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Doxorubicin loaded chitosan-ZnO hybrid nanospheres combining cell imaging and cancer therapy

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We reported the Chitosan-ZnO nanoparticles (CZNPs), which had enhanced photoluminescence stability and longer retention time in cells compared with pure ZnO QDs. These CZNPs can illuminate the cells, show the distribution of the nanospheres in the cell, and thus monitor the fate of the loaded drug, which enable these CZNPs to have a promising future in cell imaging and cancer therapy.

Photo-luminescent quantum dots (QDs) composed of II-VI semiconductor nanoparticles (NPs) have been intensively studied as biological labels in recent years due to their narrow size distribution, high luminescent efficiency and narrow symmetric emission peaks compared with organic species.[1] Among these widely used nanoparticles, ZnO QDs are the most attractive one for its nontoxicity and low price compared with CdSe or CdTe.[2] The traditional sol-gel procedure is considered as the most optimal method to fabricate ZnO QDs due to its mild experimental condition.[3] However, the obtained ZnO QDs are unstable and tend to aggregate in the aqueous solution because of their high surface energy or Ostwald ripening, which result in low quantum yields (QYs).[4] Furthermore, the fluorescence of ZnO QDs may be quickly quenched in the aqueous dispersions and biological media due to the damage of the surface defects of ZnO QDs by the attack of water molecules.[5] Besides, upon the direct contact with water, ZnO can dissolve slowly in the biological media (e.g. acidic tumor microenvironment) and or inside the cells. The release of Zn^{2+} ions will affect the function of mitochondria, leading to the apoptosis or the death of the cells.[6] Moreover, the high surface area of ZnO QDs also generate high level of intracellular reactive oxygen species, which can destroy cells.[7] Thus, the surface modification strategies, such as surface capping, have been developed to stabilize the ZnO QDs in vitro and in vivo. For instance, various capping agents, such as oleic acid (OA),[8] polymethylmethacrylate (PMMA),[9] and polystyrene (PS),[10] have been used to protect the ZnO QDs in aqueous solution. Usually, such reported surface modification should be taken in nonpolar solvent and the obtained surface modified ZnO QDs were usually unstable in water, which was not suitable for bio-imaging. Besides, most of these capping agents were synthesized through a series of complex procedures and sometimes sacrificed the environmental friendly properties.[11]

In this work, colloidal ZnO QDs with good stability, enhanced biocompatibility, high luminescent efficiency in water, and stable performance in vitro were fabricated through entrapping the ZnO QDs into chitosan (CS) nanoparticles via a nonsolvent-aided counter-ion complexation method.[13] The CS enhances the biocompatibility, stability and internalization of ZnO QDs in cells, and also provides enough space to load doxorubicin hydrochloride (DOX) while maintaining the fluorescent properties of ZnO QDs. We hope this kind of multifunctional NPs can be used for cancer therapy and monitoring the real-time distribution of NPs in cells simultaneously.

ZnO QDs with strong blue emission were first prepared by utilizing OA and Diethanolamine (DEA) as surface modification agents. Homogeneously dispersed ZnO QDs are clearly shown in transmission electron microscopy (TEM) image in Fig. 1A. Lattice space equalling 2.6 Å (Fig. 1A inset) is distinctly observed by high resolution TEM (HRTEM), which corresponds to the (002) lattice of ZnO QDs. The XRD pattern of ZnO QDs (Fig. S1) fits well with the wurtzite structure (JCPDS Card No. 89-1397). These ZnO QDs have a narrow size distribution with an average size of 3.7±0.6 nm calculated from the TEM images (Fig. 1C), which is smaller than the value (14.8 nm) obtained from the dynamic laser scattering (DLS) result (Fig. S2A). These ZnO QDs show a strong blue fluorescence (emission wavelength 402 nm) under the excitation at 337 nm, which confirms that these QDs can be utilized as cell imaging agents (Fig. S3.). The quantum yield of ZnO QDs was determined to be 56% using quinine sulfate as a reference.[8] After encapsulation of ZnO QDs in the CS NPs, uniformly spherical and mono-dispersed NPs can be seen in the TEM image (Fig. 1B) with an average diameter of 102±12.6 nm (Fig. 1D) smaller than the hydrodynamic diameter (146 nm, Fig. S2B). They showed a positive surface charge at 31.2 mV due to the coating of CS. The quantum yield of CZNPs showed a

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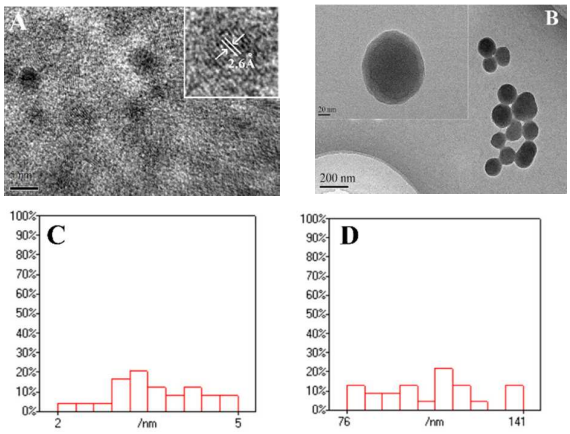

Fig. 1 The HRTEM images of ZnO QDs, (inset: Lattice space) (A); The TEM images of CZNPs (inset: HRTEM of CZNPs) (B); The size distribution of ZnO QDs (C) and CZNPs (D) calculated from the corresponding TEM images (more than 200 NPs).

little decrease to 52%. The fine structure of CZNPs was further presented in the HRTEM image (Fig. 1B, inset) with a marked variation in the contrast between the light shell and dark core. The presence of the dark core confirmed that a large amount of QDs were successfully wrapped in chitosan shell owning to the high electron density of ZnO QDs. By a close observation, small dark dots with a size less than 5 nm are clearly observed in the core, which also confirms the presence of ZnO QDs. The chemical composition, measured by energy dispersive spectrometer (EDS), showed that it was 31.34% (W_t%) of ZnO inside the hybrid NPs (Fig. S4).

An MTT assay was used to test the in vitro biocompatibility and the cytotoxicity of CZNPs with different Zn^{2+}concentrations ranging from 0.1 mg/mL to 5 mg/mL, as shown in Fig. 2A. No obvious cytotoxic effect on cell viability was found when CZNPs were incubated with the cells, even the concentration of Zn^{2+} reached up to 5 mg/mL after 48 hours incubation (87.62% survival). The low cytotoxicity can be attributed to the excellent biocompatibility of ZnO QDs and the covering layer of CS. Results of MTT assay suggest that CZNPs have a low cytotoxicity and are suitable for in-vivo application as drug carriers and fluorescence label. The cytotoxicity of DOX and DOX-loaded CZNPs (DOX-encapsulation efficiency up to 90.07%) were also tested by the MTT assay with different DOX concentrations as shown in Fig. 2B. The cytotoxicity of DOX is significant at a low concentration of 50 μg/mL with only 60.56% of cells survived after 24 hours incubation. The CZNPs also

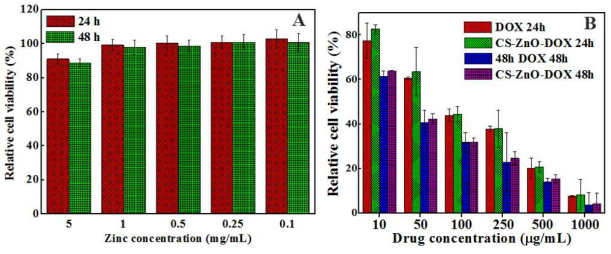

Fig. 2 In vitro viability of A549 cells in the presence of CZNPs for 24 and 48 hours (A); In vitro cytotoxicity of DOX-loaded CZNPs compared with pure DOX (B).

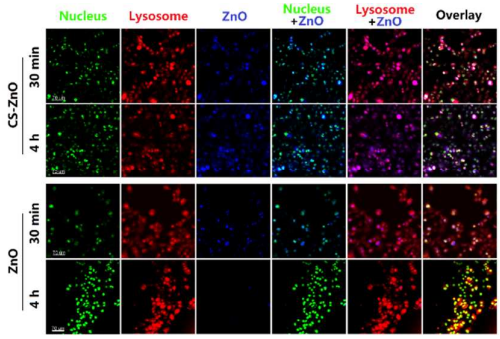

Fig. 3 CLSM images of A549 cells incubation with pure ZnO QDs and CZNPs for 4 hours. The nuclei were stained with SYTO (green). The lysosomes were stained with Lysotracker Red

exhibited a similar cytotoxicity as that of free DOX. When the incubation time was extended to 48 hours, fewer cells were alive. All these results described that DOX was successfully encapsulated inside CZNPs and the DOX-loaded CZNPs could be used in cancer therapy at an appropriate concentration.

To explore the imaging function of CZNPs, A549 cells were separately incubated with ZnO QDs solution (140 μg/mL) and CZNPs solution (1 mg/mL) and then examined by confocal microscope (Fig. 3). Because of the inherited blue fluorescence of ZnO QDs, the nuclei were selectively stained with SYTO showing a green color instead of DAPI for distinguishing. The lysosomes were stained with Lysotracker Red, giving a red color. In Fig. 3, nuclei with green fluorescence could be clearly seen in the images, indicating the successful staining of the nuclei with the SYTO. After 30 minutes co-cultured with CZNPs, blue spots were clearly observed in the image and purple sports were shown in the overlaid image of lysosomes, which indicated that CZNPs were internalized by lysosomes. After 4 hours, more blue spots were seen inside the A549 cells, strongly recommending the remarkable uptake of CZNPs by cells. In contrast, fluorescence from pure ZnO QDs could be observed only in lysosomes after 30 minutes incubation, and less purple spots were seen in the cells, which mean that less amount of ZnO QDs were engulfed by the A549 cells. Extending the incubation time to 4 hours led to extinguish the blue fluorescence, demonstrating that pure ZnO QDs degraded rapidly in the lysosome within 4 hours because of the acidic condition in lysosomes. The above results indicated that encapsulated ZnO QDs in CZNPs could protect ZnO QDs from being degraded by the lysosomes, owing to the effective protection of chitosan nanospheres. Thus, these CZNPs are more stable against the harsh intracellular microenvironment, which is especially beneficial for cell labelling compared to the blank ZnO QDs.

A549 cells were incubated with the CZNPs and the distribution of DOX loaded CZNPs in cells was characterized with CLSM. The nuclei were stained with green color (Fig. 4). After 2 hours incubation, red colour from DOX could be found in the nuclei and yellow color was clearly observed in the images when the DOX channel (red) overlaid with the SYTO channel (green), confirming the entrance of DOX into the nuclei (Fig. 4). Besides, there are lots of purple spots in the overlap image from DOX channel and ZnO channel (blue), which clearly indicates that DOX and ZnO QDs are

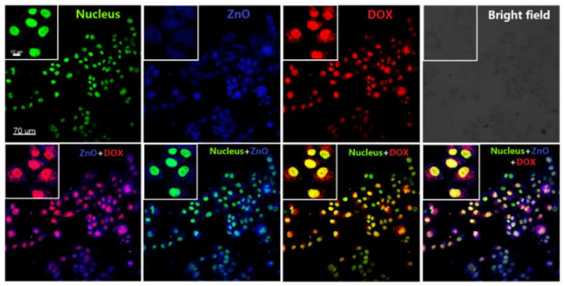

Fig. 4 CLSM images of A549 cells incubated with DOX-loaded CZNPs for 2 hours. The nuclei were stained with SYTO (green)

co-incubated in the CS NPs. However, no blue core is observed in the nuclei, proving that ZnO QDs are inside the CS nanospheres and cannot enter nuclei. It means that ZnO QDs and DOX are successfully loaded into chitosan nanospheres and DOX can be released out from the hybrid nanospheres. All these results suggested that DOX-loaded CZNPs were successfully synthesized and could be used in cancer therapy as well as cell-labelling probe.

In conclusion, DOX-loaded CZNPs were synthesized through a nonsolvent-aided counter-ion complextion procedure. These obtained hybrid CZNPs with an average diameter of about 100 nm showed excellent nontoxicity even at a high concentration of Zn^{2+}up to 5 mg/mL. In vitro CLSM images confirmed the cell imaging property of the obtained CZNPs. The encapsulated ZnO QDs in chitosan nanospheres showed higher stability in cells than the blank ZnO QDs and most of them distributed in the cytoplasm. Consequently, these nanoparticles possessed enhanced photoluminescence stability and retention time in cells, and DOX loaded CZNPs showed high cytotoxicity against A549 cells. Utilizing the abundant functional groups on the surface of CZNPs, these CZNPs can be further modified to prepare multifunctional drug delivery systems for effective cancer therapy.

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Notes and references

1 (a) X. Michalet, F. Pinaud, T. D. Lacoste; M. Dahan, M. P. Bruchez, A. P. Alivisatos, S. Weiss, *Single Molecules* 2001, **2**, 261; (b) M. Nirmal, L. Brus, *Acc. Chem. Res.* 1999, **32**, 407; (c) U. Resch-Genger, M. Grabolle, S. Cavaliere-Jaricot, R. Nitschke, T. Nann, *Nat. Methods* 2008, **5**, 763.

2 (a) N. A. Bakar, A. A. Umar, T. T. Aziz, S. H. Abdullah, M. M. Salleh, M. Yahaya, B. Majlis, In *Synthesis of CdSe quantum dots: Effect of surfactant on the photoluminescence property, Semiconductor Electronics*, 2008. ICSE 2008. *IEEE International Conference* on, IEEE: 2008; pp 528; (b) S. Cingarapu, Z. Yang, C. M. Sorensen, K. J. Klabunde, *Journal of Nanomaterials* 2012, **5**, 7.

3 (a) D. W. Bahnemann, C. Kormann, M. R. Hoffmann, *J. Phys. Chem.* 1987, **91**, 3789; (b) L. Spanhel, *J. Sol-Gel Sci. Technol.* 2006, **39**, 7; (c) G. Oskam, F. d. J. P. Poot, *J. Sol-Gel Sci. Technol.* 2006, **37**, 157.

4 (a) H.-M. Xiong, D.-P. Liu, Y.-Y. Xia, J.-S. Chen, *Chem. Mater.* 2005, **17**, 3062; (b) H.-P. Wang, H. Jiang, X.-M. Wang, *Chem. Commun.* 2010, **46**, 6900; (c) C.-H. Hung, W.-T. Whang, *J. Mater. Chem.* 2005, **15**, 267.

5 (a) N. R. Jana, H.-h. Yu, E. M. Ali, Y. Zheng, J. Y. Ying, *Chem. Commun.* 2007, 1406; (b) H.-M. Xiong, *J. Mater. Chem.* 2010, **20**, 4251.

6 D. W. Choi, J. Y. Koh, *Annual review of neuroscience* 1998, **21**, 347.

7 A. Sasidharan, P. Chandran, D. Menon, S. Raman, S. Nair, M. Koyakutty, *Nanoscale* 2011, **3**, 3657.

8 Y.-S. Fu, X.-W. Du, S. A. Kulinich, J.-S. Qiu, W.-J. Qin, R. Li, J. Sun, J. Liu, *J. Am. Chem. Soc.* 2007, **129**, 16029.

9 H. M. Xiong, Z. D. Wang, Y. Y. Xia, *Adv. Mater.* 2006, **18**, 748.

10 H.-M. Xiong, D.-P. Xie, X.-Y. Guan, Y.-J. Tan, Y.-Y. Xia, *J. Mater. Chem.* 2007, **17**, 2490.

11 L. Guo, S. Yang, C. Yang, P. Yu, J. Wang, W. Ge, G. K. Wong, *Appl. Phys. Lett.* 2000, **76**, 2901.

12 (a) Y. Lin, L. Zhang, W. Yao, H. Qian, D. Ding, W. Wu, X. Jiang, *ACS Appl. Mat. Interfaces* 2011, **3**, 995; (b) Y. Ding, X. Bian, W. Yao, R. Li, D. Ding, Y. Hu, X. Jiang, Y. Hu, *ACS Appl. Mat. Interfaces* 2010, **2**, 1456.