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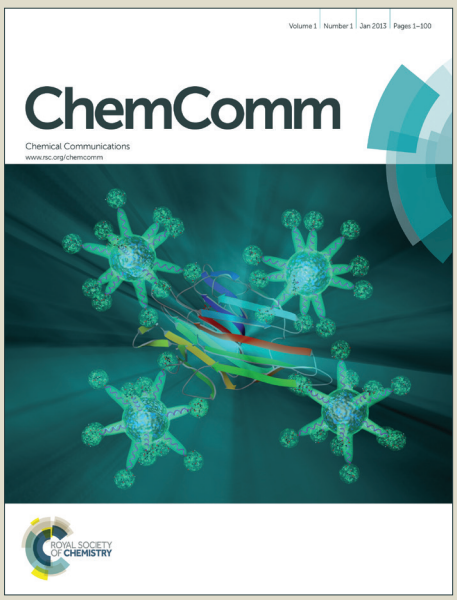

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ARTICLE TYPE

A H_2O_2-Responsive Nanocarrier for Dual-Release of Platinum Anticancer Drugs and O_2: Controlled Release and Enhanced Cytotoxicity against Cisplatin Resistant Cancer Cells

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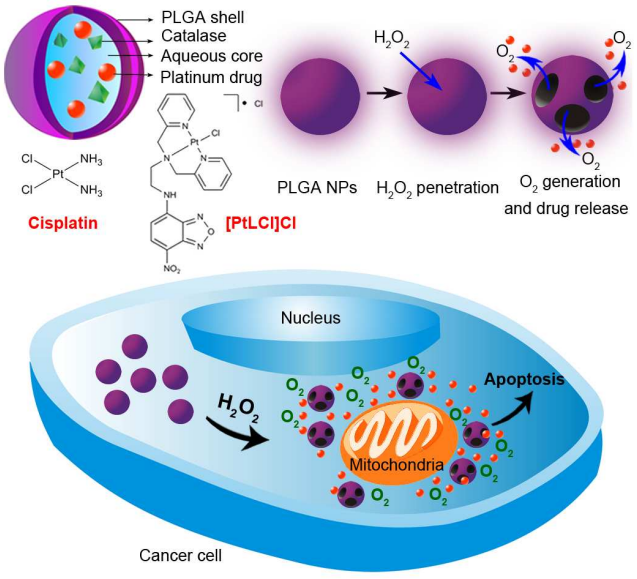
Synergistic release of platinum anticancer drugs and O_2 can be achieved in a H_2O_2-responsive nanocarrier incorporated with catalase. Such a system combines the advantages of chemotherapy and oxygen therapy and demonstrated improved therapeutic efficacy against cisplatin resistant cell lines which often appear as hypoxia condition.

Reactive oxygen species (ROS) play important roles in a variety of physiological and pathophysiological processes.[1] There are strong evidences showing that ROS can be important signalling molecules, however, their mis-management and accumulation potentially result in "oxidative stress" condition, in which the homeostasis of cellular oxidants was thrown off.[2] Many diseases associated with ROS damage have a strong oxidative stress component, especially cancer.[3] Accumulating evidences suggest that many types of cancer cells exhibit higher levels of ROS stress compared to their normal counterparts.[4] In particular, hydrogen peroxide (H_2O_2) as a major component of ROS and a common marker for oxidative stress plays a key role in carcinogenesis.[5] It is reported that large amounts of H_2O_2 could be produced in many different human carcinoma cell lines without any exogenous stimulation.[6] Increased generation of H_2O_2 has been linked to several key events in cancer cells, including DNA alterations, cell proliferation, apoptosis resistance, etc.[7] Thus, elevated cellular levels of H_2O_2 is an unique biochemical property for cancer cells.[8]

In addition to the high level of ROS, hypoxia is increasingly being recognized as another characteristic feature of cancer.[9] Hypoxia, which is caused by an inadequate oxygen supply, is a poor prognostic factor for patient outcome.[10] It poses a major therapeutic problem, as hypoxia-induced cellular changes can result in more clinically aggressive phenotypes, and the reduced partial O_2 pressure in tumor creates an obstacle for cancer therapy.[11] Especially, hypoxia can be a direct cause of multi-drug resistance (MDR) because some drugs require oxygen to be maximally cytotoxic.[12] MDR, the principal mechanism by which many cancers develop resistance to chemotherapeutic drugs, is a significant challenge in the clinical treatment of cancer.[13]

Since most tumor cells are under persistent endogenous ROS stress, H_2O_2 could be useful as a cancer-related stimulus for targeted drug release to diseased tissue. While many drug release systems using pH or reduction-mediated release have been developed,[14] there are limited examples of controlled-release systems specifically responsive to physiological level of H_2O_2 (50–100 μM).[15] Furthermore, as hypoxia is one of the major problem in chemotherapy, smart system which combines O_2 evolving and anticancer drug release would further improve the anticancer efficacy of the drugs. So far there has been no literature report that integrates a H_2O_2-responsive, O_2-evolving agent into the drug delivery system, although it is known that O_2 can be therapeutically useful to overcome hypoxia-induced drug resistance.[16]



Scheme 1. Schematic illustration of the PLGA NPs structure and the mechanism of intracellular drug release.

Herein, we report a nanocarrier system which combines catalase and platinum anticancer agents together and synergistically releases both drug molecules and O_2 when triggered by biologically relevant concentration of H_2O_2. In this system, poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) was chosen as drug carrier, and catalase was incorporated into the aqueous core of PLGA NPs as an O_2-generating agent together with platinum anticancer agents (Scheme 1). Catalyzed by catalase, O_2 is evolved when intracellular H_2O_2 penetrates into the NPs, which causes the shell rupture of NPs owing to the

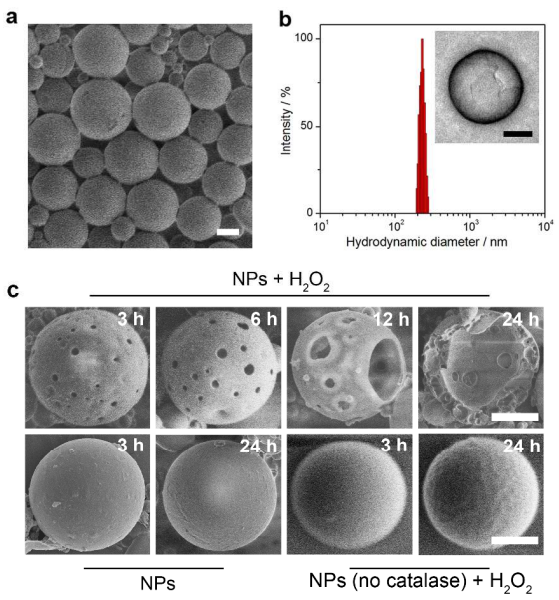


Fig. 1 (a) SEM micrographs of NPs. Scale bar: 100 nm. (b) Size distribution of the NPs characterized by DLS at 25 °C. Insets: TEM micrograph of PLGA NP. Scale bars: 100 nm. (c) The SEM micrographs of PLGA NPs incubated with and without 100 μM H_2O_2, or PLGA NPs (no catalase) incubated with 100 μM H_2O_2 during 24 h. Scale bars: 100 nm.

increase in internal pressure. As a result, the NPs could selectively unload the encapsulated drugs in virtue of the high levels of ROS stress in cancer cells, resulting in cellular apoptosis. Moreover, the O_2 produced in the drugs release process can be helpful for overcoming hypoxia-induced MDR and enhancing the efficiency of cancer chemotherapy. This is for the first time that chemotherapy and oxygen therapy are integrated in a synergetic way.

Scanning electron microscopy (SEM) indicated that the as-prepared PLGA NPs were spherical in shape with a smooth surface (Fig. 1a). The average size of PLGA NPs was approximately 230 nm as determined by dynamic light scattering (DLS) and the transmission electron microscopy (TEM) image of the NP clearly revealed its core-shell structure (Fig. 1b). Zeta potential of the NPs was –18 mV (Fig. S1), suggesting the stability in aqueous medium. The glass transition temperature (T_g) was measured to be 43 °C and the polymer can retain its payload at physiological temperature without suffering from significant leakiness (Fig. S2). No significant size change was observed for 7 days (Fig. S3), further demonstrating the long-term-stability of the nanocarrier in culture media. The detailed morphological changes of the PLGA NPs containing catalase incubated with and without 100 μM H_2O_2 were investigated by SEM. Exposure to 100 μM H_2O_2 for 3 h, small pores were clearly observed on the surface of the nanoparticle and the pore size was found to expand with the increase of incubation time. After 24-h incubation with H_2O_2, the PLGA shells of NPs were completely ruptured, as shown by the presence of fragments from the broken particles. In contrast, the NPs without H_2O_2 incubation were spherical and intact, and the NPs (no catalase) in the presence of H_2O_2 showed negligible degradation during 24 h (Fig. 1c and Fig. S4). Taking together, these results demonstrate that once the environmental H_2O_2 penetrates the PLGA shell and interacts with the catalase in

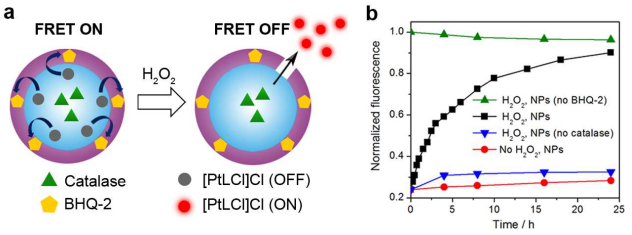


Fig. 2 (a) The mechanism in real-time monitoring of fluorescent [PtLCl]Cl release at 37 °C. (b) Plots of change in fluorescent intensity of [PtLCl]Cl (λ_{ex}= 470 nm, λ_{em}= 526 nm) vs incubation time measured from the NPs in the presence and absence of 100 μM H_2O_2, and the NPs without the BHQ-2 or catalase in the presence of 100 μM H_2O_2.

the aqueous core, O_2 bubbles are generated instantly to disrupt the NPs.

To visualize and localize the intracellular payload release in real time, a fluorescence resonance energy transfer (FRET)-based method has been employed. A fluorescent platinum(II) complex ([PtLCl]Cl)[17] was encapsulated in the aqueous core as the donor and Black Hole Quencher-2 (BHQ-2) was doped into the PLGA shells as the acceptor. The fluorescence of [PtLCl]Cl is quenched (OFF) when incorporated into the nanoparticle owing to the FRET mechanism, and will become activated (ON) in the presence of H_2O_2, leading to fluorescence increase along with the course of [PtLCl]Cl release (Fig. 2a). Spectroscopic evaluation of PLGA NPs in 100 μM H_2O_2 solution was performed by measuring the fluorescence of [PtLCl]Cl at different time points, showing that the fluorescence intensity enhanced gradually with the increasing incubation time (Fig. S5). Moreover, the fluorescent changes of [PtLCl]Cl from the NPs in the absence of H_2O_2, and the NPs without BHQ-2 or catalase in the presence of H_2O_2 were also measured. As shown in Fig. 2b, negligible increase in fluorescence was observed under these conditions, further validating that the increase in fluorescence was due to the H_2O_2-triggered release. Thus, this activatable fluorescent capability enabled us to monitor H_2O_2-triggered release from our nanocarriers.

The H_2O_2-triggered intracellular release behaviour of [PtLCl]Cl were investigated by confocal laser scanning microscopy. HeLa cells were stimulated by phorbol myristate acetate (PMA) to increase ROS level through an oxidative stress pathway.[18] For NPs containing catalase, green fluorescence indicative of [PtLCl]Cl release was observed in the cytoplasm after incubation for 4 h. As the incubation time prolonged to 16 h, the green fluorescence in the cells was much brighter, suggesting a greater release of [PtLCl]Cl (Fig. 3a). In contrast, in the case of PLGA NPs without catalase, the intracellular fluorescence still appeared very weak even incubation for 16 h (Fig. 3b). Imaging of cells incubated with 2 μM free [PtLCl]Cl (the same concentration as that PLGA NPs contain) was also performed, which suggest that the uptake efficiency is similar for the free [PtLCl]Cl and the NP-encapsulated [PtLCl]Cl. To further validate the [PtLCl]Cl release was specifically induced by intracellular H_2O_2, imaging of cells without PMA stimulation or pretreated with an ROS scavenger (*N*-acetylcysteine, NAC)[19] followed by incubation with NPs (containing catalase) was carried out, showing that the release of Pt (II) complex from the PLGA-NP was blocked when the ROS production was quenched (Fig. 3b). These results demonstrate the specific H_2O_2-mediated payload release induced by activating ROS production in cells, as further confirmed by three-dimensional visualization (ESI Video 1).

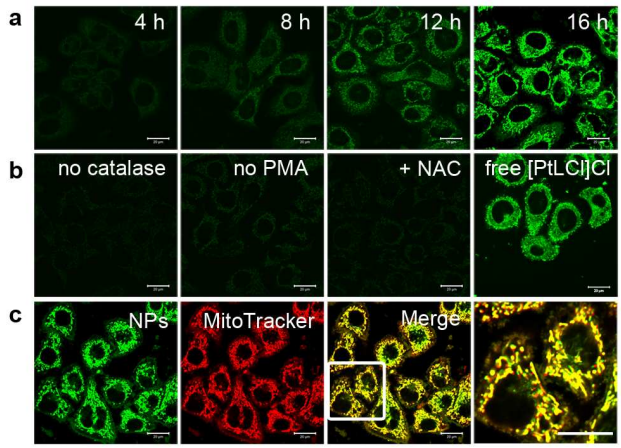


Fig. 3 Confocal fluorescence images of (a) the intracellular release of [PtLCl]Cl from NPs (contain 2 μM [PtLCl]Cl): PMA-stimulated HeLa cells incubated with NPs for 4 h, 8 h, 12 h and 16 h. (b) PMA-stimulated HeLa cells incubated with NPs containing no catalase for 16 h; HeLa cells (without PMA stimulation) incubated with NPs for 16 h; HeLa cells pre-treated with NAC, followed by incubation with NPs for 16 h; HeLa cells (without PMA stimulation) incubated with 2 μM free [PtLCl]Cl (the same concentration as that inside PLGA NPs) for 16 h. (c) PMA-stimulated HeLa cells incubated with the NPs for 16 h and then incubated with 50 nM Mito Tracker Red FM. Scale bars: 20 μm.

Many evidences have suggested that a major source of ROS is produced in the mitochondria.[20] In order to investigate the release sites of [PtLCl]Cl from PLGA NPs, intracellular colocalization with MitoTracker Red, LysoTracker Red and Hoechst 33342 was performed, showing that the released [PtLCl]Cl accumulates mainly in mitochondria and only a small portion of [PtLCl]Cl is retained in lysosome (Fig. 3c and Fig. S7).

As one of the most widely used anticancer drugs in the world, cisplatin is very effective in the treatment of different solid tumors.[21] However, its further clinical use is restricted by severe toxicity and drug resistance.[22] It was recently reported that catalase could decrease cisplatin-induced nephrotoxicity while improving the anticancer efficiency of cisplatin.[23] This motivated us to choose cisplatin as a model drug combining with catalase to

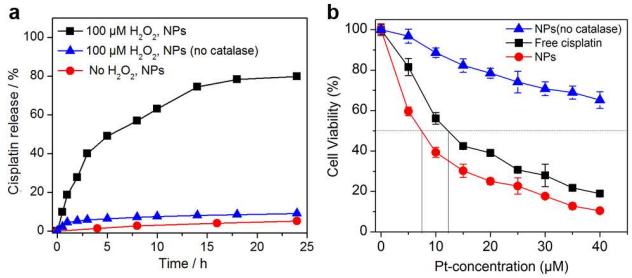


Fig. 4 (a) The in vitro release profiles of cisplatin from PLGA NPs (with or without catalase) incubated with and without 100 μM H_2O_2. (b) MTT assay of MCF-7 cells in the presence of different concentrations of free cisplatin, cisplatin inside NPs and NPs (no catalase).

construct a H_2O_2-responsive nanoparticle for targeted drug delivery and release. We first quantified cisplatin loading capacity of the NPs and release kinetics with and without H_2O_2 using inductively coupled plasma optical emission spectrometry (ICP-OES). As shown in Fig. 4a, negligible cisplatin release from the NPs was observed in the absence of H_2O_2 within 24 h, signifying the degradation process of PLGA is rather slow. In contrast, the release rate of cisplatin from NPs in the presence of H_2O_2 was much faster and a higher amount of cisplatin was released. The release reached a plateau after incubation with 100 μM H_2O_2 for about 15 h, about 80% of cisplatin originally encapsulated within the NPs was released into the medium. To further validate that the significantly higher drug release was triggered by the interaction between catalase and H_2O_2, a control experiment without catalase was also carried out, which revealed that only a small amounts of cisplatin were released from the NPs (no catalase) after 24-h incubation with H_2O_2. Furthermore, the release behavior of catalase was also investigated (Fig. S8). These results confirm that the physiological concentration of H_2O_2 induces the release of particle payloads, which is of particular interest for intracellular delivery of drugs.

Next, we compared the in vitro cytotoxicity of the cisplatin-loaded PLGA NPs and free cisplatin. As shown in Fig. 4b, the quantity of cisplatin released from NPs that afforded 50% cell death was significantly lower than that required to achieve the same level of cell death for free cisplatin, indicating the enhancement of cisplatin efficiency by the incorporation inside NPs. It should be noted that the PLGA NPs without catalase showed a much lower in vitro cytotoxicity. The results suggested that combining catalase with cisplatin by an environmentally sensitive nanocarrier improved the antitumor activity of cisplatin. Similar results were observed from the same experiment on cytotoxicity of the [PtLCl]Cl-loaded PLGA NPs and free [PtLCl]Cl (Fig. S9-S11). The enhancement was attributed to the H_2O_2-triggered prompt release, which increase the local drug concentration to effectively kill cells.

Since hypoxia contributes to the development of MDR[12] and catalase can catalyze the breakdown of H_2O_2 to generate O_2, the synergy between cisplatin and catalase may be a new avenue to overcome MDR in cancer cells. First, the intracellular release of O_2 from PLGA NPs was assessed by using an commercially available O_2 probe ($[Ru(dpp)_3]Cl_2$), showing the O_2 levels increased gradually with time when incubated with PLGA NPs (Fig. S12). To investigate the ability of free cisplatin and cisplatin-loaded NPs to promote apoptosis in cancer cells, a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine-iodide (JC-1) assay[24] was adopted. According to Fig. S13, both free cisplatin and NPs induced drug-sensitive SGC-7901 cells apoptosis. However, for drug-insensitive SGC-7901/DDP cells, free cisplatin could hardly induce any apoptosis after 24 h. In contrast, treatment with cisplatin-loaded NPs dramatically caused the collapses of the mitochondrial membrane potential, indicating cisplatin-induced apoptosis in SGC-7901/DDP cells. Furthermore, the NPs (without catalase) could not cause apoptosis on drug-sensitive or drug-insensitive cells. Similar results were observed from the same experiment on drug-sensitive A549 and drug-insensitive A549/CDDP cells (Fig. S14). The JC-1 assay showed that the H_2O_2-responsive PLGA NPs could release cisplatin promptly into MDR cells and overcome the drug resistance effect, which was further confirmed by flow cytometry (Fig. S15) and cytotoxicity assay (Fig. S16).

Conclusions

In conclusion, we have successfully engineered a novel H_2O_2-responsive nanocarrier for improving the therapeutic efficacy and reducing systemic toxic side effects of anticancer drug cisplatin. To the best of our knowledge, this is the first time that a stimuli-responsive carrier has been developed to release drugs as well as

O_2 in a spatiotemporally controlled manner through integration of catalase and anticancer drugs. Moreover, the new nanocarrier was successfully applied to solve the hypoxic problems such as MDR in cancer cells. We anticipate that the method may prove to be an attractive new general strategy to improve therapeutic outcomes by combining chemotherapy and oxygen therapy.

Notes and references

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1 T. Finkel and N. J. Holbrook, *Nature*, 2000, **408**, 239.
2 M. Giorgio, M. Trinei, E. Migliaccio and P. G. Pelicci, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 722.
3 D. Trachootham, J. Alexandre and P. Huang, *Nat. Rev. Drug Discov.*, 2009, **8**, 579.
4 S. Toyokuni, K. Okamoto, J. Yodoi and H. Hiai, *FEBS Lett.*, 1995, **358**, 1.
5 M. López-Lázaro, *Cancer Lett.*, 2007, **252**, 1.
6 T. P. Szatrowski and Nathan, C. F. *Cancer Res.*, 1991, **51**, 794.
7 W. Dröge, *Physiol. Rev.*, 2002, **82**, 47.
8 B. Kumar, S. Koul, L. Khandrika, R. B. Meacham and H. K. Koul, *Cancer Res.*, 2008, **68**, 1777.
9 M. Höckel, K. Schlenger, B. Aral, M. Mitze, U. Schäffer and P. Vaupel, *Cancer Res.,* 1996, **56**, 4509.
10 S. Thomas, M. A. Harding, S. C. Smith, J. B. Overdevest, M. D. Nitz, H. F. Frierson, S. A. Tomlins, G. Kristiansen and D. Theodorescu, *Cancer Res.*, 2012, **72**, 5600.
11 X. Song, X. Liu, W.Chi, Y. Liu, L. Wei, X. Wang and J. Yu, *Cancer Chemother. Pharmacol.*, 2006, **58**, 776.
12 A. L. Harris, *Nat. Rev. Cancer*, 2002, **2**, 38.
13 T. Tsuruo, M. Naito, A. Tomida, N. Fujita, T. Mashima, H. Sakamoto and N. Haga, *Cancer Sci.*, 2003, **94**, 15.
14 R. Hong, G. Han, J. M. Fernández, B. J. Kim, N. S. Forbes and V. M. Rotello, *J. Am. Chem. Soc.*, 2006, **128**, 1078.
15 M. S. Shim and Y. Xia, *Angew. Chem. Int. Ed.*, 2013, **52**, 6926; K. E. Broaders, S. Grandhe and J. M. Fréchet, *J. Am. Chem. Soc.*, 2011, **133**, 756; C. de Gracia Lux, S. Joshi-Barr, T. Nguyen, E. Mahmoud, E. Schopf, N. Fomina and A. Almutairi, *J. Am. Chem. Soc.*, 2012, **134**, 15758.
16 S. I. Gundersen and A. F. Palmer, *Biotechnol. Prog.*, 2008, **24**, 1353.
17 S. Wu, C. Zhu, C. Zhang, Z. Yu, W. He, Y. He, Y. Li, J. Wang and Z. Guo, *Inorg. Chem*., 2011, **50**, 11847.
18 L. L. Qu, D. W. Li, L. X. Qin, J. Mu, J. S. Fossey and Y. T. Long, *Anal. Chem.*, 2013, **85**, 9549.
19 L. Liu, Z. Zhang and D. Xing, *Free Radic. Biol. Med.*, 2011, **51**, 53.
20 V. Adam-Vizi and C. Chinopoulos, *Trends Pharmacol. Sci.*, 2006, **27**, 639.
21 E. R. Jamieson and S. J. Lippard, *Chem. Rev.*, 1999, **99**, 2467.
22 Y. Jung and S. J. Lippard, *Chem. Rev.*, 2007, **107**, 1387.
23 S. F. Ma, M. Nishikawa, K. Hyoudou, R. Takahashi, M. Ikemura, Y. Kobayashi, F. Yamashita and M. Hashida, *Kidney Int.*, 2007, **72**, 1474.
24 S. Dhar and S. J. Lippard, *Proc. Natl. Acad. Sci. USA*, 2009, **106**, 22199.

Table of Contents

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