared with other hosts, such as cyclodextrins and calixarenes, the binding constant of CB[*n*] with its guests is much larger, especially to the cationic species, driven by a combination of ion–dipole interactions, hydrogen bonds, and the hydrophobic effect.[5] Herein, the large molecular volume and hydrophilic exterior of CB[*n*] molecules have encouraged us to explore the possibility of using CB[*n*] as bulky "noncovalent building blocks" to weaken the close stacking of porphyrins, thus suppressing the self-quenching of the excited states and improving the antibacterial efficiencies even upon aggregation. In addition, the rich host–guest chemistry of CB[*n*] can enable the bulky substituents to be noncovalently attached to the porphyrins, which is environmentally friendly and can greatly decrease the required steps of chemical synthesis. For this purpose, a new kind of supramolecular photosensitizer has been designed as shown in Figure 1. Porphyrins are readily modified with four positive charges (TPOR), so as to efficiently adsorb onto the negative charged surface of bacteria. The other building block, CB[7], is selected as the bulky hydrophilic heads of the supramolecular photosensitizers. The strong host–guest interaction between CB[7] and naphthalene–methylpyridinium moiety[5e] on TPOR is used as the driving force for the construction of the supramolecular photosensitizers.

For the construction of the desired supramolecular photosensitizers, CB[7] was added to the aqueous solutions of TPOR in a molar ratio of TPOR:CB[7] = 1:4. Different methods were employed to confirm the formation of the desired supramolecular photosensitizers. Firstly, isothermal titration calorimetry (ITC) was carried out to provide information about the binding ability of CB[7] with TPOR. The obtained titration isotherm is shown in Figure 2a. The binding stoichiometry between TPOR and CB[7] is calculated to be 1:4, indicating that the desired supramolecular photosensitizers shown in Figure 1 have been obtained. By fitting the data, the binding constant of the naphthalene–methylpyridinium subgroup with CB[7] is calculated to be $K = 6.6 \times 10^7\,\text{M}^{-1}$, indicating that the driving force is quite strong and efficient interactions can take place for the noncovalent construction of the TPOR/$(CB[7])_4$ supramolecular photosensitizers. Secondly, the formation of the supramolecular photosensitizers is confirmed by dynamic light scattering

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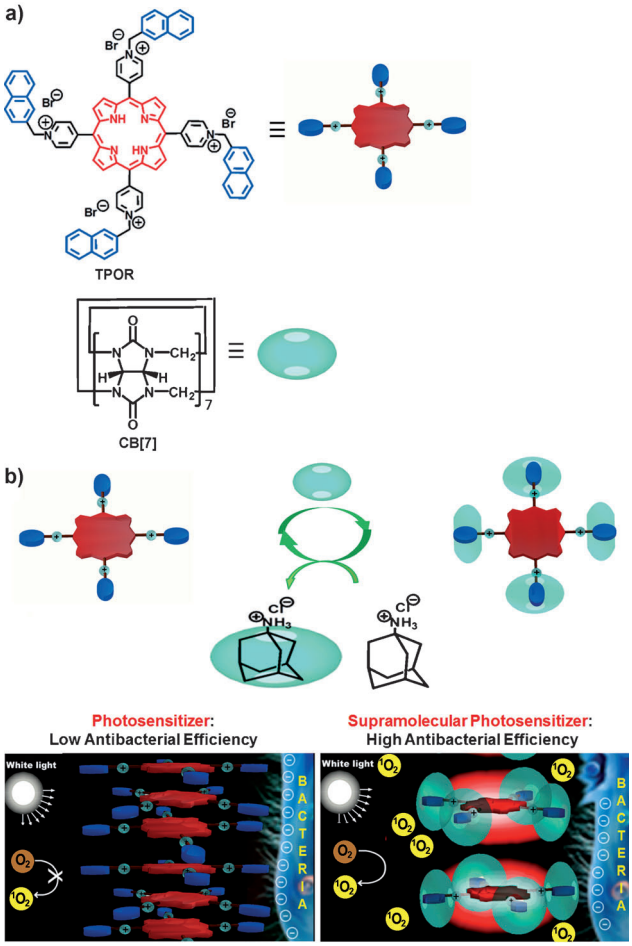

Figure 1. a) Chemical structures of the TPOR (photosensitizers) and CB[7]. b) The construction of TPOR/$(CB[7])_4$ supramolecular photosensitizers and the mechanism for the enhanced antibacterial efficiency of TPOR/$(CB[7])_4$ compared with that of TPOR.

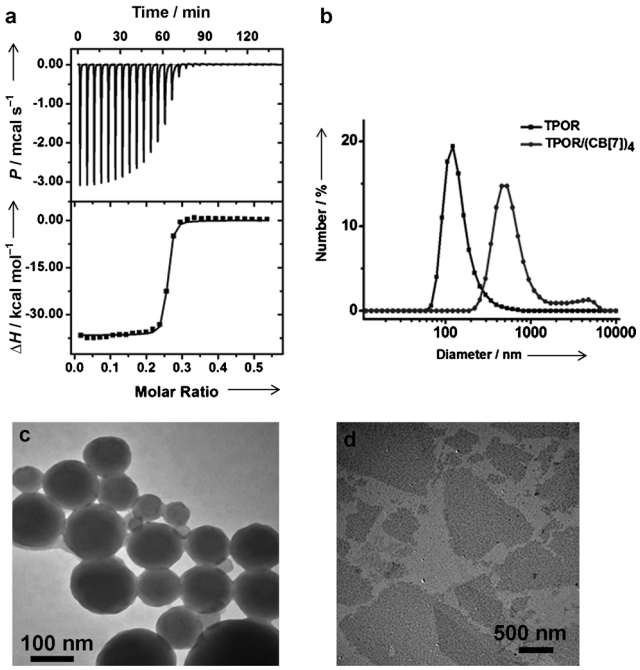

Figure 2. a) ITC data for the titration of CB[7] with TPOR, the "molar ratio" is defined as TPOR:CB[7]. b) DLS measurements of the TPOR and the TPOR/$(CB[7])_4$ aqueous solution. TEM images of c) TPOR and d) TPOR/$(CB[7])_4$ assemblies. The concentration of TPOR is 3.6×10^{-4} M and that of CB[7] is 1.44×10^{-3} M.

(DLS) measurements. Because of the amphiphilic nature of TPOR and TPOR/$(CB[7])_4$, both of them can self-assemble in water to form well-defined aggregates.[8] As indicated by Figure 2b, the hydrodynamic diameter of the TPOR self-assemblies is determined to be around 100 nm. However, upon adding CB[7] into the TPOR aqueous solution, the TPOR self-assemblies disappear completely. Instead, assemblies with a larger hydrodynamic diameter of about 600 nm can be detected, indicating that the TPOR/$(CB[7])_4$ supramolecular photosensitizers show quite different self-assembling behavior compared with that of their building blocks. Thirdly, electron microscopy observation indicates that the addition of CB[7] into the TPOR aqueous solution has changed the morphology of the self-assemblies. TPOR self-assembles into spherical-like aggregates, as indicated by TEM (Figure 2c), scanning electron microscopy (SEM; Figure S1a), and cryo-TEM images (Figure S1b). There is no clear contrast between the rim and the center of the spheres, indicating that the spherical-like aggregates are micelle-like structures rather than hollow vesicles. However, the TPOR/$(CB[7])_4$ supramolecular photosensitizers self-assemble into nanosheets, as indicated by TEM (Figure 2d) and cryo-TEM images (Figure S2). No spherical assemblies can be observed, indicating that the added CB[7] building blocks are quite efficient to affect the assembling behavior of TPOR.

Interestingly, an obvious increase of the fluorescence intensity of porphyrins is observed upon the binding of CB[7] to TPOR. As shown in Figure 3a, in the TPOR assemblies, the fluorescence of porphyrins is severely quenched, which is ascribed to the commonly observed "aggregation-induced fluorescence quenching" of π-conjugated chromophores. However, by adding CB[7] into the aqueous solution, the resulting TPOR/$(CB[7])_4$ solution emits more intense fluorescence, although the porphyrin chromophores still remain in the aggregated state. In other words, the aggregation-induced fluorescence self-quenching of porphyrins is largely suppressed in their aggregates by the bulky CB[7] molecules that are noncovalently attached on the porphyrin aromatic rings.

It can be envisaged that, in the TPOR/$(CB[7])_4$ supramolecular photosensitizers, the hydrophobic porphyrin chromophores tend to stack closely based on strong hydrophobic and π–π interactions. However, the bulky and space-demanding CB[7] molecules have impeded the close stacking of porphyrins in the assemblies, thus suppressing the quenching of their fluorescence. We have employed UV/Vis spectroscopy to support this assumption. As shown in Figure 3b, the molar extinction coefficient of the porphyrin increases upon adding CB[7] into the TPOR aqueous solution. It has been well studied that the molar extinction coefficient of the chromophores, is dependent on the distance of the adjacent chromophores.[9] The observed increase of the molar extinc-

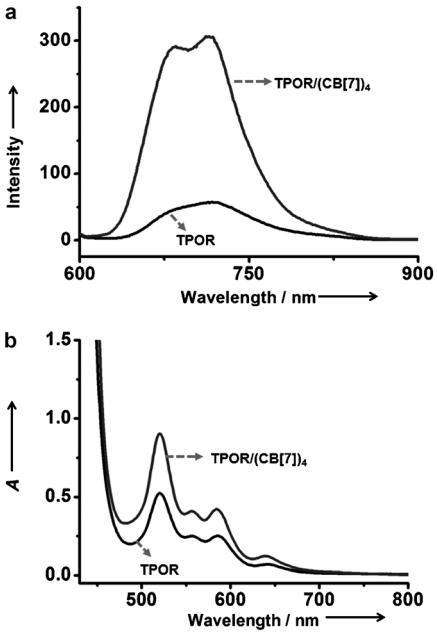

Figure 3. a) Fluorescence and b) UV/Vis spectra of TPOR and TPOR/$(CB[7])_4$ aqueous solution. The concentration of TPOR is 3.6×10^{-4} M and that of CB[7] is 1.44×10^{-3} M.

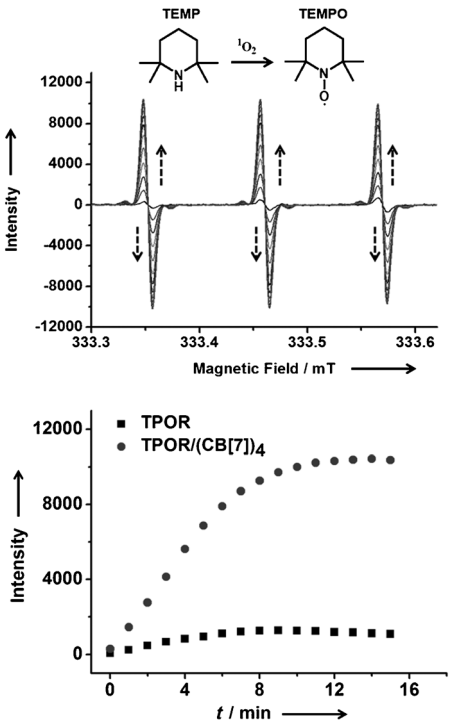

Figure 4. a) EPR spectra of the TPOR/$(CB[7])_4$ aqueous solution in which trace TEMP acts to capture the $^1O_2$ generated upon photoirradiation. The arrows indicate the changes of the spectra with irradiation time increases. b) The change of intensity of the EPR peak (positive part) with irradiation time shown in (a). The concentration of TPOR is 3.6×10^{-4} M, and that of CB[7] is 1.44×10^{-3} M.

tion coefficient should be then ascribed to the increased distance between the adjacent porphyrin chromophores. Thus, the interaction between the chromophores is weakened, leading to the suppressed self-quenching of the excited state and recovery of the fluorescence of porphyrins.

As a logical next step, we investigated the ability of our supramolecular system to generate $^1O_2$. Interestingly, we have found that the construction of TPOR/$(CB[7])_4$ supramolecular photosensitizers can greatly enhance the $^1O_2$ generation ability of porphyrins. Upon irradiation with visible light, the porphyrins can be excited to the singlet excited state and subsequently converted to its triplet state through intersystem crossing. In the presence of oxygen, the energy of the triplet state of porphyrins can be transferred to the triplet of oxygen, leading to singlet oxygen formation.[2] However, the aggregation of porphyrins can decrease their ability to generate $^1O_2$, as the absorbed energy can be released as heat by the closely stacked state.[3] Electron paramagnetic resonance (EPR) spectroscopy was employed to monitor the $^1O_2$ generation ability of TPOR/$(CB[7])_4$ upon photoirradiation. Trace amounts of 2,2,6,6-tetramethylpiperidine (TEMP), a diamagnetic and water-soluble molecule, was used to capture $^1O_2$ by yielding a paramagnetic product, the stable nitroxide radical TEMPO,[10] as shown in Figure 4a. The unpaired electron located on the NO group of TEMPO can lead to the hyperfine splitting of the EPR signal into three narrow lines, arising from the interaction between the unpaired electronic spin and the nitrogen nucleus. As shown in Figure 4a, the EPR spectral pattern of three lines of equal intensity, which is characteristic for the TEMPO nitroxide radical, was observed when an oxygen-saturated aqueous solution of TPOR/$(CB[7])_4$ was irradiated in the presence of TEMP at room temperature. The g value of the radicals was found to be $g = 2.002$, consistent with the previously reported values.[10] Upon photoirradiation, the intensity of the EPR signal increases gradually, indicating that the $^1O_2$ molecules are generated in the aqueous solution. To get a better understanding on the kinetics of $^1O_2$ generation, the intensities of the EPR peak, which would be a reflection of the amount of $^1O_2$ generated in this process, are plotted against the photoirradiation time. As indicated by Figure 4b, the amount of $^1O_2$ molecules increases linearly at the first stage upon irradiation, and reaches an equilibrium value in about 10 minutes. As a control experiment, the irradiation for TPOR aqueous solution with the same concentration of porphyrin sensitizers was carried out. Very interestingly, the $^1O_2$ generation rate for the TPOR/$(CB[7])_4$ supramolecular photosensitizers is 7.5 times faster compared with that of TPOR (see Figure S3 in the Supporting Information), by comparing the slope of the linear region of the curve shown in Figure 4b. In addition, the equilibrium value, which is closely related to the efficacy for $^1O_2$ generation in the aqueous solution, is increased. As a control experiment, the CB[7] molecules alone cannot generate $^1O_2$ upon irradiation. Thus, the enhanced efficiency of the supramolecular photosensitizers to generate $^1O_2$ should be attributed to the suppressed self-quenching of the excited states of porphyrins, as indicated by the increased fluorescence of TPOR/$(CB[7])_4$ shown in Figure 3a.

The bulky CB[7] "noncovalent substituents" in the supramolecular photosensitizers have ensured a dramatically

increased efficiency for the $^1O_2$ generation of the porphyrins, regardless of aggregation at high concentrations. It is thus anticipated that the supramolecular photosensitizers can attain an elevated concentration of local $^1O_2$ when irradiated by white light, leading to an improved photocytotoxicity for application in photodynamic therapy. For this purpose, we investigated the antibacterial activities of TPOR and TPOR/$(CB[7])_4$ toward *Escherichia coli* (*E. coli*), a Gram-negative bacteria, by a traditional surface plating method.[11] The *E. coli* was incubated with TPOR and TPOR/$(CB[7])_4$, respectively, in the dark at a fixed concentration of porphyrin sensitizer, after which the bacteria were illuminated with a white light at a flux rate of 25 mW cm^{-2} for the duration of 40 s (corresponding to fluences of 1 J cm^{-2}). Colony counting shows that the inhibition ratio towards *E. coli* is about 17% for TPOR, whereas the value increases to 97% for the TPOR/$(CB[7])_4$ supramolecular photosensitizers (Figure 5). We have

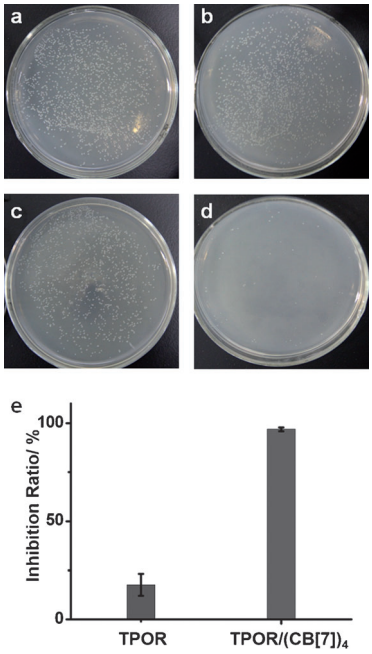

Figure 5. Plate photographs for *E. coli* on YTD agar plate treated with a) TPOR in the dark, b) TPOR under photo-irradiation, c) TPOR/$(CB[7])_4$ in the dark, d) TPOR/$(CB[7])_4$ under photoirradiation, and e) biocidal activities of TPOR and TPOR/$(CB[7])_4$ toward *E. coli*. The values represent the mean standard deviation of three separate experiments. The error bars represent standard deviations of data from three separate measurements. The concentration of TPOR is fixed at 7.2×10^{-7} M for killing *E. coli*.

also demonstrated that CB[7] alone has no photocytotoxicity upon photoirridiation. Thus, the antibacterial efficiency is greatly enhanced after the porphyrins have been incorporated into the supramolecular photosensitizers. The result is consistent with the enhanced $^1O_2$ generation of the supramolecular photosensitizers (Figure 4), indicating that the improved antibacterial efficiency should be ascribed to the enhanced ability of porphyrins to sensitize oxygen. To the best of our knowledge, the TPOR/$(CB[7])_4$ supramolecular photosensitizer is amongst the most efficient porphyrin-based antibacterial PDT systems reported to date,[12] in terms of a lower concentration of the sensitizer (0.72 μM, amongst the lowest concentration reported to date), low fluences of irradiation (only 1 J cm^{-2}, compared with several tens J/cm^2 in previous published results), and a high inhibition ratio towards bacteria (about 97%).

The improved antibacterial efficiency of the supramolecular photosensitizers is reasonable considering the following aspects (Figure 1b). 1) In TPOR there are four cationic substituents on the periphery of porphyrins, which can lead to efficient adsorption and accumulation of the sensitizers onto the negatively charged surface of *E. coli* even at low concentrations. This point is quite important as the life of $^1O_2$ is very short and it is the close contact that facilitates the $^1O_2$ to kill bacteria. 2) On the other hand, the accumulation of TPOR on the surface of *E. coli* results in a high local concentration of porphyrins, leading to the close stacking of the porphyrin sensitizers and therefore a dramatic quenching of their excited states upon irradiation. However, CB[7] can recognize and bind firmly with the cationic naphthalene-methylpyridinium moiety on TPOR, so the TPOR/$(CB[7])_4$ supramolecular photosensitizers can be efficiently fabricated. The four noncovalently attached bulky CB[7] molecules have weakened the close stacking of porphyrins, for which the aggregation-induced self-quenching of the excited states of the sensitizer is greatly suppressed even at high local concentrations, thus leading to the improved $^1O_2$ generation ability and much higher antibacterial efficiency of the supramolecular photosensitizers.

Compared with the photosensitizers fabricated on the basis of covalent chemistry, the supramolecular approach established here not only has decreased the required synthetic steps, but also allows for the construction of a responsive smart material, inheriting from the reversible host–guest interactions. 1-Adamantanamine hydrochloride (AD), which has a binding constant as high as 4.2×10^{12} M^{-1} for complexation with CB[7],[5c] was added into the TPOR/$(CB[7])_4$ supramolecular photosensitizers with a molar ratio of TPOR/$(CB[7])_4$:AD = 1:4. As shown in Figure S4a, the TPOR/$(CB[7])_4$ assemblies, that is the nanosheets, disappeared, whereas spherical-like aggregates similar to the TPOR assemblies reappeared again. This indicates that the preferred binding of CB[7] to the AD guests can lead to the dissociation of the TPOR/$(CB[7])_4$ supramolecular photosensitizers. Furthermore, after adding the competitive AD, the fluorescence of the solution recovers to the initial highly quenched state, as shown in Figure S4b. The fluorescence of the TPOR/$(CB[7])_4$/AD_4 solution is nearly the same as TPOR, indicating that the supramolecular photosensitizers are highly reversible and adaptive.

In summary, we have successfully fabricated a novel supramolecular photosensitizer which shows a greatly improved antibacterial efficiency. The fabrication of the supramolecular photosensitizer based on host–guest interaction is facile, highly efficient, environmentally friendly and has greatly decreased the number of required synthetic steps. In addition, it also allows for an adaptive system with switchable photophysical properties. The TPOR/$(CB[7])_4$ supramolecular photosensitizer is amongst the most efficient

porphyrin-based antibacterial PDT systems reported to date, in terms of the low porphyrin concentration and low fluences of light energy required. It is anticipated that such supramolecular photosensitizer might also be effective for improving the anticancer properties of porphyrins or other photosensitizers in photodynamic therapy systems.

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