ChemComm

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# A charge-switchable, four-armed polymeric photosensitizer for photodynamic cancer therapy†

Cite this: *Chem. Commun.*, 2014, 50, 4354

Received 28th January 2014,
Accepted 4th March 2014

DOI: 10.1039/c4cc00746h

www.rsc.org/chemcomm

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**A water-soluble, charge-switchable, four-armed polymeric photosensitizer (C4P-PS), in which charge switching is pH dependent, has been designed as a new class of photosensitizer for photodynamic cancer therapy.**

Photodynamic therapy (PDT) is a minimally invasive therapeutic strategy for a variety of diseases.[1] The general procedure of PDT involves the administration of a photosensitizer (PS) followed by selective light irradiation.[1,2] PSs are nontoxic to cells in the absence of laser irradiation. When irradiated with specific activating wavelengths, they generate cytotoxic reactive oxygen species, such as singlet oxygen ($^1O_2$), and free radicals that can kill cancer cells. However, the use of conventional PSs is clinically limited due to their poor water-solubility and low specificity for tumors.[3]

To overcome these limitations, various polymer functionalized PSs (*i.e.*, polymeric PS) have been developed.[3b,4] Recently, a PS functionalized by a stimuli-responsive polymer has been extensively explored.[5] In particular, pH-responsiveness has been the most frequently used stimulus for cancer-specific targeting. The extra-cellular pH of tumor tissues ($pH_e$) is more acidic (~pH 6.5) than the pH of the conventional blood-stream (~pH 7.4), which is caused by the up-regulation of glycolysis generating lactates and protons in the extra-cellular environment.[6] In this regard, various pH-responsive polymeric PSs have been developed for cancer-targeted PDT.[5c,7] However, the synthetic procedures for pH-responsive polymeric PSs involve multiple laborious steps, which are hard to reproduce, and entail high costs. Very recently, Wang and Lee groups reported a tumor-sensing ionic molecule containing 2,3-dimethylmaleic acid, which is a cleavable ionic molecule.[8] The cleavable linkages are relatively stable at neutral and alkaline pH values, but are hydrolyzed promptly under the $pH_e$ value, resulting in the exposure of charged groups. However, these types of acid-labile linkages are susceptible to chemical damage, which makes it difficult to store them for a long-term.[9]

In this study, we describe a charge-switchable, four-armed polymeric PS (C4P-PS) as a new class of photosensitizer for applications in cancer PDT. C4P-PS was synthesized using a facile two-step synthetic process: (1) ring opening polymerization (ROP) of α-amino acid *N*-carboxyanhydrides (NCAs) using *meso*-tetra(4-aminophenyl) porphyrin (TAPP) as an initiator and (2) introduction of pH-responsive ionizable groups (*e.g.*, imidazole groups) through an aminolysis reaction.[10] The newly synthesized C4P-PS was expected to be highly soluble and photoactivatable in water compared to the conventional PSs due to the presence of ionizable functional groups (Scheme 1a). In addition, the C4P-PS could change its net charge in response to $pH_e$; while the C4P-PS molecules are negatively charged under physiological conditions (~pH 7.4), they become positively charged in tumor tissues (~pH 6.5) *via* protonation of the imidazole groups. Since cell membranes are generally negatively charged,[8a,11] the positively charged C4P-PS at $pH_e$ will enhance their internalization to tumoral cells and therefore enhance their therapeutic effects (Scheme 1b). Moreover, we expect that molecules capable of pH-responsive charge-switching *via* the protonation of chemical pendant groups are more chemically stable than molecules containing acid-labile linkages.

To prove this concept, C4P-PS was synthesized *via* a simple chemical reaction as shown in Scheme S1 (see ESI†). First, poly(benzyl-L-aspartic acid) was polymerized from the four-terminal primary amine groups of TAPP to generate TAPP-pBLA. Then, the pH-responsive groups (*i.e.*, 1-(3-aminopropyl) imidazole, API) were introduced through aminolysis between the primary amine groups of API (nucleophile) and the benzyl ester groups of TAPP-pBLA. The chemical structure of C4P-PS was confirmed using $^1$H-NMR spectroscopy and GPC (Fig. S1, see ESI†). Table S1 (see ESI†) clearly demonstrates that the actual API content in the polymers is increased when the molar ratio of API used in the reaction is increased. In this study, compound **2** was selected

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† Electronic supplementary information (ESI) available: Materials, detailed synthesis procedures, and characterization of synthetic compounds, and experimental details. See DOI: 10.1039/c4cc00746h

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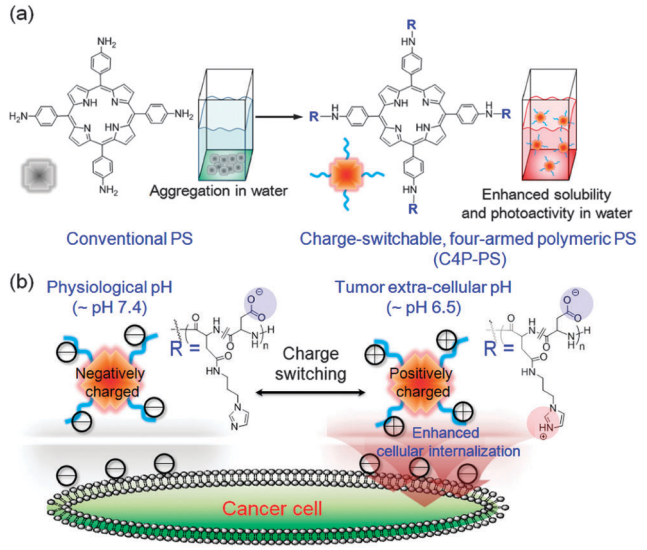


Scheme 1 (a) Schematic illustration of charge-switchable, four-armed polymeric PSs (C4P-PSs) and their enhanced water-solubility. (b) Schematic illustration of pH-dependent charge-switching and enhanced cellular internalization at the tumor extra-cellular pH ($pH_e$). Inset: protonation of the imidazole groups in C4P-PS at different pH.

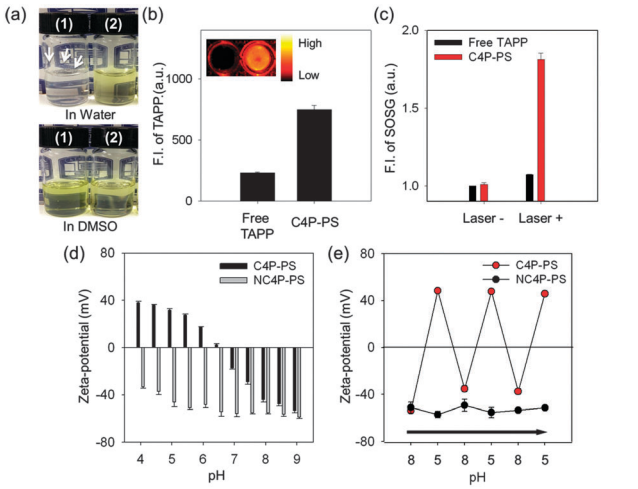


Fig. 1 (a) Photographs of (1) free TAPP and (2) C4P-PS dissolved in water or DMSO (the white arrow indicates aggregates of free TAPP), (b) fluorescence emission intensity of free TAPP and C4P-PS in water. Inset: fluorescence image from wells containing free TAPP and C4P-PS in water, (c) singlet oxygen generation (SOG) of free TAPP and C4P-PS in water with or without laser irradiation, (d) variation of the zeta-potential measurements of C4P-PS and NC4P-PS as a function of pH, and (e) reversible variation of the zeta potentials of NC4P-PS and C4P-PS at two representative pH (pH 8 and 5).

as a representative C4P-PS because of its strong charge-switchability between pH 7.4 and 6.5, which can be attributed to the quantitative balance of ionic groups (*e.g.*, carboxyl and imidazole groups) in the polymer (Table S1, see ESI†). A noncharge-switchable, four-armed polymeric photosensitizer (NC4P-PS) was also synthesized as a control.

Fig. 1a displays the solubility of free TAPP and C4P-PS in water and an organic solvent (dimethyl sulfoxide, DMSO), respectively. While free TAPP rapidly aggregated in water, the water-solubility of C4P-PS was significantly increased. However, both samples could be well dissolved in DMSO. To investigate the photoactivity of C4P-PS in water, its fluorescence emission and singlet-oxygen generation (SOG) properties were examined. Consistent with observations from the water-solubility experiments, the fluorescence emission ($\lambda_{ex}$ 650, $\lambda_{em}$ 675) of C4P-PS was much higher than that of free TAPP in water (Fig. 1b). However, both samples showed similar fluorescence emission in DMSO (Fig. S2, ESI†). The SOG of C4P-PS was also confirmed chemically using singlet oxygen sensor green (SOSG) as a probe upon laser irradiation (670 nm). C4P-PS exhibited significantly increased SOG when compared to free TAPP in water (Fig. 1c). These results indicate that the introduction of ionizable groups (*e.g.*, carboxyl and imidazole groups) effectively enhanced the solubility and photoactivity in water.

To confirm the charge-switchable behavior of C4P-PS, the zeta-potential was measured at various pH values and compared with those of a noncharge-switchable PS (NC4P-PS). The zeta-potential of C4P-PS changed from negative (~−30 mV) to positive (~+10 mV) as the pH of the solution decreased from pH 7.4 to 6.5 (Fig. 1d). The negative value was due to the unmodified carboxyl groups of the poly(L-aspartic acid) backbone at pH 7.4 and was offset by protonation of the imidazole groups at pH 6.5. In contrast, NC4P-PS did not exhibit an appreciable change in the zeta-potential at various pH values. These results indicate that C4P-PS had the ability to change its zeta-potential in response to environmental pH. As shown in Fig. 1e, the zeta-potential of C4P-PS switched between −40 and +50 mV at pH 8 and 5 in a highly reversible manner. Interestingly, this feature was retained after incubation for 4 weeks in PBS buffer (50 mM, pH 7.4, Fig. S3, see ESI†). Moreover, long-term stability of the chemical structure of C4P-PS was also confirmed using $^1$H-NMR (Table S1, see ESI†). These results suggest that C4P-PS has a reversible charge-switching ability and long-term stability, and thus has potential as a favourable pharmaceutical product because it can be delivered in a target-specific fashion and stored successfully for long periods of time.

To investigate the cellular internalization behavior of C4P-PS at pH 7.4 and 6.5, we used flow cytometry and confocal laser scanning microscopy (CLSM) with human colon cancer (HCT-116) cells. Enhanced cellular uptake (~3-fold increased) was detected at pH 6.5 when compared to the cellular uptake of C4P-PS at pH 7.4 (Fig. 2a). This observation was further confirmed by CLSM. At pH 6.5, the red fluorescence of C4P-PS was observed in HCT-116 cells and distributed extensively in the cytoplasm, whereas at pH 7.4, weak red fluorescence was observed in cells (Fig. 2b). These results can be attributed to the charge-switching of C4P-PS, which becomes positively charged at pH 6.5, which should strengthen the interaction of C4P-PS with cells and enhance its cellular internalization. In contrast, no significant difference was observed between pH 7.4 and 6.5 using flow cytometry and CLSM analysis when NC4P-PS was incubated with the cells (Fig. S4, see ESI†).

To verify the feasibility using C4P-PS for PDT, a quantitative *in vitro* cell cytotoxicity test was performed using the CCK-8 assay at pH 7.4 and 6.5 (Fig. 3a). As laser irradiation power increased ($\geq$6 J cm$^{-2}$), C4P-PS showed significantly enhanced cytotoxicity at pH 6.5 compared to that at pH 7.4 ($^*P < 0.005$, $^{**}P < 0.001$), while no difference in cytotoxicity was observed under dark conditions. The results of the

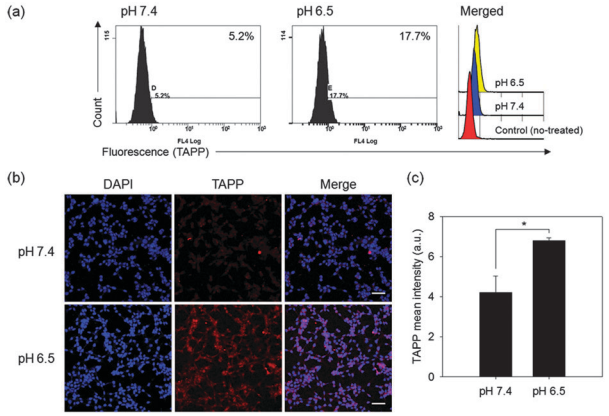


Fig. 2 (a) Flow cytometry quantification of cellular internalization of C4P-PS at pH 7.4 and 6.5. (b) CLSM image of HCT-116 cells treated with C4P-PS at pH 7.4 and 6.5 (scale bar = 50 μm). (c) The TAPP mean fluorescence value of HCT-116 cells treated with C4P-PS at pH 7.4 and 6.5 (*$P < 0.05$, $n = 3$).

live/dead assay agreed with the CCK-8 assay for C4P-PS (Fig. 3b). Significantly increased cell death (red fluorescence) was observed upon laser irradiation (24 J cm$^{-2}$) of cancer cells treated with C4P-PS at pH 6.5, in contrast to those cells after the same treatment but at an incubation pH of 7.4. Without laser irradiation, most cells remained alive after being treated with C4P-PS at different pH. Additionally, no significant cytotoxicity was observed in the NC4P-PS treated group (Fig. S5, see ESI†). These results indicate that the charge-switchability of the C4P-PS enhanced its cellular internalization, which led to remarkably enhanced efficiency in killing cancer cells.

Finally, to further demonstrate the effectiveness of C4P-PS on tumor growth inhibition, we performed an *in vivo* tumor suppression experiment. Under laser irradiation (670 nm laser source, 150 J cm$^{-2}$), mice treated with C4P-PS showed successful tumor growth inhibition compared to those of the control groups (*$P < 0.05$, **$P < 0.01$) with no significant change in body weight (Fig. 4 and Fig. S6, see ESI†).

In conclusion, we developed a charge-switchable, four-armed polymeric PS (C4P-PS) for efficient PDT treatment. C4P-PS was synthesized through a facile two-step approach consisting of ROP and aminolysis reactions. The synthesized C4P-PS has the properties of water-solubility, pH-responsive charge-switchability and long-term stability. In particular, C4P-PS is capable of reversing its charge from negative to positive at $pH_e$ to facilitate cellular internalization, which led to enhanced cytotoxicity in cancer cells. Therefore, we conclude that C4P-PS have considerable potential as a new class of PSs for photodynamic cancer therapy.

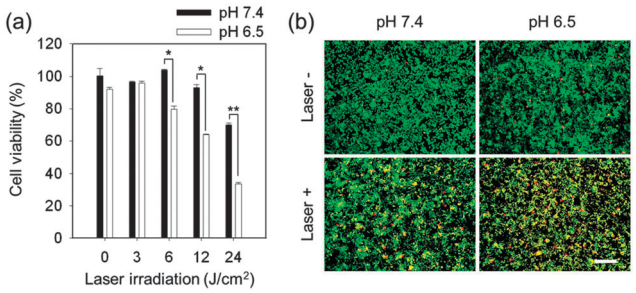


Fig. 3 (a) Cell viability of HCT-116 cells treated with C4P-PS with or without laser irradiation (3 to 24 J cm$^{-2}$) at pH 7.4 and 6.5 (*$P < 0.005$, **$P < 0.001$), and (b) live/dead assay of HCT-116 cells treated with C4P-PS with or without laser irradiation (24 J cm$^{-2}$) at pH 7.4 and 6.5 (scale bar = 200 μm).

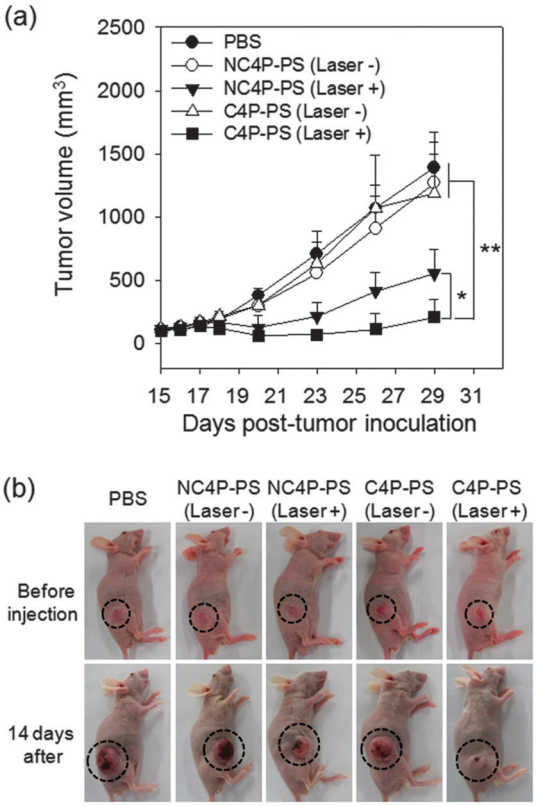


Fig. 4 *In vivo* tumor therapy of a subcutaneous tumor model injected with each sample. (a) Tumor growth inhibition after tail-vein injection of PBS, NC4P-PS, and C4P-PS (dose: 10 mg kg$^{-1}$ of TAPP, $n = 4$, *$P < 0.05$, **$P < 0.01$) with or without laser irradiation (670 nm laser source, 150 J cm$^{-2}$). Values are mean ± SD in (a). (b) Representative images of mice from each group. Black circles indicate tumor region.

We acknowledge the financial support of the Korean Ministry of Education, Science and Technology through Strategic Research (2011-0028726).

## Notes and references

1 D. E. Dolmans, D. Fukumura and R. K. Jain, *Nat. Rev. Cancer*, 2003, **3**, 380–387.
2 J. F. Lovell, T. W. Liu, J. Chen and G. Zheng, *Chem. Rev.*, 2010, **110**, 2839–2857.
3 (*a*) M. Hamblin, J. Miller, I. Rizvi, H. Loew and T. Hasan, *Br. J. Cancer*, 2003, **89**, 937–943; (*b*) W. Park, S.-j. Park and K. Na, *Biomaterials*, 2011, **32**, 8261–8270.
4 (*a*) S. J. Lee, H. Koo, H. Jeong, M. S. Huh, Y. Choi, S. Y. Jeong, Y. Byun, K. Choi, K. Kim and I. C. Kwon, *J. Controlled Release*, 2011, **152**, 21–29; (*b*) H. Park and K. Na, *Biomaterials*, 2013, **34**, 6992–7000.
5 (*a*) J. Chen, K. Stefflova, M. J. Niedre, B. C. Wilson, B. Chance, J. D. Glickson and G. Zheng, *J. Am. Chem. Soc.*, 2004, **126**, 11450–11451; (*b*) C.-S. Lee, W. Park, S.-j. Park and K. Na, *Biomaterials*, 2013, **34**, 9227–9236; (*c*) S. Y. Park, H. J. Baik, Y. T. Oh, K. T. Oh, Y. S. Youn and E. S. Lee, *Angew. Chem., Int. Ed.*, 2011, **50**, 1644–1647.
6 (*a*) K. Engin, D. Leeper, J. Cater, A. Thistlethwaite, L. Tupchong and J. McFarlane, *Int. J. Hyperthermia*, 1995, **11**, 211–216; (*b*) E. S. Lee, Z. Gao and Y. H. Bae, *J. Controlled Release*, 2008, **132**, 164–170.

 

7 (*a*) H. Koo, H. Lee, S. Lee, K. H. Min, M. S. Kim, D. S. Lee, Y. Choi, I. C. Kwon, K. Kim and S. Y. Jeong, *Chem. Commun.*, 2010, **46**, 5668–5670; (*b*) X. Zhu, W. Lu, Y. Zhang, A. Reed, B. Newton, Z. Fan, H. Yu, P. C. Ray and R. Gao, *Chem. Commun.*, 2011, **47**, 10311–10313.
8 (*a*) J. Z. Du, T. M. Sun, W. J. Song, J. Wu and J. Wang, *Angew. Chem., Int. Ed.*, 2010, **49**, 3621–3626; (*b*) N. M. Oh, D. S. Kwag, K. T. Oh, Y. S. Youn and E. S. Lee, *Biomaterials*, 2012, **33**, 1884–1893.
9 (*a*) P. Xu, E. A. Van Kirk, Y. Zhan, W. J. Murdoch, M. Radosz and Y. Shen, *Angew. Chem., Int. Ed.*, 2007, **46**, 4999–5002; (*b*) Z. Zhou, Y. Shen, J. Tang, M. Fan, E. A. Van Kirk, W. J. Murdoch and M. Radosz, *Adv. Funct. Mater.*, 2009, **19**, 3580–3589.
10 M. Nakanishi, J.-S. Park, W.-D. Jang, M. Oba and K. Kataoka, *React. Funct. Polym.*, 2007, **67**, 1361–1372.
11 E. C. Cho, J. Xie, P. A. Wurm and Y. Xia, *Nano Lett.*, 2009, **9**, 1080–1084.