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Platinum (IV) Prodrugs with Long Lipid Chains for Drug Delivery and Overcoming Cisplatin Resistance

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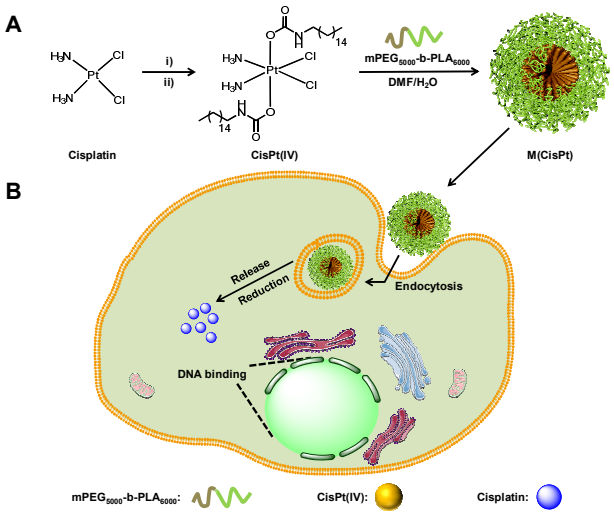
Platinum(IV) prodrugs of clinically used cisplatin and oxaliplatin with two axial long lipid chains were developed for nanoparticle delivery to combat cisplatin resistance.

Because of their exceptional anti-cancer efficacy, cisplatin and oxaliplatin have been widely used as first-line anti-cancer regimens either alone or in combination therapies.[1-3] Nonetheless, the therapeutic outcomes are compromised by drug resistance and great side effects.[4-6] Drug resistance against platinum drugs firstly arises from the difficulty of drugs in entering the cancer cells.[7-9] Moreover, platinum drugs are susceptible to deactivation by intracellular reductants, i.e. methionine, glutathione (GSH), and metallothionein.[10-13] Therefore, they have to be administrated at higher doses once drug resistance arises, leading to further formidable systemic toxicity. One possible way to overcome resistance and reduce toxicity is engineering platinum drugs into nanoparticle-based drug delivery systems.[3, 14-16] Nanoparticles can ameliorate pharmacokinetic profiles, enhance cell internalization, and improve drug stability by modulating size, surface properties, and targeting capacities.[17-22] However, it remains a challenging task to load cisplatin and oxaliplatin into conventional nano-carriers because of limited functional groups for drug conjugation and poor hydrophilicity/hydrophobicity for drug encapsulation.

To address these issues, starting from cisplatin and oxaliplatin, we developed Pt(IV) prodrugs (CisPt(IV) and OxaPt(IV)) with two long lipid chains into axial positions (Scheme 1) and encapsulated these prodrugs with FDA approved biodegradable polymer methoxyl poly - (ethylene glycol) - block - poly (lactic acid) ($mPEG_{5000}$-b-PLA_{6000}) .[23, 24] The lipid chain endows lipophilicity into Pt(IV) drugs, providing with the possibility of incorporating them into amphiphilic nano-carriers, which ultimately improves cellular uptake of platinum drugs.[25] In addition, compared to Pt(II), Pt(IV) prodrugs are more resistant to deactivation by reductants and sequestration owing to their exceptional chemical stability.[26] Once in the cells, with abundant intracellular GSH, inert Pt(IV) prodrugs can be triggered to be reduced to toxic Pt(II) and then kill the cancer cells.[27]

To demonstrate this strategy, $mPEG_{5000}$-b-PLA_{6000} was employed to encapsulate Pt(IV) prodrugs of cisplatin and oxaliplatin to form nanoparticles (M(CisPt) and M(OxaPt)). These Pt(IV) prodrugs containing nanoparticles could be endocytosed by the cancer cells, naturally circumventing the cellular pathway of internalizing small molecule-based Pt drugs by cells which adopts passive diffusion as well as copper transporter mediated active transportation as the major internalization pathway[1]. Moreover, the nanoparticle encapsulation brings with additional protection of the Pt drugs by polymers, which reduces the thiol-mediated detoxification of them. Here, optimized formulation, triggered drug release and subsequent biological evaluation on resistant ovarian cancer cells were performed.

CisPt(IV) and OxaPt(IV) were synthesized by oxidizing cisplatin and oxaliplatin with hydrogen peroxide (H_2O_2) and subsequently attaching two axial long lipid chains to the Pt(IV)



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spheres (Figure S1). CisPt(IV) and OxaPt(IV) were successfully synthesized and proved by characterization of ^{1}HNMR (Figure S2), ESI-MS (Figure S3) and IR (Figure S4). The long lipid chains endow CisPt(IV) and OxaPt(IV) with possibility for encapsulation of drugs. Here various formulations via drug encapsulation by $mPEG_{5000}$-b-PLA_{6000} were studied and appropriate nanoparticle size and loading efficiency were monitored (Figure 1, Figure S5). The mean diameters of M(CisPt) and M(OxaPt) increased correspondingly as the drug to polymer feed ratio increased from 0.01 to 0.4 (Figure 1A and Figure S5A). The polymer dispersity index (PDI) for M(CisPt) and M(OxaPt) ranged from ~0.1 to ~0.4, which indicated all nanoparticles formed have moderate polydispersity (Figure 1B and Figure S5B). The zeta potentials for both M(CisPt) and M(OxaPt) were almost kept at around -20 mV (Figure 1C and Figure S5C), suggesting that the drug to polymer feed ratio in the range of 0.01 to 0.4 has no significant impact on the zeta potential for both systems. Furthermore, the Pt loading ratios were increased correspondingly as the drug to polymer feed ratio increased from 0.01 to 0.4, but became similar when the drug to polymer ratio was at 0.2 and 0.4 (Figure 1D and Figure S5D). Based on the above characterization results, we selected the drug to polymer ratio at 0.2 as the optimized ratio in the following study.

To prove the formation of nanoparticles, the morphology and diameter of micelle obtained were evaluated using TEM and DLS (Figure 2). Specifically, both M(CisPt) and M(OxaPt) were spherical in shape and had a smooth surface without any aggregation, with a diameter of 140 nm and 120 nm, respectively. This was further confirmed by DLS (Figure 2C), showing average diameters of 220 nm and 178 nm for M(CisPt) and M(OxaPt), respectively. The discrepancy in diameter between TEM and DLS could be contributed to shrinkage of samples during the drying process in the TEM sample preparation.

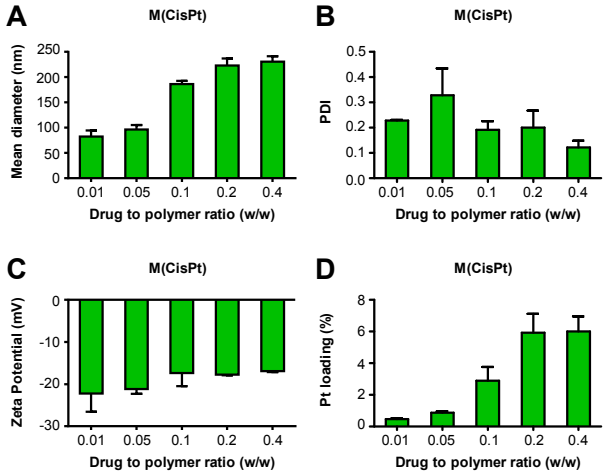

Figure 1. Formulation optimization of the nanoparticles of M(CisPt). Mean diameter (A), PDI (B) Zeta potential (C) and Pt loading at various drug to polymer ratios (D) were shown.

In vitro platinum release behavior of M(CisPt) was studied at pH 7.4, pH 5.0 and in the presence of 10 mM GSH respectively (Figure 2D). At pH 7.4 which mimics the pH values in the blood circulation and normal tissue[28], less than 15% platinum was released even up to 12 h, while there is 43% of M(CisPt) at pH 5.0. However, in the reductive environment (10 mM GSH), 100% of platinum released after 36 h. The results indicated that the release behavior of M(CisPt) was highly dependent on the reducing agents and acid hydrolysis. The acid and GSH could benefit the platinum release. In the acid environment (pH 5.0), the polymer could be easily degraded to diffuse the drugs out of the biopolymer. In contrast, it was relatively stable at pH 7.4. In the presence of GSH, the platinum could be released rapidly in a short time. That was because GSH could reduce hydrophobic CisPt(IV) into relatively hydrophilic cisplatin, which promotes the release of Pt in the polymer matrix. Above results indicated that the CisPt(IV) loaded drug delivery system showed sensitivity for both acid and GSH. As acidic environment and high concentration of GSH are present in tumor cells, such system is expected to release the active component rapidly during the treatment process.

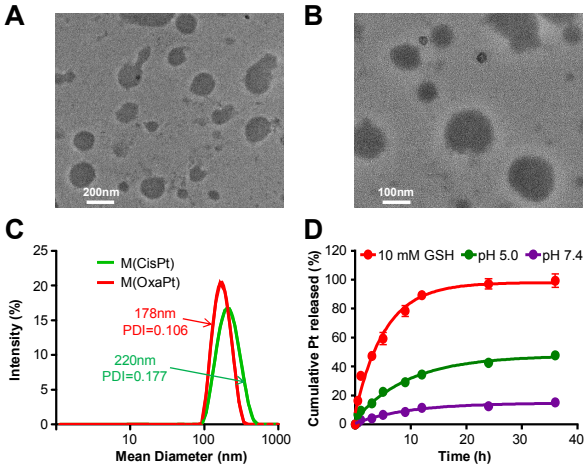

Figure 2. Characterization of M(CisPt) and M(OxaPt). TEM images of representative nanoparticles of M(CisPt) (A), M(OxaPt) (B) and DLS of M(CisPt) and M(OxaPt) (C) as well as the drug release profiles of M(CisPt) at pH 7.4, pH 5.0 and in the presence of 10 mM GSH (D).

To gain further insight into the toxicity of M(CisPt) and M(OxaPt) against cancer cells, A2780 (cisplatin sensitive) and A2780DDP (cisplatin resistant) cells were chosen. Dose dependent behaviors of cellular viability of A2780 and A2780 DDP at 48 h were shown in Figure 3A, respectively. On A2780 cells, cisplatin and M(OxaPt) showed almost similar toxicity against the cells. M(OxaPt) was much more toxic than oxaliplatin on the cells. However, M(CisPt) was the most effective. Nevertheless, on the resistant A2780DDP cells, cisplatin was less effective. Oxaliplatin and M(OxaPt) were almost the same in cell killing. Notably, M(CisPt) was the most effective on the resistant cells. To further describe this, the half inhibitory concentration (IC_{50}) based on Pt as well as the resultant drug resistance fold for all drugs were shown in Figure 3B. Cisplatin showed IC_{50} values of 2.7 ± 0.6 μM and 24 ± 1.45 μM Pt on A2780 and A2780DDP cells, which indicated a drug resistance fold of 9. For oxaliplatin, the IC_{50} were 0.86 ± 0.1 μM and 2.08 ± 0.47 μM on A2780 and A2780DDP respectively with a drug resistance fold of 2.4. It seemed that cells resistant to cisplatin did not show resistance to oxaliplatin. Notably, M(CisPt) had the lowest IC_{50} values on both cells lines (0.0067 ± 0.001 μM on A2780 and 0.0205 ± 0.0034 μM on A2780DDP). The resistance fold of M(CisPt) was 3.1.

The platinum drugs could finally target DNA in the cancer cells *via* formation of Pt-DNA adduct, disrupting the DNA duplicates and eventually leading to apoptosis. The uptake of Pt by A2780 and A2780DDP cells were measured by ICP-MS after treatment of the cells at 5 h and 9 h. It could be observed that drug uptake by the cell lines were in a cell line and time dependent manner (Figure 4A). For almost all drug treated groups, as the time increased, more drugs were internalized for both cell lines. However, it seemed that A2780

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took more cisplatin and oxaliplatin than A2780DDP cells at the same time point. The less uptake of free drugs cisplatin and oxaliplatin may explain the drug resistance for them. Moreover, the drug uptake of M(CisPt) and M(OxaPt) had shown almost the same on both A2780 and A2780DDP cells, which were more than cisplatin and oxaliplatin. Thus, the nanoparticles helped to increase the drug internalization greatly and may circumvent the intracellular pathways and biological barriers of the free drugs, which resulted in the increase of intracellular drug accumulation and the enhanced cellular cytotoxicity.

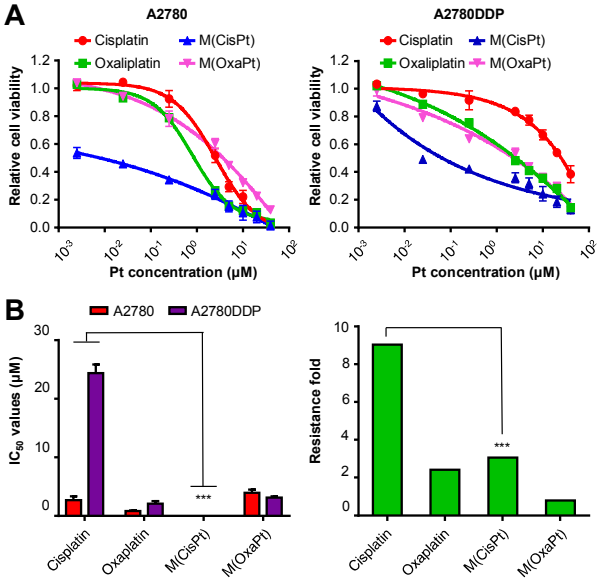

Figure 3. *In vitro* evaluation of M(CisPt) and M(OxaPt) on ovarian cancer cells. Cytotoxic assay by MTT of cisplatin, oxaliplatin, M(CisPt) and M(OxaPt) on cisplatin sensitive A2780 cells and cisplatin resistant A2780DDP cells (A). The IC_{50} values and the drug resistance fold were then derived (B). Significance is defined as *** p<0.001.

To further study the specific internalization pathway, uptake of M(CisPt) in the presence of various endocytosis inhibitors were performed (Figure 4B). Results showed that minimal uptake by the cells was found at 4 °C, indicating this process is ATP dependent endocytosis.[29] Moreover, chlorpromazine, genistein and sodium azide were used as endocytosis inhibitors to further determine the internalization pathway of M(CisPt). Sodium azide works as an electron transport chain inhibitor[19], while chlorpromazine and genistein are routinely used to inhibit clathrin-mediated endocytosis and caveolae-mediated endocytosis, respectively[30]. It could be clearly found that the cell lines cultured with sodium azide and genistein had less uptake compared to the PBS treated control group. However, cells treated with chlorpromazine exhibited the least uptake, indicating the pathway of M(CisPt) by cancer cells is majorly clathrin-mediated endocytosis. Taken together, M(CisPt) adopts different internalization pathways from small molecule-based Pt drugs.

To visualize the intracellular internalization pathway of M(CisPt). A fluorescent dye Rhodamine B (RhB) was co-loaded to label the nanoparticles (M(CisPt/RhB)). Treatment of the cells with M(CisPt/RhB) for 2 h and imaging of the cells by confocal laser scanning microscopy (CLSM) were performed (Figure 4C). It showed that M(CisPt/RhB) were in the cell cytoplasm, indicating the nanoparticles were in the cell plasma. To further quantify the intracellular uptake, cells were treated with M(CisPt/RhB) for 2 h to 4 h and monitored by flow cytometry (Figure 4D). Results showed that as time gone by, more and more M(CisPt/RhB) were endocytosed into cells and this internalization was continuous and progressive from 2 h to 4 h.

Cisplatin is considered to induce cell and cycle arrest and apoptosis.[19] To evaluate whether M(CisPt) and M(OxaPt) had similar impact on the cell fate, especially for cell cycle arrest and apoptosis, flow cytometry experiments and cell cycle analysis were carried out (Figure S6 and Figure S7). Results showed that the PBS treated cells were mostly in the G_1 phase. However, when dosed with M(CisPt) and M(OxaPt), the ratio of cells in S phase increased significantly, while G_1 phase decreased dramatically. In the G_1 phase, the biosynthetic activities of the cell reach a high rate and cell stores more proteins, enlarges the number of organelles, and grows bigger.[31] During the S phase, DNAs start to duplicate and the amount of DNA is doubled. Most cells stay in S phase when exposed to Pt based drug indicated that the DNA replication process is disrupted in the presence of M(CisPt) and M(OxaPt) and fail to move to the next phase. The S phase disruption effect could be due to the association of platinum drugs to DNA structure and blockade of the DNA helix, which could lead to inhibition of cell division and finally results in cell apoptosis.

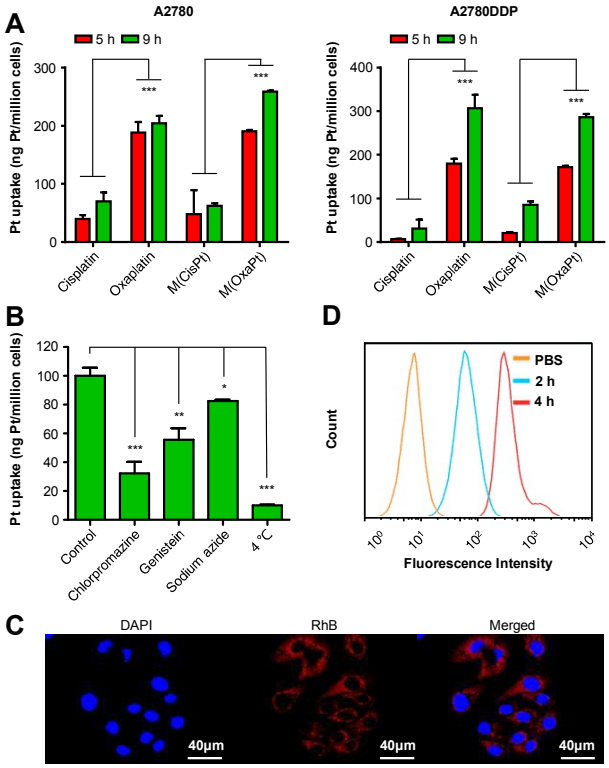

Figure 4. Intracellular uptake of Pt(IV) loaded nanoparticles. Pt uptake of M(CisPt) and M(OxaPt) by A2780 and A2780DDP cells were measured by ICP-MS (A). Different from the internalization pathway of cisplatin via passive diffusion, the mechanism of uptake of M(CisPt) was studied in the presence of various endocytosis inhibitors (B). To visualize the nanoparticles, A2780DDP cells were treated with M(CisPt/RhB) and imaged at 4 h. DAPI was used to stain the cell nucleus (blue). The red fluorescence comes from RhB in the nanoparticles (C). To quantify the nanoparticle uptake, flow cytometry was used to monitor the fluorescence intensity in the A2780 cells at 2 h and 4 h (D). Significance is defined as * p < 0.05, ** p < 0.01; *** p<0.001.

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In summary, an acid and reduction sensitive Pt(IV) prodrug delivery system was developed and optimized to overcome cisplatin resistance. The Pt(IV) prodrug micelle exhibited rapid drug release under acid and high concentration reducing agent conditions. These systems could release toxic Pt drugs rapidly in human ovarian cancer cells and showed greater toxicity to A2780DDP cells. What's more, the endocytic mechanisms of this system were ATP dependent and multiple receptor-mediated. Further, most cells were arrested in S phase after treatment of M(CisPt) and M(OxaPt), which resulted in apoptosis. Therefore, the present study suggested a new strategy for overcoming cisplatin-resistance by delivering and activating platinum drugs in cancer cells.

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Notes and references

1. T. C. Johnstone, K. Suntharalingam and S. J. Lippard, *Chemical reviews*, 2016, **116**, 3436-3486.
2. D. Wang and S. J. Lippard, *Nature Reviews Drug Discovery*, 2005, **4**, 307.
3. P. a. Ma, H. Xiao, C. Li, Y. Dai, Z. Cheng, Z. Hou and J. Lin, *Materials Today*, 2015, **18**, 554-564.
4. R. Qi, Y. Wang, P. M. Bruno, H. Xiao, Y. Yingjie, T. Li, S. Lauffer, W. Wei, Q. Chen, X. Kang, H. Song, X. Yang, X. Huang, A. Detappe, U. Matulonis, D. Pepin, M. T. Hemann, M. J. Birrer and P. P. Ghoroghchian, *Nature communications*, 2017, **8**, 2166.
5. H. Maeda, G. Y. Bharate and J. Daruwalla, *European journal of pharmaceutics and biopharmaceutics*, 2009, **71**, 409-419.
6. H. Xiao, D. Zhou, S. Liu, Y. Zheng, Y. Huang and X. Jing, *Acta Biomaterialia*, 2012, **8**, 1859-1868.
7. D. Zhou, Y. Cong, Y. Qi, S. He, H. Xiong, Y. Wu, Z. Xie, X. Chen, X. Jing and Y. Huang, *Biomaterials Science*, 2015, **3**, 182-191.
8. M. A. Fuertes, C. Alonso and J. M. Pérez, *Chemical Reviews*, 2003, **103**, 645-662.
9. Y. Zhang, G. Guo, B. Ma, R. Du, H. Xiao, X. Yang, W. Li, Y. Gao, Y. Li and X. Jing, *Anti-cancer drugs*, 2015, **26**, 698-705.
10. J. Reedijk and J. M. Teuben, in *Cisplatin: Chemistry and biochemistry of a leading anticancer drug*, Verlag Helvetica Chimica Acta, 2006, DOI: 10.1002/9783906390420.ch13, pp. 339-362.
11. Z. Guo and P. J. Sadler, in *Adv. Inorg. Chem.*, ed. A. G. Sykes, Academic Press, 1999, vol. 49, pp. 183-306.
12. J. Reedijk, *Chemical Reviews*, 1999, **99**, 2499-2510.
13. Q. Yang, R. Qi, J. Cai, X. Kang, S. Sun, H. Xiao, X. Jing, W. Li and Z. Wang, *RSC Advances*, 2015, **5**, 83343-83349.
14. Q. Cheng and Y. Liu, *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 2017, **9**, e1410.
15. W. Zhang, C. Li, C. Shen, Y. Liu, X. Zhao, Y. Liu, D. Zou, Z. Gao and C. Yue, *Drug Delivery*, 2016, **23**, 2575-2580.
16. R. Du, H. Xiao, G. Guo, B. Jiang, X. Yan, W. Li, X. Yang, Y. Zhang, Y. Li and X. Jing, *Colloids and Surfaces B: Biointerfaces*, 2014, **123**, 734-741.
17. Y. Wen, H. R. Kolonich, K. M. Kruszewski, N. Giannoukakis, E. S. Gawalt and W. S. Meng, *Molecular pharmaceutics*, 2013, **10**, 1035-1044.
18. M. E. Davis, Z. Chen and D. M. Shin, *Nature Reviews Drug Discovery*, 2008, **7**, 771-781.
19. W. Li, M. Jiang, Y. Cao, L. Yan, R. Qi, Y. Li and X. Jing, *Bioconjugate chemistry*, 2016, **27**, 1802-1806.
20. K. Wang, Q. Huang, F. Qiu and M. Sui, *Current medicinal chemistry*, 2015, **22**, 4118-4136.
21. H. Wang, Z. Lu, L. Wang, T. Guo, J. Wu, J. Wan, L. Zhou, H. Li, Z. Li and D. Jiang, *Cancer research*, 2017, **77**, 6963-6974.
22. Y. Yang, X. Wang, G. Liao, X. Liu, Q. Chen, H. Li, L. Lu, P. Zhao and Z. Yu, *J. Colloid Interface Sci.*, 2018, **509**, 515-521.
23. J. W. Singer, B. Baker, P. De Vries, A. Kumar, S. Shaffer, E. Vawter, M. Bolton and P. Garzone, *Advances in experimental medicine and biology*, 2003, **519**, 81-99.
24. H. Guan, M. J. McGuire, S. Li and K. C. Brown, *Bioconjugate Chem.*, 2008, **19**, 1813-1821.
25. Y. R. Zheng, K. Suntharalingam, T. C. Johnstone, H. Yoo, W. Lin, J. G. Brooks and S. J. Lippard, *Journal of the American Chemistry Society*, 2014, **136**, 8790-8798.
26. H. Xiao, H. Song, Y. Zhang, R. Qi, R. Wang, Z. Xie, Y. Huang, Y. Li, Y. Wu and X. Jing, *Biomaterials*, 2012, **33**, 8657-8669.
27. H. Xiao, H. Song, Q. Yang, H. Cai, R. Qi, L. Yan, S. Liu, Y. Zheng, Y. Huang, T. Liu and X. Jing, *Biomaterials*, 2012, **33**, 6507-6519.
28. Y. Zhang, F. Wang, M. Li, Z. Yu, R. Qi, J. Ding, Z. Zhang and X. Chen, *Advanced Science*, 2018, 1700821.
29. L. Kastl, D. Sasse, V. Wulf, R. Hartmann, J. Mircheski, C. Ranke, S. Carregal-Romero, J. A. Martínez-López, R. Fernández-Chacón and W. J. Parak, *ACS nano*, 2013, **7**, 6605-6618.
30. G. Sahay, D. Y. Alakhova and A. V. Kabanov, *Journal of controlled release*, 2010, **145**, 182-195.
31. D. A. Foster, P. Yellen, L. Xu and M. Saqcena, *Genes & Cancer*, 2010, **1**, 1124-1131.