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A Tumor-targeting Ru/Polysaccharide/Protein Supramolecular Assembly with High Photodynamic Therapy Ability

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Supramolecular assembly with tumor-targeting property or photodynamic therapy (PDT) ability has recently become a focus of interest in biomaterial fields because of their high therapeutic efficacy against tumor cells. Herein, we reported a new type of targeted supramolecular nanoparticles for photodynamic therapy of tumor cells constructed by adamantane-functionalized transferrin protein (Ad-TRF) and β-cyclodextrin-functionalized ruthenium complex (Ru-HOP-CD), where Ad-TRFs acted as the targeted sites for tumor cells, the coordinated Ru(II) centers acted as the PDT active sites, and the biocompatible polysaccharide β-cyclodextrins acted as the non-covalent linkers. Significantly, the resultant Ru/polysaccharide/protein exhibited not only the specific targeting property towards tumor cells but also the high PDT ability under the irradiation of visible light. Furthermore, the assembly showed the selective killing towards tumor cells along with the negligible toxicity towards normal cells.

The development of advanced systems for malignant tumors therapy is still one of the most challenging tasks in medical fields.[1] As an effective method, supramolecular chemistry provides a new way to construct controllable drug release of targeted drug delivery system without complicated modification process.[2] Among the various macrocyclic compounds used in the design of supramolecular materials, cyclodextrins (CDs), a class of cyclic oligosaccharides with six to eight D-glucopyranose units linked by α-1,4-glucoside bonds, are extensively studied, because CDs are nontoxic, water-soluble, and commercially available at low cost, and more importantly, can associate various molecules with high shape/size selectivity both in solid state and aqueous solution.[3] Therefore, CDs have been widely used as convenient building blocks for constructing bioactive and nanoscale functional materials.[4] However, the construction of multi-functional integrated diagnosis systems with multifarious therapeutic approaches is still imperative, and this kind of systems is anticipated to be applied in vitro and vivo.[5] Mao et al recently reported a three-in-one nanoplatform for two-photon imaging, dual-drug delivery and chemo-photodynamic synergistic therapy using native CD monomers and adamantane-functionalized ruthenium complexes.[6] We reported a magnetism, photo dual-controlled supramolecular assembly with biocompatible iron oxide magnetic nanoparticles, targeting peptide and polysaccharide-modified CD for suppression of tumor invasion and metastasis.[7] In addition, we also constructed a photo-controlled assembly that could induce a pronounced cell morphological change and cell death by reversibly control its aggregation behavior.[8] Li et al designed and fabricated a kind of non-covalently connected copolymers using Fc-CPT and mPEG-β-CD segments which showed a significantlyhyper-fast CPT release at tumor cells than normal cells, exhibiting equal cellular proliferation inhibition toward A549 tumor cells.[9]

On the other hand, tumor cell targeting is one of the perfect strategies to enable the insignificant toxicity to normal tissues and high therapeutic efficacy toward malignant tumors.[10] Targeted therapy can significantly increase the drug concentration in the lesion, thereby reducing the dose administered and ultimately reducing the systemic side effects of the drug on the patient. Due to its biocompatibility, easy to modification and biodegradability, transferrin (TRF) was used as a carrier and targeting reagent in anticancer systems, because the concentration of TRF receptors on the surface of tumor cells is 2-7 times as high as that on the surface of normal cells.[11] In addition, the affinity of TRF receptor in tumor cells to TRF is 10-100 times higher than that of TRF receptor in normal cells.[12] Therefore, TRF could strongly bind to the TRF receptor on the surface of tumor cell and then enter the tumor cell by endocytosis.

Photodynamic therapy (PDT), which utilizes photosensitizer (PS) to generate light-activated chemical[13] such as singlet oxygen to kill cells, has attracted tremendous attention, owing to its high selectivity and noninvasive feature under temporal and spatial control,[14] and ruthenium-based compounds have been widely investigated as anticancer candidates due to theirs unique physical, mechanical, and optical properties.[15] As alternatives to platinum-based prodrugs, lots of ruthenium complexes were constructed and exhibited significant anticancer activity in vitro and in vivo. However, there are also some drawbacks such as complicated synthetic routes, multi-stepped work-up, insufficient targeting capability, cytotoxicity of the drug carriers and undefined

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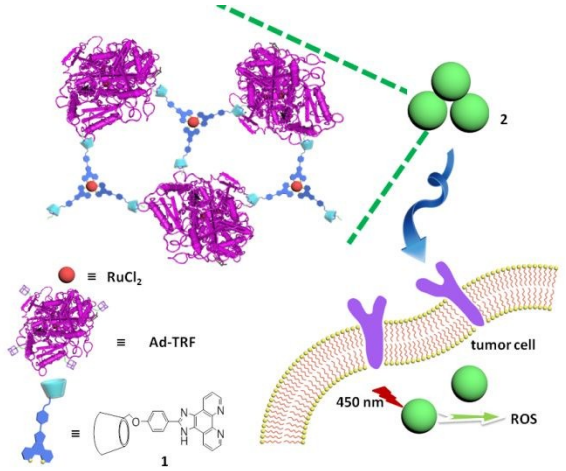
mechanism of drug release that would not facilitate their further application in anticancer therapy.[16]

By incorporating the advantage of biocompatibility, tumor-targeting property and PDT ability, we herein constructed a multifunctional supramolecular assembly by simply mixing the β-CD-functionalized Ru(II) complex and adamantane-functionalized TRF in water at room temperature (Scheme 1) and investigated its targeted PDT behaviors towards tumor cells. There are several potential advantages for this supramolecular system: (1) the existence of numerous CD groups not only enables the good water solubility but also provides the non-covalent linking of targeting sites and PDT sites through the strong binding of adamantane and β-CD cavity[17]. (2) The use of β-CD and TRF as main building blocks enables the good biocompatibility and biodegradability. (3) The existence of TRF enables the good tumor-targeting property via the selective reorganization of TRF-receptor on the surface of tumor cells, which facilitated its PDT capability by damaging lysosomes. (4) The existence of numerous Ru(II) complex centers enable the good PDT ability towards tumor cells.



Scheme 1. Illustration of phenanthroline-modified β-CD derivative (**1**), adamantane-functionalized transferrin (Ad-TRF), Ad-TRF/Ru-HOP-CD supramolecular assembly (**2**), and its tumor-targeting PDT property.

Phenanthroline-modified β-CD derivative **1** was synthesized according to our reported procedure with a slight modification.[18] Then, Ru-HOP-CD was synthesized by refluxing $RuCl_3$ with 3 equiv. of **1** in EtOH/H_2O for 12 h under nitrogen atmosphere (Scheme S1). The obtained product was further purified by Sephadex G-25 column chromatography in 40% yield. Owing to the good water solubility, the photo physical properties of **1** and Ru-HOP-CD could be investigated by means of UV-vis and fluorescence spectroscopy in aqueous solution. The UV-vis spectra of **1** and Ru-HOP-CD in water both showed an intense absorption at 220 – 400 nm assigned to the spin-allowed intra-ligand transition. In addition, the UV-vis spectra of Ru-HOP-CD also displayed an absorption maximum peak at 476 nm assigned to the metal-to-ligand charge-transfer band.[19] When excited 450 nm, Ru-HOP-CD displayed the intense red fluorescence.

Owing to the strong binding of β-CD cavity with adamantane group,[17] the supramolecular assembly **2** was constructed conveniently by mixing Ru-HOP-CD and adamantane-modified TRF with a 1:1 (m:m) stiochiometry in water at room temperature. As shown in Figure S7 and Figure S8, with the increase of the concentration of Ad-TRF, both of the UV/Vis spectra showed a slight bathochromic shift and increase of signals (see Supporting Information for details). Furthermore, the binding constants of Ad-TRF with Ru-HOP-CD and HOP-CD were calculated as (2.8 ± 0.4) × 10^5 M^{-1} and (1.7 ± 0.2) × 10^5 M^{-1} by analyzing the nonlinear least-squares fit of the titration data via UV-vis spectral titrations (Figures S7-S8). High-resolution transmission electronic microscopy (HR-TEM), zeta potential and dynamic light scattering (DLS) experiments were applied to investigate the size and morphological characteristics of Ru-HOP-CD and supramolecular assembly **2**. More importantly, the UV/vis spectra of Ad-TRF/Ru-HOP-CD assembly nearly unchanged in serum within 100 h which indicated the good stability of the nanoparticles (Figure S9).

As shown in Figures 1, TEM images of Ru-HOP-CD showed a number of discrete nanoparticles with an average diameter of 112 nm. In the TEM image of supramolecular assembly **2**, these discrete nanoparticles tended to aggregate together to a large microstructure with a dimension of several micrometers. Moreover, DLS experiments gave an average hydrodynamic diameter (Dh) of Ru-HOP-CD and the assembly as ca. 124 nm and 130 nm with a narrow distribution (Figures S5), which was similar to the diameter measured by TEM. In addition, the zeta potential of Ru-HOP-CD was measured as 43.4 mV (Figures S6), suggesting that the positively charged surface of Ru-HOP-CD may facilitate its entrance into cells.

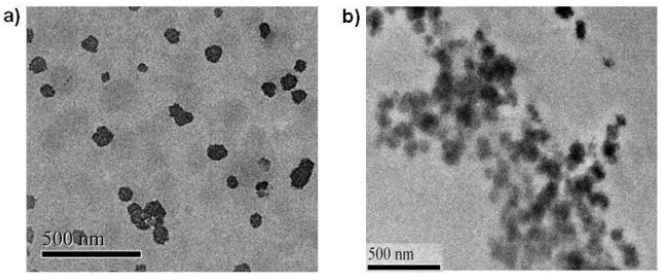


Figure 1. TEM images of (a) Ru-HOP-CD and (b) Ad-TRF/Ru-HOP-CD supramolecular assembly.

It was well documented that the Ru complexes could generate reactive oxygen species (ROS) under visible light irradiation and display high affinity for DNA ($K_b > 10^6$). [20] Therefore, the agarose-gel electrophoresis was performed to investigate the photo-induced DNA cleavage ability of the Ru-HOP-CD with pBR322 DNA as a model substrate. When irradiated at 450 nm, Ru-HOP-CD showed an obvious DNA cleavage ability (Figure 2), which enhanced with the increase of Ru-HOP-CD concentration. For example, supercoiled DNA could be completely cleaved to the linear DNA in the presence of 0.1 mg/mL Ru-HOP-CD. In contrast, Ru-HOP-CD showed no DNA cleavage in the dark environment even the concentration of Ru-HOP-CD reached 0.5 mg/mL, suggesting the generated ROS played an important role in the DNA photocleavage.

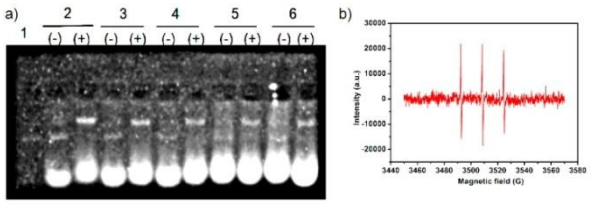


Figure 2. (a). Photocleavage of pBR322 DNA (5 μg/mL). Lane 1: the blank control; Lanes 2–6 ([Ru-HOP-CD] = 0.1, 0.2, 0.3, 0.4, 0.5 mg/mL), upon irradiation at 450 nm for 1 h; in the absence (-) and presence (+) of visible light. (b). TEMP trapping EPR spectra of Ru-HOP-CD upon irradiation at 450 nm for 1 h ([Ru-HOP-CD] = 0.2 mM).

Electron paramagnetic resonance (EPR) spin-trapping experiments were also carried out to further confirm the production of ROS in Ru-HOP-CD. The radical species spectrum in the presence of triacetonamine (TEMP) under irradiation at 450 nm (Figure 2b) showed a typical EPR spectrum for the TEMPO-$^1O_2$ adducts with hyperfine coupling parameters α_N = 16.0 G [21] and a three-line signal of 1:1:1, which confirmed the production of ROS in solution. In addition, the generation of ROS was also measured in vitro by means of fluorescence spectrum (Figure. S10). Both Ru-HOP-CD and Ad-TRF/Ru-HOP-CD nanoparticles were found able to generate ROS after irradiation. The highest fluorescence intensity belonged to Ad-TRF/Ru-HOP-CD, indicating that the Ad-TRF/Ru-HOP-CD assembly was an efficient ROS generator.

The tumor cell targeting efficiency of the supramolecular assembly was investigated by fluorescence confocal microscopy. Human lung adenocarcinoma cells (A549 cells, obtained from the cell Center of Peking Union Medical College) were used as the TRF receptor positive group, and 293T cells (human normal embryonic kidney cells, obtained from the cell Center of Peking Union Medical College) were used as the TRF receptor negative group. Herein, A549 cells and 293T cells were treated with Ad-TRF/Ru-HOP-CD supramolecular assembly (80 mg/L) for 4 h. As shown in Figure 3a, only A549 cells displayed a bright red fluorescence of Ru-HOP-CD in the cytoplasm. In contrast, 293T cells exhibited no fluorescence due to the less TRF receptors on the cellular surface. The targeting efficiency of Ad-TRF/Ru-HOP-CD was further confirmed by the fluorescence intensity of cells. Under the control condition (assembly free), both the tumor cells and normal cells had extremely low fluorescence density. However, after the treatment with the assembly, the tumor cells exhibited much higher fluorescence (about 18-fold higher) density than the normal cells (Figure 3b). These results indicated that the supramolecular assembly can specifically internalized by tumor cells through the TRF-receptor mediated cellular endocytosis, which might have potential application in the cancer diagnosis.

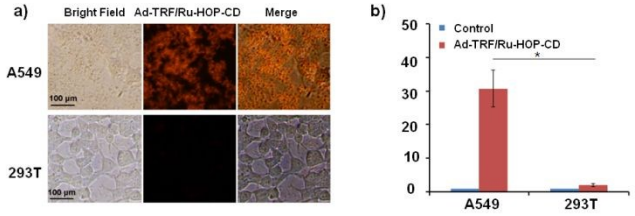


Figure 3. (a). Confocal fluorescence images of A549 cells, 293T cells treated with Ad-TRF/Ru-HOP-CD ([Ad-TRF] = [Ru-HOP-CD] = 80 mg/L) (λex = 450 nm, λem = 600 nm). (b). Fluorescence intensity of A549 cells (control), 293T cells (control), A549/Ad-TRF/Ru-HOP-CD and 293T/Ad-TRF/Ru-HOP-CD (λex = 450 nm, λem = 600 nm).

In order to evaluate the antitumor activity of Ad-TRF/Ru-HOP-CD in vitro, the cytotoxicity experiments were performed in A549 cells and 293T cells by MTT assays. Figure 4a demonstrated the viability results of both cells under the irradiation or not. In dark, Ad-TRF/Ru-HOP-CD even at high concentration of 160 mg/L had no obvious toxicity to both A549 cells and 293T cells after incubation for 48 h, indicating the good biocompatibility of the supramolecular assembly. However, after 450 nm irradiation for 10 min, the supramolecular assembly displayed remarkable antitumor activity towards A549 cells, and the viability of tumor cells decreased about 50% or 70% at a concentration of supramolecular assembly as 80 mg/L or 160 mg/L, respectively. This indicated that the supramolecular assembly was as effective as some clinical anticancer drugs such as photofrin[22] and cisplatin.[23] To our delight, Ad-TRF/Ru-HOP-CD supramolecular assembly exhibited a relatively low cytotoxicity toward 293T cells when irradiated at 450 nm. We may conclude that the hydrophilic TRF moiety of Ad-TRF/Ru-HOP-CD specifically recognizes A549 cells by strongly binding to TRF receptors on the cell surface, and then it enters the tumor cells through receptor-mediated endocytosis. Subsequently, the supramolecular assembly generates ROS when irradiated by visible light. Therefore, Ad-TRF/Ru-HOP-CD can be used as the tracer reagent for tumor cells and tissues, as well as a therapeutic reagent to kill tumor cells with high efficiency and hypotoxic to normal cells. In addition, flow cytometry revealed that the Ad-TRF/Ru-HOP-CD assemblies bound much more strongly to TRF-tagged tumor cells (98.2 %) than Ru-HOP-CD (67.4 %)(Figure S11), indicating that the TRF-containing assemblies actively targeted the tumor cells. Finally, the assemblies showed the higher cytotoxicity to tumor cells.

Reduced glutathione (GSH), as a universal scavenger, was used to evaluate the role of ROS produced by Ad-TRF/Ru-HOP-CD in the PDT activity. As shown in Figure 4b, under a dark condition, Ad-TRF/Ru-HOP-CD and GSH had no impact on the viability of tumor cells. It was noteworthy that GSH could remarkably recover the cell viability under the treatment of Ad-TRF/Ru-HOP-CD with irradiation. This can be explained by the ROS scavenging effect of GSH resulting in detoxification of the assembly.

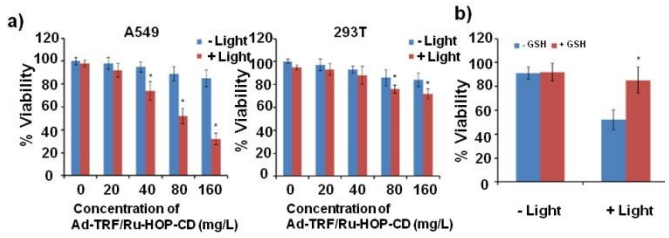


Figure 4. Cellular viability of (a). A549 tumor cells and 293T normal cells after the treatment with the supramolecular assembly at various concentrations after irradiation (450 nm, 10 min) and in the dark. (b). A549 cells after treatment with (red) or without (blue) GSH in the dark or light condition. ([Ad-TRF/Ru-HOP-CD] = 80 mg/L, n_{GSH} = 1 mM).

In conclusion, we synthesized β-CD-modified Ru(II) complex (Ru-HOP-CD) and adamantane-modified TRF (Ad-TRF) through a coordination reaction and an esterification reaction respectively. Under the visible light, Ru-HOP-CD showed a promising DNA photocleavage ability. Benefiting from the ultra-strong host-guest interaction between β-CD and adamantane, the Ad-TRF/Ru-HOP-CD supramolecular assembly was conveniently constructed and exhibited the excellent PDT selectivity towards A549 tumor cells and low toxicity towards normal cells. Under 450 nm irradiation, Ad-TRF/Ru-HOP-CD produces sufficient ROS to damage lysosomes and thus shows a high cytotoxicity to tumor cells in vitro. The present methodology provides a convenient and efficient way to construct tumor-oriented supramolecular assembly used in PDT.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1. M. E. Caldorera-Moore, W. B. Liechty and N. A. Peppas, *Acc. Chem. Res.* 2011, **44**, 1061-1070.
2. G. Yu, W. Yu, Z. Mao, C. Gao and F. Huang, *Small*, 2015, **11**, 919-925.
3. G. Yu, K. Jie and F. Huang, *Chem. Rev.*, 2015, **115**, 7240-7303.
4. (a) Y. Chen and Y. Liu, *Chem. Soc. Rev.*, 2010, **39**, 495-505; (b) N. Cheng, Y. Chen, J. Yu, J. J. Li and Y. Liu, *Bioconjug Chem.*, 2018, **29**, 1829-1833.
5. (a) W.-H. Chen, Q. Lei, G.-F. Luo, H.-Z. Jia, S. Hong, Y.-X. Liu, Y.-J. Cheng and X.-Z. Zhang, *ACS Appl. Mater. Interfaces*, 2015, **7**, 17171-17180; (b) J.-N. Liu, W. Bu and J. Shi, *Chem. Rev.*, 2017, **117**, 6160-6224; (c) X. Xu, Z. Huang, Z. Huang, X. Zhang, S. He, X. Sun, Y. Shen, M. Yan and C. Zhao, *ACS Appl. Mater. Interfaces*, 2017, **9**, 20361-20375; (d) Q.-L. Li, Y. Sun, L. Ren, X. Wang, C. Wang, L. Li, Y.-W. Yang, X. Yu and J. Yu, *ACS Appl. Mater. Interfaces*, 2018, **10**, 29314-29324.
6. G. G. Yang, L. Hao, Q. Cao, H. Zhang, J. Yang, L. N. Ji and Z. W. Mao, *ACS Appl. Mater. Interfaces*, 2018, **10**, 28301-28313.
7. Q. Yu, Y.-M. Zhang, Y.-H. Liu, X. Xu and Y. Liu, *Science Advances*, 2018. DOI: 10.1126/sciadv.aat2297.
8. Y.-M. Zhang, N.-Y. Zhang, K. Xiao, Q. Yu and Y. Liu, *Angew. Chem. Int. Ed.*, 2018, **57**, 8649-8653.
9. Y. Kang, X. Ju, L.-S. Ding, S. Zhang and B.-J. Li, *ACS Appl. Mater. Interfaces*, 2017, **9**, 4475-4484.
10. (a) D. Peer, E. J. Park, Y. Morishita, C. V. Carman and M. Shimaoka, *Science*, 2008, **319**, 627-630; (b) G. Liu, K. Y. Choi, A. Bhirde, M. Swierczewska, J. Yin, S. W. Lee, J. H. Park, J. I. Hong, J. Xie, G. Niu, D. O. Kiesewetter, S. Lee and X. Chen, *Angew. Chem. Int. Ed.*, 2012, **51**, 445-449; (c) Y. Yang, Y. M. Zhang, Y. Chen, J. T. Chen and Y. Liu, *J. Med. Chem.*, 2013, **56**, 9725-9736.
11. Y. Niitsu, Y. Kohgo, T. Nishisato, Y. Urushizaki, H. Kondo and I. Urushizaki, in *Protides of the Biological Fluids*, ed. H. Peeters, Elsevier, 1985, vol. 32, pp. 583-586.
12. J. H. Brock, in *Metalloproteins: Part 2: Metal Proteins with Non-redox Roles*, ed. P. M. Harrison, Palgrave Macmillan UK, London, 1985, DOI: 10.1007/978-1-349-06375-8_5, pp. 183-262.
13. (a) D. E. J. G. J. Dolmans, D. Fukumura and R. K. Jain, *Nature Reviews Cancer*, 2003, **3**, 380; (b) S. Chakrabortty, B. K. Agrawalla, A. Stumper, N. M. Vegi, S. Fischer, C. Reichardt, M. Kogler, B. Dietzek, M. Feuring-Buske, C. Buske, S. Rau and T. Weil, *J. Am. Chem. Soc.*, 2017, **139**, 2512-2519.
14. (a) Y. Yuan, C.-J. Zhang, R. T. K. Kwok, S. Xu, R. Zhang, J. Wu, B. Z. Tang and B. Liu, *Adv. Funct. Mate.*, 2015, **25**, 6586-6595; (b) L. Zeng, P. Gupta, Y. Chen, E. Wang, L. Ji, H. Chao and Z. S. Chen, *Chem. Soc. Rev.*, 2017, **46**, 5771-5804.
15. (a) G. Sava, S. Zorzet, C. Turrin, F. Vita, M. Soranzo, G. Zabucchi, M. Cocchietto, A. Bergamo, S. DiGiovine, G. Pezzoni, L. Sartor and S. Garbisa, *Cancer Res.*, 2003, **9**, 1898-1905; (b) C. G. Hartinger, M. A. Jakupec, S. Zorbas-Seifried, M. Groessl, A. Egger, W. Berger, H. Zorbas, P. J. Dyson and B. K. Keppler, *Chem. Biodiversity*, 2008, **5**, 2140-2155; (c) C. A. Puckett and J. K. Barton, *Biochemistry*, 2008, **47**, 11711-11716; (d) R. R. Ye, Z. F. Ke, C. P. Tan, L. He, L. N. Ji and Z. W. Mao, *Chemistry*, 2013, **19**, 10160-10169; (e) E. Baggaley, M. R. Gill, N. H. Green, D. Turton, I. V. Sazanovich, S. W. Botchway, C. Smythe, J. W. Haycock, J. A. Weinstein and J. A. Thomas, *Angew. Chem. Int. Ed.*, 2014, **53**, 3367-3371; (f) C. M. Clavel, E. Paunescu, P. Nowak-Sliwinska, A. W. Griffioen, R. Scopelliti and P. J. Dyson, *J. Med. Chem.*, 2015, **58**, 3356-3365.
16. (a) M. Kryjewski, T. Goslinski and J. Mielcarek, *Coord. Chem. Rev.,* 2015, **300**, 101–120; (b) B. Rosal, B. Jia and D. Jaque, *Adv. Funct. Mater.,* 2018, **28**, 1803733; (c) A. B. Mihoub, L. Larue, A. Moussaron, Z. Youssef, L. Colombeau, F. Baros, C. Frochot, R. Vanderesse and S. Acherar, *Molecules*, 2018, **23**, 1936.
17. D. Zhao, Y. Chen and Y. Liu, *Chem. Asian. J.*, 2014, **9**, 1895-1903.
18. Y. Liu, Y. Chen, Z.-Y. Duan, X.-Z. Feng, S. Hou, C. Wang and R. Wang, *ACS Nano*, 2007, **1**, 313-318.
19. R. B. Elmes, K. N. Orange, S. M. Cloonan, D. C. Williams and T. Gunnlaugsson, *J. Am. Chem. Soc.*, 2011, **133**, 15862-15865.
20. C. S. Burke, A. Byrne and T. E. Keyes, *Angew. Chem. Int. Ed.*, 2018, **57**, 12420-12424.
21. Y. Chen, W. Lei, G. Jiang, Y. Hou, C. Li, B. Zhang, Q. Zhou and X. Wang, *Dalton Trans.*, 2014, **43**, 15375-15384.
22. E. Delaey, F. van Laar, D. De Vos, A. Kamuhabwa, P. Jacobs and P. de Witte, *J. of Photochem. and Photobiol. B: Biol.*, 2000, **55**, 27-36.
23. E. L. Wong, G. S. Fang, C. M. Che and N. Zhu, Chem. Commun. 2005, 4578-4580.

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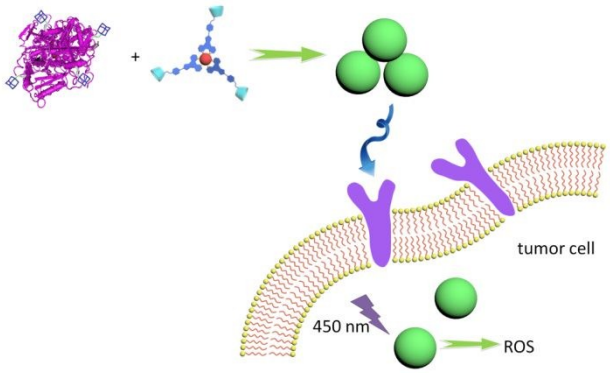

A supramolecular assembly was conveniently constructed and exhibited the excellent photodynamic therapy selectivity towards tumor cells and negligible toxicity towards normal cells.