ChemComm

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: X. Lv, Y. Zhu, H. Ghandehari, A. Yu and Y. Wang, *Chem. Commun.*, 2019, DOI: 10.1039/C9CC00358D.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/chemcomm

ChemComm

View Article Online
DOI: 10.1039/C9CC00358D

COMMUNICATION

ROS-responsive and self-accelerating drug release nanoplatform for overcoming multidrug resistance†

Xueming Lv,[a] Yiyong Zhu,[a] Hamidreza Ghandehari,[b] Ao Yu*[c] and Yongjian Wang*[a]

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

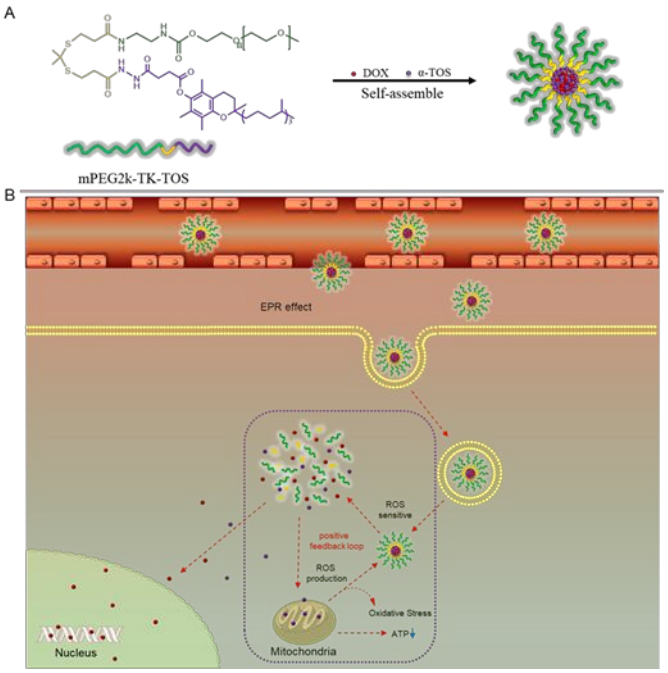
An 'on-demand' drug release and ROS-responsive nanoparticale was prepared by chemically conjugating hydrophobic α-tocopheryl succinate to hydrophilic poly(ethylene glycol) *via* thioketal linker. This nanoparticale encapsulated with doxorubicin and α-tocopheryl succinate exhibited remarkable efficiency in reversing multidrug resistance both *in vitro* and *in vivo*.

Cancer is still the leading cause of death all over the word.[1] Although emerging anticancer strategies such as radiotherapy, photothermal therapy and immunotherapy have been utilized in clinic to treat cancer, conventional chemotherapy is still an indispensable technique for cancer treatment. However, its therapeutic efficiency is severely restricted by the development of multidrug resistance (MDR) and the off-target toxicity of chemotherapeutics. The mechanisms of MDR are rather complicated and elusive, often include increased drug efflux, decreased drug influx, enhanced DNA repair, altered cell cycle regulation and blocked cell apoptosis.[2,3] Over the past decades, numerous studies have been carried out to improve the therapeutic efficacy of traditional anticancer drugs by circumventing MDR. Nanotechnology is the most exploited strategy to combat MDR due to its ability to bypass exposure of drugs to the membrane efflux transporters.[4,5] Although the nanosized drug delivery systems (DDSs) can specifically deliver chemotherapeutics to tumour through the enhanced permeability and retention (EPR) effect, premature drug release during circulation and insufficient drug release inside the tumour cells still hinders the application of DDSs in clinic. Therefore, DDSs developed for overcoming MDR should be not only responsive to unique tumour microenvironment to realize targeted drug delivery, but also have enhanced intracellular drug release profile.

To achieve tumour targeted delivery and site-specific drug release, stimuli responsive nanosized DDSs have been developed in response to the stimuli signals in tumour microenvironments, such as acidic pH, redox potential and reactive oxygen species (ROS).[6,7] ROS including superoxide anions (O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) are mostly generated in mitochondria due to the partial reduction of oxygen.[8,9] Excess production of ROS is related to a series of pathological disorders, including inflammatory diseases, cardiovascular diseases, diabetes and cancer.[10-12] Abnormal high intracellular ROS levels in cancer cells (up to 10×10^{-5} M) are a distinguishable property from those in normal cells (around 20×10^{-9} M),[12] which provide a unique target for cancer therapy and a new direction to devise intelligent DDSs. In contrast to the lysosomal acidic pH and intracellular high concentration of glutathione which exist both in normal and cancerous cells, the ROS level in cancer cells are higher than that in normal tissues. This endows ROS-responsive DDSs possessing better tumour selectivity. Although a variety of ROS-responsive units, such as arylboronic ester, selenium, thioether, and thioketal groups, have been integrated into DDSs for ROS-triggered drug release, the current ROS-responsive DDSs are far from optimal due to the fact that those developed ROS-sensitive DDSs cannot release their payloads sufficiently under the inherent intracellular ROS level.[13,14]

Several studies have been carried out to construct DDSs capable of amplifying ROS signals to facilitate responsive intracellular drug release. Incorporation of photo-sensitizers into DDS is one of the prevalent approaches to generate ROS.[15-17] However, external long period irradiation usually causes non-specific ROS elevation in both normal and cancer cells which could give rise to lethal damage to healthy tissues. In contrast to addition of external triggers, ROS-responsive and self-accelerating DDSs by co-delivery ROS-inducing agents with anticancer drugs have become a major focus and exhibit

[a] Key Laboratory of Bioactive Materials, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, China. E-mail: yongjian_wang@nankai.edu.cn
[b] Departments of Pharmaceutics and Pharmaceutical Chemistry, and of Bioengineering, Utah Center for Nanomedicine, Nano Institute of Utah, University of Utah, Salt Lake City, UT, USA
[c] College of Chemistry, Nankai University, Tianjin 300071, China. E-mail: esr@nankai.edu.cn
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x



Scheme 1 Schematic representation of the ROS-responsive and self-accelerating micelle.

remarkably improved therapeutic efficiency.[18,19] α-Tocopheryl succinate (α-TOS), a kind of vitamin E derivative, is a commonly used ROS-inducing agent. α-TOS acts as a competitive inhibitor of complex II in mitochondria respiratory chain, thus causes the accumulation of high concentrations of ROS.[20] Herein, we hypothesize that encapsulating α-TOS into ROS-responsive DDSs can self-induce the amplification of tumour intracellular ROS signal and achieve efficient drug release.

As a proof of concept, we developed a novel ROS-responsive DDS encapsulated with both α-TOS and DOX (defined as PTKTD) which can selectively self-induce tumour cell ROS amplification to facilitate drug release and overcome MDR. As shown in Scheme 1, a small amount of α-TOS could be released from PTKTD with the stimulation of tumour intrinsic ROS. The released α-TOS would interact with mitochondrial respiratory complex II to generate ROS, which in turn accelerates the collapse of PTKTD micelles and release more α-TOS. This positive feedback loop ensures an efficient and thorough release of α-TOS and DOX to induce cell apoptosis. This novel ROS-responsive and self-accelerating drug delivery system demonstrated strong cell-killing ability against MCF-7/ADR cells *in vitro* and conspicuous tumour growth inhibition capability *in vivo*, which makes it a promising approach for cancer therapy.

In this study, a ROS-responsive and self-accelerating drug release nanosystem (PTK) was synthesized by covalently conjugating α-TOS to mPEG2k through the ROS-cleavable thioketal (TK) linker. The synthetic routes of PTK were shown in scheme S1 (ESI†). The chemical structures of the intermediates and final product were characterized using ^{1}H NMR (Fig. 1 and S1, ESI†). The ^{1}H NMR spectrums of Fig. 1A and 1B showed the characteristic peak at 1.57 ppm which belongs to the -CH_3 protons of TK linker. The peaks at 3.64 ppm and 0.88 ppm

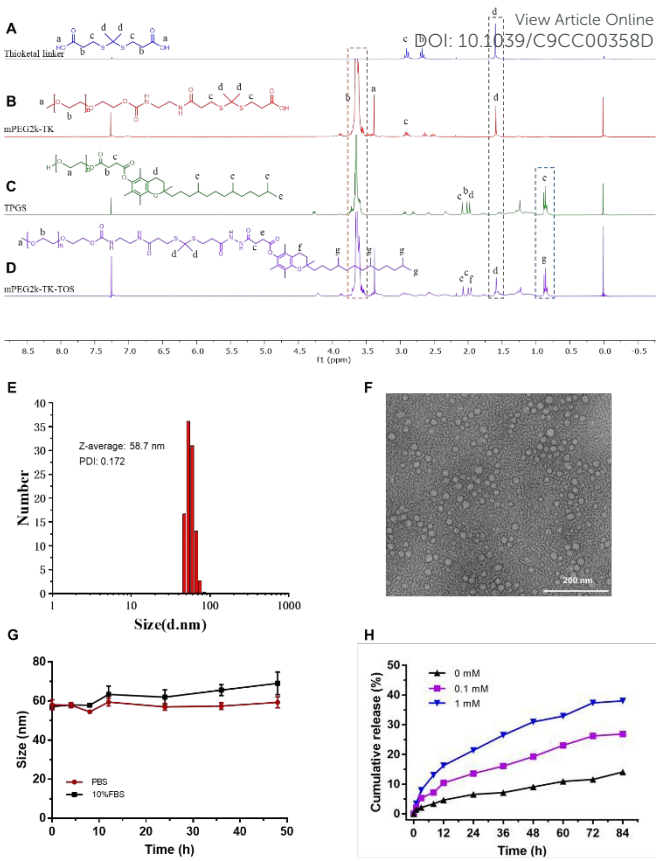


Fig. 1 ^{1}H NMR spectra of TK linker, mPEG2k-TK, TPGS and mPEG2k-TK-TOS (A-D). DLS analysis and TEM image of PTKTD micelles (E, F). Scale bar: 200 nm. Colloidal stability of PTKTD micelles in PBS and PBS containing 10% FBS (G). Cumulative release profile of DOX in the presence of different concentrations of H_2O_2 (H).

represented the characteristic peaks of PEG and α-TOS, respectively (Fig. 1C). The ^{1}H NMR spectrum of Fig. 1D confirmed the successful synthesis of PTK. The PTKTD micelles were prepared by thin-film hydration method. The mean diameter of the PTKTD micelles were 58.7 nm with a narrow polydispersity (0.172) characterized by dynamic light scattering (DLS) as shown in Fig. 1E. Meanwhile, the stability of PTKTD micelle was also assessed by DLS. No obvious variation in the particle size was observed for PTKTD micelles both in PBS and PBS containing 10% FBS for 48 h, suggesting excellent stability of PTKTD micelles (Fig 1G). Noncovalent intermolecular interactions[21-23] between DOX and α-TOS, like hydrogen bond and π-π stacking might contribute to the micellar stability (Fig S2, ESI†). The morphology of PTKTD micelles were confirmed by TEM (Fig. 1F) which showed a generally spherical morphology with an average diameter of 20-25 nm. The average diameter determined by TEM was smaller than that characterized by DLS due to the shrinkage of TEM samples under dry condition. The diameter and morphology of PTK and PTKT micelles were also confirmed by DLS and TEM (Fig. S3 and S4, ESI†). DOX loading content (DL%) of PTKTD micelles is 9.2% and the encapsulation efficiency (EE%) is 87.6%, which were investigated with UV-vis.

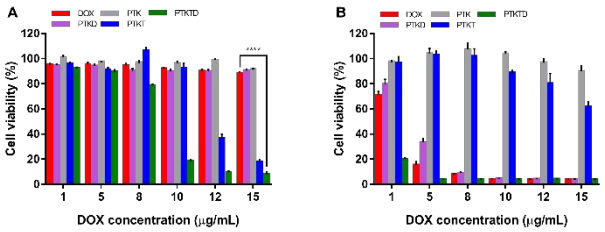


Fig. 2 Cell viability of MCF-7/ADR (A) and MCF-7 (B) after being treated with DOX, PTK, PTKD, PTKT and PTKTD for 48 h. The experiment was repeated three times and data was expressed as average ± SD, ****P < 0.0001.

To verify the ROS-responsive property of PTKTD micelles, the *in vitro* DOX release profile of PTKTD micelle was studied. As shown in Fig. 1H, less than 15% of DOX was released from the PTKTD micelles without adding extra H_2O_2 even after 84 h incubation. In contrast, ~27% and ~38% of DOX were released from the PTKTD micelles after incubation with 0.1 mM and 1 mM H_2O_2 respectively, indicating the ROS concentration dependent release profile of DOX and also suggesting the rationale for the 'self-accelerating release' concept of the PTKTD micelle. The ROS-responsiveness of PTK was further verified by ^{1}H NMR as shown in Fig. S5 (ESI†). Disappearance of the characteristic peak at 1.57 ppm confirmed that TK was efficiently cleaved by ROS.

The cytotoxicity of the free DOX and PTKTD micelles at equivalent doses was evaluated using DOX-resistant human breast adenocarcinoma cells (MCF-7/ADR) and human breast adenocarcinoma cells (MCF-7) by MTT cell proliferation assay (Fig. 2). PTK micelles or PTK micelles encapsulated with α-TOS (PTKT) or DOX (PTKD), respectively, were used as controls to illustrate the efficiency of PTKTD in reversing MDR. PTK micelles showed negligible growth inhibitory effect against both MCF-7/ADR and MCF-7 cells at the experimental concentrations. Nevertheless, PTKT micelles showed noticeable cytotoxicity against MCF-7/ADR and low toxicity against MCF-7 cells at relatively high concentrations. It is reported that ROS concentration in MCF-7/ADR cells was approximately 4 times that in MCF-7 cells.[24] Therefore, much more α-TOS would be released from PTKT micelles in MCF-7/ADR cells in contrast to that in MCF-7 cells, which in turn accelerate the disintegration of PTKT micelles to realize thorough release of α-TOS and oxidative stress amplification.[25] PTKD micelles did not show noticeable cytotoxicity against MCF-7/ADR cells (Fig. 2A), which may be caused by the insufficient drug release under the initial relatively low ROS concentration in MCF-7/ADR cells. As expected, the PTKTD micelles exhibited conspicuous cytotoxicity against MCF-7/ADR cells due to the self-accelerating drug release mode and the synergistic effect of DOX and α-TOS, indicating the potent capability of the PTKTD micelles to reverse MDR. As for MCF-7 cells (Fig. 2B), PTKTD micelles showed remarkable cytotoxicity. Unlike most reported work in literature that the cytotoxicity of drug loaded nanomedicine against sensitive tumour cells is usually lower than that of the free drug because of the delayed drug release,[26,27] PTKTD micelles exhibited significant higher

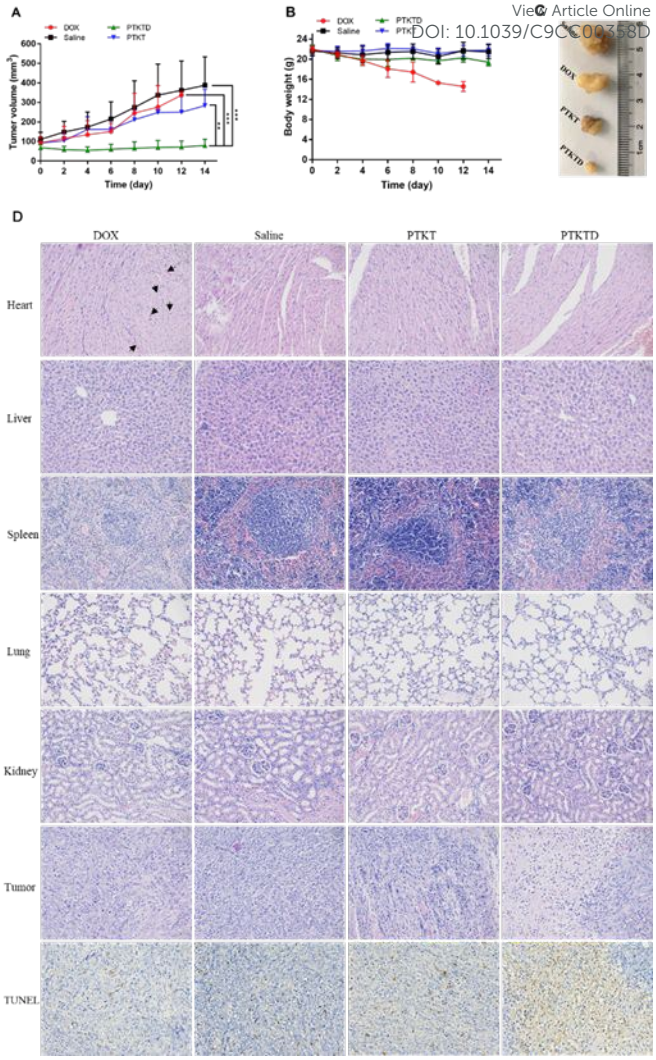


Fig. 3 Tumour inhibition study. Relative tumour volume (A) and body weight changes (B) of MCF-7/ADR tumour bearing mice after treatment with saline, DOX, PTKT and PTKTD within 14 days evaluation period, **P < 0.01, ***P < 0.001. Photograph of tumour tissues at the end of the treatment (C). H&E staining images of heart, liver, spleen, lung, kidney and tumour, and TUNEL staining images of tumour (200 × magnification) (D).

cytotoxicity against MCF-7 cells than free DOX which may benefit from the self-accelerating drug release profile, and the synergistic effect of DOX and α-TOS.[28]

To investigate ROS regenerating ability of different formulations, DCFH-DA was used to monitor the intracellular oxidative species.[29] As shown in Fig. S6 (ESI†), the ROS level was significantly elevated in cells treated with PTKT or PTKTD micelles, while no obvious ROS variations were observed for DOX, PTK and PTKD groups, suggesting excellent ROS inducing ability of PTKT and PTKTD micelles.

The *in vivo* antitumor activities of free DOX and its different formulations were evaluated in MCF-7/ADR tumour bearing BALB/c nude mice. The mice were randomly divided into four groups (n=6) and treated with saline, free DOX, PTKT and PTKTD micelles, respectively, at an identical DOX dosage of 5mg/kg. All formulations were intravenously injected into tail veins every

three days and tumour volume and body weight were measured every two days during the 14 days treatment period. The antitumor efficacy of different formulations was shown in Fig. 3. Saline did not show any effect on tumour growth and thus tumour volumes increased rapidly. Free DOX had comparable antitumor performance compared with saline, suggesting that free DOX cannot efficiently inhibit the growth of drug-resistant tumour. Consistent with the *in vitro* studies, the PTKT micelle exhibited moderate tumour inhibition activity, indicating that oxidative stress induced by PTKT micelles could slow down MDR tumour growth. Significantly, the PTKTD micelles showed preferable antitumor efficacy compared with other groups and the tumours almost stopped growing (Fig. 3A). This result indicated the synergistic antitumor effect endowed by the combination of ROS-responsiveness, oxidative stress and chemotherapeutics. During the treatment period, no obvious body weight changes were found in saline, PTKT and PTKTD micelle groups as shown in Fig 4B. In contrast, mice treated with free DOX exhibited notable body weight loss, suggesting severe side effects of free DOX at this test dosage. The major organs (heart, liver, spleen, lung and kidney) of the mice as well as the tumour were collected and sectioned for further immunohistochemical analysis. As shown in Fig. 3D, the heart section of free DOX group exhibited degradation, necrosis of myocardial cells and myocardial fibre breakage, suggesting the possible cardiotoxicity of free DOX. In contrast, no noticeable tissue damage was detected in PTKTD micelle group for this test dosage, reflecting the negligible systemic toxicity and better safety profiles of PTKTD micelles. It is worth noting that there was large percentage of cell shrinkage and nuclear condensation and fragmentation in tumour sections treated with the PTKTD micelles. However, free DOX group displayed negligible tumour necrosis area which was similar as the saline group. Consistently, TUNEL staining also reflected the remarkable apoptotic efficacy induced by PTKTD micelles. Overall, the *in vivo* experiments indicated that PTKTD micelles exerted anti-MDR efficacy.

In summary, a novel ROS-responsive and self-accelerating nanoplatform (PTKTD) was fabricated to co-deliver DOX and α-TOS for circumventing multidrug resistance and enhancing tumour chemotherapy. This PTKTD micelle system can specifically amplify tumour intracellular ROS concentrations and in turn promote micelle disintegration, thus realizing sufficient release of DOX and α-TOS. The *in vitro* and *in vivo* studies indicated that, with the treatment of PTKTD micelles, the DOX resistance of MCF-7/ADR cells was remarkably reversed through enhanced intracellular DOX retention and on-demand release of DOX and α-TOS under the stimuli of tumour intracellular ROS. These results demonstrated that our PTKTD micellar system may provide a promising approach for fighting tumour multidrug resistance in clinic.

The financial support from the National Natural Science Foundation of China (21577071), and the National Key R&D Program of China (No. 2017YFC1104400) is acknowledged.

Conflicts of interest

There are no conflicts to declare.

View Article Online
DOI: 10.1039/C9CC00358D

References

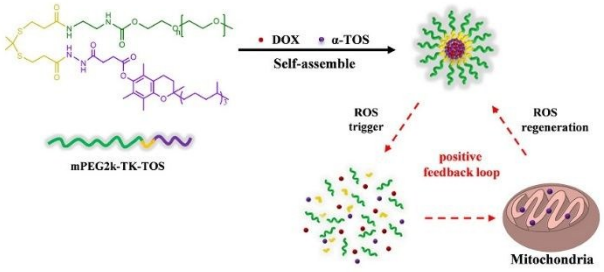
1 F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre and A. Jemal, *CA-Cancer J. Clin.*, 2018, **68**, 394-424..
2 G. Szakacs, J. K. Paterson, J. A. Ludwig, C. Booth-Genthe and M. M. Gottesman, *Nat. Rev. Drug Discov.*, 2006, **5**, 219-234.
3 W. Li, H. Zhang, Y. G. Assaraf, K. Zhao, X. Xue, J. Xie, D. H. Yang and Z. S. Chen, *Drug Resistance Updates*, 2016, **27**, 14-29.
4 A. R. Kirtane, S. M. Kalscheuer and J. Panyam, *Adv. Drug Deliver. Rev.*, 2013, **65**, 1731-1747.
5 M. Ye, Y. Han, J. Tang, Y. Piao, X. Liu, Z. Zhou, J. Gao, J. Rao and Y. Shen, *Adv. Mater.*, 2017, **29**, 1702342.
6 Y. Chen, M. Su, Y. Li, J. Gao, C. Zhang, Z. Cao, J. Zhou, J. Liu and Z. Jiang, *ACS Appl. Mater. Inter.*, 2017, **9**, 30519-30535.
7 R. Li and Y. Xie, *J. Control. Release*, 2017, **251**, 49-67.
8 G. Manda, G. Isvoranu, M. V. Comanescu, A. Manea, B. D. Butuner and K. S. Korkmaz, *Redox Biology*, 2015, **5**, 347-357.
9 S. H. Lee, M. K. Gupta, J. B. Bang, H. Bae and H. J. Sung, *Adv. Healthc. Mater.*, 2013, **2**, 908-915.
10 C. C. Benz and C. Yau, *Nat. Rev. Cancer*, 2008, **8**, 875-879.
11 H. Tsutsui, S. Kinugawa and S. Matsushima, *Cardiovasc. Res.*, 2009, **81**, 449-456.
12 X. Xu, P. E. Saw, W. Tao, Y. Li, X. Ji, S. Bhasin, Y. Liu, D. Ayyash, J. Rasmussen, M. Huo, J. Shi and O. C. Farokhzad, *Adv. Mater.*, 2017, **29**, 1700141.
13 J. J. Hu, Q. Lei, M. Y. Peng, D. W. Zheng, Y. X. Chen and X. Z. Zhang, *Biomaterials*, 2017, **128**, 136-146.
14 K. Kim, C. S. Lee and K. Na, *Chem. Commun. (Camb)*, 2016, **52**, 2839-2842.
15 C. Yue, C. Zhang, G. Alfranca, Y. Yang, X. Jiang, F. Pan, J. M. de la Fuente and D. Cui, *Theranostics*, 2016, **6**, 456-469.
16 Z. Li, M. Wu, H. Bai, X. Liu and G. Tang, *Chem. Commun.*, 2018, **54**, 13127-13130.
17 J. Li, C. Sun, W. Tao, Z. Cao, H. Qian, X. Yang and J. Wang, *Biomaterials*, 2018, **170**, 147-155.
18 Z. Su, M. Chen, Y. Xiao, M. Sun, L. Zong, S. Asghar, M. Dong, H. Li, Q. Ping and C. Zhang, *J. Control. Release*, 2014, **196**, 370-383.
19 G. G. Yang, H. Zhang, D. Y. Zhang, Q. Cao, J. Yang, L. N. Ji and Z. W. Mao, *Biomaterials*, 2018, **185**, 73-85.
20 L. F. Dong, V. J. Jameson, D. Tilly, J. Cerny, E. Mahdavian, A. Marin-Hernandez, L. Hernandez-Esquivel, S. Rodriguez-Enriquez, J. Stursa, P. K. Witting, B. Stantic, J. Rohlena, J. Truksa, K. Kluckova, J. C. Dyason, M. Ledvina, B. A. Salvatore, R. Moreno-Sanchez, M. J. Coster, S. J. Ralph, R. A. Smith and J. Neuzil, *J. Biol. Chem.*, 2011, **286**, 3717-3728.
21 S. Li, Q. Zou, Y. Li, C. Yuan, R. Xing and X. Yan, *J Am Chem Soc*, 2018, **140**, 10794-10802.
22 L Y. Li, Q. Zou, C. Yuan, S. Li, R. Xing and X. Yan, *Angewandte Chemie-International Edition*, 2018, **57**, 17084-17088.
23 J. Wang, K. Liu, R. Xing and X. Yan, *Chemical Society Reviews*, 2016, **45**, 5589-5604.
24 C. Ge, B. Cao, D. Feng, F. Zhou, J. Zhang, N. Yang, S. Feng, G. Wang and J. Aa, Sci. Rep., 2017, 7, 3791
25 D. Liang, A. Wang, Z. Yang, Y. Liu, and X. Qi, Mol. Pharm., 2015, 12, 2189−2202
26 L. Li, Q. Yang, Z. Zhou, J. Zhong and Y. Huang, *Biomaterials*, 2014, **35**, 5171-5187.
27 H. Deng, X. Zhao, J. Liu, L. Deng, J. Zhang, J. Liu and A. Dong, *J. Mater. Chem. B*, 2015, **3**, 9397-9408.
28 X. Zhang, X. Peng, W. Yu, S. Hou, Y. Zhao, Z. Zhang, X. Huang and K. Wu, *Cancer Letters*, 2011, **307**, 174-181.
29 R. G. Tuguntaev, S. Chen, A. S. Eltahan, A. Mozhi, S. Jin, J. Zhang, C. Li, P. C. Wang and X. J. Liang, *ACS Appl Mater Interfaces*, 2017, **9**, 16900-16912.

View Article Online
DOI: 10.1039/C9CC00358D

ChemComm

COMMUNICATION

View Article Online
DOI: 10.1039/C9CC00358D



A novel ROS-responsive and self-accelerating nanoplatform was fabricated for circumventing multidrug resistance and enhancing tumour chemotherapy.