RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Two dimensional TiO_2 nanosheets: *in vivo* toxicity investigation

Sha-Sha Song,[a,⊥] Bao-Yu Xia,[b,⊥] Jie Chen,[a] Jiang Yang,[d] Xiu Shen,[a] Sai-Jun Fan,[a] Mei-li Guo,[c,*] Yuan-Ming Sun,[a,*] Xiao-Dong Zhang[a,*]

Titanium dioxide (TiO_2) nanosheets had received attentions in photodynamic therapy due to unique electronic structure and high surface activity. However, it is still not clear about the biological response and toxicity of two dimensional (2D) nanomaterials. Herein, *in vivo* toxicity of TiO_2 nanosheets, such as biodistribution, hematology, biochemistry and pathology, were evaluated at the dose of 10 mg/kg by intraperitoneal injection up to 30 days. It was found that TiO_2 could gradually accumulate in liver and spleen with increase of exposure time, which is due to large size induced absorption from reticuloendothelial system (RES). Furthermore, the hematological data indicated that no significant difference was found. However, the biochemistry showed that liver indicator, AST, presented significant difference after 30 days compared with control mice. The present work revealed that 2D TiO_2 nanosheets did not cause the appreciable toxicity, but induced the accumulation in the liver and slight abnormality of liver with increasing exposure time.

.

* E-mail: meiliguo314@gmail.com

* E-mail: yuanmings1962@163.com

* E-mail: Xiaodongzhang@tju.edu.cn

⊥These authors contributed equally to this work.

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Introduction

Titanium dioxide (TiO_2) has attracted wide attentions in different areas, such as cosmetics,[1] paint industries,[2] pharmaceutics and biomedical,[3] due to their high stability, anticorrosion[4] and photocatalysis.[5, 6] Highly active TiO_2 can be used for the photothermal therapy contrast agent.[7] Meanwhile, TiO_2 can show an effective bactericidal activity even under very weak UV light illumination, and its biological activity has been confirmed by *in vivo* experiment.[8, 9] Unfortunately, *in vivo* toxicity of TiO_2 is still a big challenge, and it is necessary to understand their biological response.

The *in vivo* biodistribution and toxicity of nanomaterials are closely related to the physical dimension and surface chemistry.[10-18] In the past several years, several groups investigated the *in vitro* and *in vivo* experiments of TiO_2 nanoparticles (NPs).[19, 20] Park, *et al.* showed that surface area of TiO_2 nanowires may play an important role in biological response. They found that the increase of autophagosome-like vacuoles may be an important reason for cytotoxicity due to NPs induced reactive oxygen species (ROS).[21] Warheit, *et al.* revealed that anatase TiO_2 nanorods and nanodots did not lead to lung inflammation or pathological changes.[22] Jin, *et al.* reported that spontaneous ROS was generated by anatase-TiO_2 exposure and then could induce cellular apoptosis, but was not found in rutile-TiO_2 NPs.[23] Similarly, dose-dependent effects of TiO_2 were widely investigated. Fabian, *et al.* reported that TiO_2 with lower dose (5 mg/kg) showed no damage to all of the organs.[24] The biodistribution showed that the lots of Ti could be found in the liver, kidney, and spleen after injecting anatase TiO_2 nanoparticles (5, 10, 50 mg/kg). As a contrast, TiO_2 with higher dose (100, 150 mg/kg) caused serious damage to the liver, kidney, and myocardium of mice.[25] A similar study showed injected dose of TiO_2 performed the significant influence on toxicity of mice.[26, 27]

Despite of lots of *in vivo* toxicity investigations, the biological response of two dimensional (2D) nanosheets is still less reported. Compared with traditional nanoparticles, nanorods, and nanowires, TiO_2 nanosheets with high surface area can introduce many interesting chemical and biological properties.[28, 29] Therefore, it is necessary to investigate the biological effects of 2D TiO_2 nanosheets. In this work, we present the *in vivo* toxicity of TiO_2 nanosheets systematically. *In vivo* biodistribution, the immunogenicity, hematological toxicity, and damages in liver and spleen, were investigated in detail.

Materials and Methods

Fabrication

The anatase TiO_2 nanosheets were synthesized through a modified hydrothermal method.[30-32] In a typical synthesis, 5 mL of titanate isopropoxide (97%, Sigma-Aldrich) was added into a 40 mL Teflon-lined autoclave. Then 0.6 mL of 48% HF solution was added drop-wise. The mixed solution was sealed and kept in an electric oven at 180 °C for 24 h. Then it was naturally cooled down to room temperature. After that, N-(Carbonyl-methoxypolyethyleneglycol 5000)-1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG_{5000}) was used to coat the TiO_2 nanosheets. Typically, 20 mg DSPE-PEG_{5000} was dispersed in 20 ml pure water, and then added the TiO_2 nanosheets (1 mg) into the solution, and then washed 6 times.[33] Finally, we got the DSPE-PEG_{5000} coated TiO_2 nanosheets. Transmission electron microscope (TEM) analysis was conducted at 200 kV with JEOL models (JEM-2100F and JEM-2011HC).

Animal Administration

Animals were purchased, maintained, and handled with protocols approved by the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (IRM, CAMS). The C57 mice, obtained from IRM laboratories at 8 weeks of age, were housed in a 12 h/12 h light/dark cycle. The temperature was maintained at 20 ± 2 °C, relative humidity at 60 ± 10 %, based on the previously identified factor.[34] Distilled water and sterilized food for mice were available ad libitum. Mice were acclimated to this environment for 3 days. Then, mice were randomly divided into five groups (seven in each group) for one control group and four experimental groups, respectively. The experimental mice received 200 μL of TiO_2 solution at a dose of 1 mg/mL. The mice were weighted and assessed for behaviour and symptom every other day for 30 days post-injection.

Hematology, Biochemistry and Sample Collection

Mice were sacrificed, and blood, serum and organs were collected for hematological, biochemical, immune, and histopathologic analysis after 1, 7, and 30 days post-injection. Using a standard blood collection technique, 20 μL blood was drawn from the saphenous vein into a potassium ethylenediamine tetra-acetic acid collection tube for hematological analysis. Mice were sacrificed using isoflurane anesthetic and exsanguinated with phosphate-buffered saline using an angiocatheter. Serum was harvested by centrifuging blood at 6000 rpm for 5 minutes. During necropsy, heart, liver, spleen, lung, kidney, thymus, and testis were collected. One mouse from each group was fixed with 10% buffered formalin following exsanguination. To explicitly examine the immune response, spleen and thymus indexes (S_x) can be defined as:

$$S_x = \frac{\text{Weight of experimental organ } (mg)}{\text{Weight of experimental animal } (g)}$$

Major organs from those mice were processed routinely into paraffin, and stained with hematoxylin and eosin (H&E). Pathology was examined using a digital microscope.[35]

Results and Discussions

Characterization of 2D TiO_2 Nanosheet by TEM and SEM

The DSPE-PEG_{5000} coated TiO_2 was fabricated by pervious reported methods.[36-38] The characterization of size and morphology of TiO_2 was presented by transmission electron microscope (TEM) and scanning electron microscope (SEM) in Fig. 1. It could be seen that DSPE-PEG_{5000} coated TiO_2 nanosheets performed the uniform dispersion. Average size of TiO_2 nanosheets was 92.5 nm measured by TEM and SEM. According to our pervious investigation, the phase of TiO_2 nanosheets was anatase.[30, 31] Anatase TiO_2 showed the very high photocatalytic activity and wide application in energy and environment, and toxicity of anatase TiO_2 will also be interesting.[39] We next move to *in vivo* toxicity of TiO_2 nanosheets.

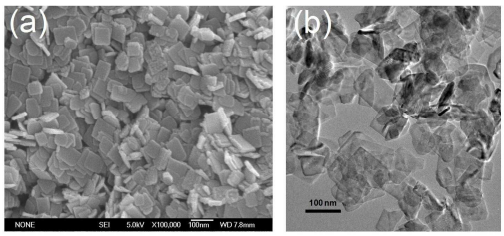

Fig. 1 The SEM (a) and TEM (b) images of TiO_2 nanosheets.

Body Weight and Immune Response

The body weight is an important indicator for evaluating the toxicological effects of the DSPE-PEG_{5000} coated TiO_2. Fig. 2 showed the time-dependent variation of body weight of control and TiO_2 treated mice in the period of 30 days. The body weight of mice was recorded every 2 days. No changes, such as vocalizations, labored breathing, were detected in each group. Difference of body weight between normal mice and treated mice was not found after 1-12 days injection. However, after injection of 14 days, the body weight of normal mice performed more obvious increase than that of TiO_2 treated mice. Using Student's t-test, no significant statistical difference between normal mice and treated mice was found. The *P* values were 1.55 for 14 days and 0.179 for 30 days, respectively. The small weight difference between the two groups indicated that the TiO_2 could lead to potential toxicity in mice. Similar trend was observed after injection 30 days.

Fig. 3 presented the immune response of mice treated with TiO_2 based on thymus and spleen indexes. We calculated the spleen and thymus indexes in order to examine immune system damage. For the spleen index, no significant statistical difference was detected between the control and the treated groups by using Student's t-test. Generally, the increase of spleen index indicated the immune response of mice, which had been widely reported elsewhere.[40] It could be concluded that the spleen and thymus indexes of the treated mice had no significant changes, which indicated no obvious immune response was found.

The indexes of spleen and thymus are important indicators for immune system of mice.[41] In spite of no significant statistical difference, the DSPE-PEG_{5000} coated TiO_2 still caused the slight change of spleen index in treated mice. The change may be related to the preferable retention of TiO_2 in the spleen. These changes inspire our interest to explore more findings, such as biodistribution, hematology, and biochemistry.

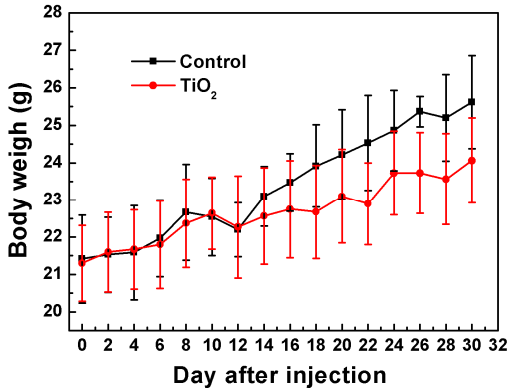

Fig. 2 Body weight changes in mice for the control and treated groups injected TiO_2 at a dose of 1mg/mL at different time points. The body weight was measured every 2 days. Each point represents the mean and standard deviation of seven mice per group. Data were analyzed using SPSS 13.0, and the differences between the treated groups and control for body weigh were not significant (P>0.05). *P* values were 1.55 for 14 days and 0.179 for 30 days of treated mice, respectively.

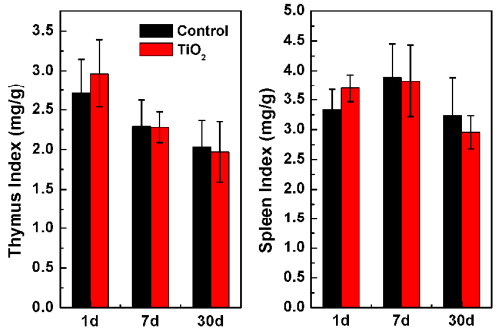

Fig. 3 Thymus index and spleen index of mice between control and treated groups. Bars present mean and standard deviation from seven mice. Data were analyzed using SPSS 13.0, and the differences between the treated and control groups were not significant (P>0.05).

Biodistribution

The biodistribution of TiO_2 treated mice was presented in Fig. 4. It was observed that 4.32% ID/g could be found in liver after 24 h, and then reduced to 4.03% ID/g after 7 days. After 30 days, the uptake of liver decreased to 1.21% ID/g and uptake of spleen sharply increased to 10.42% ID/g. The main reason is that spleen is the largest organ of the immune system.[34] When exogenous nanoparticles enter in body, they will be firstly absorbed by reticuloendothelial system (RES) and thus induce the high distribution in the spleen which was consistent with previous results.[25] The accumulation of TiO_2 in spleen may come from gradual absorption of TiO_2. Meanwhile, the decrease of uptake in liver indicated that partial TiO_2 were excreted by the liver. Recently, our groups reported that Bi_2Si_3 NPs mostly was cleared

from mice body by 90 days post-injection, and the residual amount was less than 5%.[42] Meanwhile, PEG-coated gold nanoparticals preferred to stay in liver at an exposure time of 30 days.[43] Compared with these results, 2D TiO_2 nanosheets show long retention time. [44]

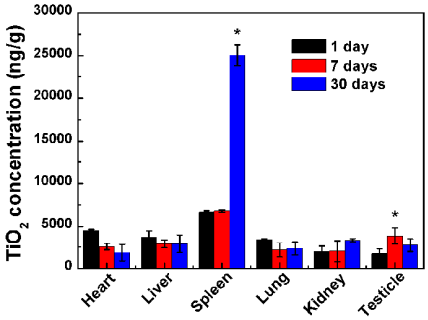

Fig. 4 Biodistribution of mice injected TiO_2 at a dose of 1 mg/mL (10mg/kg). TiO_2 preferred to stay in liver and spleen. Error bars were based on mean and standard deviation of seven mice per group. Data were analyzed by Student's t-test, and stars present significant statistical analysis (P<0.05).

Hematology and Biochemistry

TiO_2 will firstly interact with the blood components when they are injected into mice. Thus, it is very important to investigate the hematologic factors change, such as white blood cell (WBC).[34] Fig. 5 performed the hematologic analysis of mice both control and treated groups. We analyzed standard hematologic markers, such as WBC, red blood cell (RBC), platelet (PLT), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). As is well known, WBC and RBC are the most important indicators for *in vivo* response, which are directly related to infection and inflammation. The WBC and RBC did not show significant changes and maintain a normal range, which indicated that TiO_2 did not cause serious infections. PLT showed a slight increase after 7 days (P=0.01). HTC showed an obvious decrease after 30 days (P=0.001), and MCH and MCHC showed significant increase after 30 days (P=0.001), which indicated that TiO_2 induced slight damage in blood system. Other parameters, such as HGB and MCV, had no significant difference. Thus, it was clear that TiO_2 nanosheets did not cause the significant infection but induce a slight toxic response after 30 days.

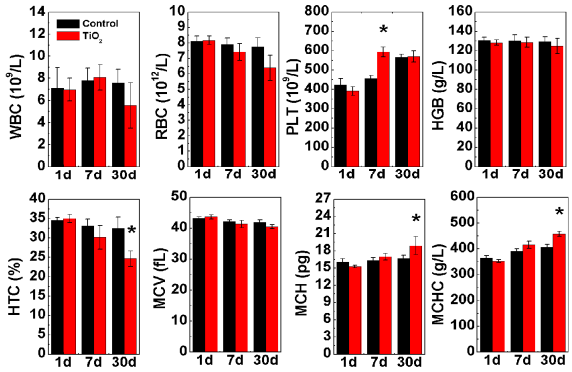

Fig. 5 Hematological results of mice treated with TiO_2 at the dose of 1mg/mL after 1, 7 and 30 days after intraperitoneal injection. The same control data were collected from the age match untreated mice. Error bars present mean and standard deviation of seven mice. Data were analyzed by Student's t-test, and stars present significant statistical analysis (P<0.05). PLT at 7 days, HTC, MCH, MCHC at 30 days showed the significant difference and corresponding P values were 0.01, 0.001, 0.001, and 0.001, respectively.

It is necessary to investigate whether TiO_2 cause toxicity in liver and kidney. Thus, we investigated the blood biochemistry. We presented the biochemical parameters, such as, alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), creatinine (CREA), total protein (TP), albumin (ALB), globulin (GLOB), and albumin to globulin (A/G) in Fig.6. We firstly focused on AST, ALT, and CREA because they are closely related to the function of liver and kidney, respectively. For the treated mice, the AST and ALT levels significantly decreased after 30 days, and statistical differences could be found compared with control group, and corresponding P values were 0.001 and 0.021, respectively. Meanwhile, CREA also showed slight increase, but no significant statistical difference could be found. No significant statistical difference was found for other indicators, such as BUN, ALB, TP, A/G, and GLOB. Thus, it could be concluded that injection of TiO_2 caused the significant toxicity in liver after 30 days.[45] It has been shown that TiO_2 preferred to stay in liver and spleen by blood circulation. Furthermore, TiO_2 could react with proteins in blood and further form the complexes of protein corona.[46] Hybrid corona in the blood could accumulate in liver and cause the low liver damage.

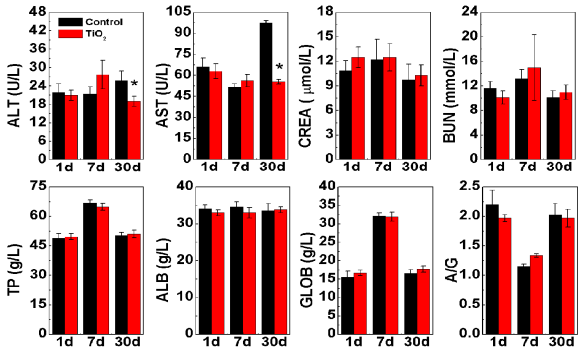

Fig.6 Biochemical data of mice treated with TiO_2 at the dose of 1mg/mL after 1, 7 and 30 days after intraperitoneal injection. The same data were collected from the age match untreated mice at the same time points as control. Biochemical data indicated liver and spleen kept relatively good working order after injecting TiO_2. Statistical analysis was based on seven mice per time points. Data were analyzed by Student's t-test, and stars present significant statistical analysis (P<0.05). ALT and AST after 30 days presented significant statistical analysis, and corresponding P values were 0.021 and 0.001.

Histopathology

To investigate toxicity, histological assessments were performed to determine whether TiO_2 could cause tissue damage,

inflammation or lesions. Finally, histological images of harvested organs, including heart, liver, spleen, lung, kidney and testicle, were performed to evaluate potential toxicity. It could be seen from Fig. 7 that no apparent damages and histopathological abnormalities were observed for the mice treated with TiO_2. The pathological results provided macroscopic and visual evidence of toxicity. Appreciable pathological changes or organs damage were not found in the interested organs.

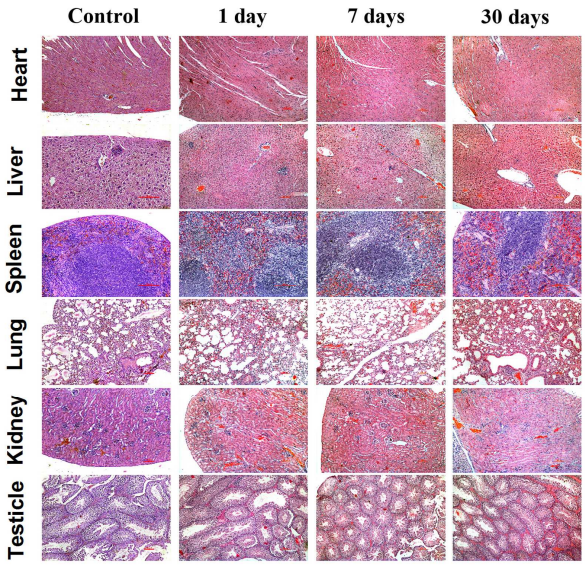

Fig. 7 Pathological images from the control and TiO_2 injected mice at various time points post-injection.

Oxidative Stress Response

To further confirm the damage of liver and lung, we performed the time dependent malondialdehyde (MDA) and superoxide dismutase (SOD). Oxidative stress response is related to the emergence of ROS.[47] The content of MDA and SOD reflected the damaged level of organism resulting from free radicals. As seen from Fig. 8, the level of MDA decreased in liver, inversely, the SOD increased, which indicated that the clearance ability of free radicals was enhanced after 24 h. After 7 days, the SOD and MDA of liver and lung were recovered, which showed that the damaged level of free radicals was recovered. Statistical differences could be found for SOD and MDA after 24 h by using the Student's t-test, and corresponding *P* values were 0.0005 and 0.001. Therefore, injection of TiO_2 could induce acute influence on ROS by decreasing level of MDA and increasing SOD after 24 h.

From these results, we concluded that TiO_2 could accumulate in liver, and thus induced liver toxicity. Furthermore, hematological and blood biochemical analysis presented significant changes in liver. Finally, the decreased level of MDA and increased SOD after 24 h indicated that active defense for free radical could be found. Therefore, we concluded that the accumulation of TiO_2 induced liver toxicity at the present dose level.

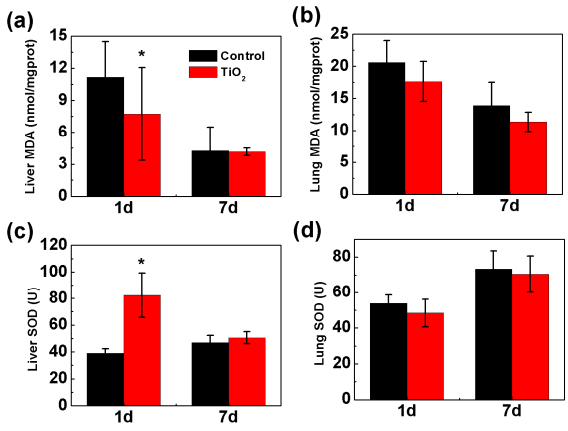

Fig. 8 The activity changes of MDA and SOD from liver (a, c) and lung (b, d) between treated and control mice after 1 day and 7 days, respectively. Bars present mean and standard deviation from 8 mice. Data were analyzed by Student's t-test, and stars present significant statistical analysis ($P<0.05$).

These results suggested that further metabolism and toxicity of TiO_2 should be investigated. Nanomaterials may generate both acute and chronic toxic effects. We speculate the toxicity of TiO_2 is closely related to protein binding. In addition, some interactions, such as enhanced binding and conformational change of the proteins, may influence the *in vivo* toxicity.[34] Furthermore, it is not clear whether TiO_2 in the liver will cause abnormal gene expression. The change of key indicators, such as ALT, AST and CERA, are still not clear for a longer time. Thus, further study is necessary to preferably understand toxicity.

Conclusion

We completed systematic examinations of the potential toxicity of DSPE-PEG_{5000} coated TiO_2 at different exposure times. The DSPE-PEG_{5000} coated TiO_2 had not caused the significant decrease of spleen and thymus indexes. However, TiO_2 caused appreciable toxicity in liver at the concentration of 1 mg/mL by blood markers analysis and histological assessment. Using oxidative stress response, we found that TiO_2 could induce the increase of SOD and the decrease of MDA after 24 h, but it recovered after 7 days. These findings will provide some useful information to TiO_2 in nanotoxicology.

Notes and references

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No 81000668 and 11304220), Natural Science Foundation of Tianjin (Grant No 13JCQNJC13500), the Subject Development Foundation of Institute of Radiation Medicine, CAMS (Grant No SF1440, SZ1336).

Notes

[a] *Tianjin Key Laboratory of Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China*
[b] *School of Chemical and Biomedical Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore*
[c] *Department of Physics, School of Science, Tianjin Chengjian University, Tianjin, China*
[d] *Department of Biological Systems Engineering, University of Wisconsin-Madison, Madison, WI, USA*

References

1 B. Trouiller, R. Reliene, A. Westbrook, P. Solaimani and R. H. Schiestl, *Cancer Res.,* 2009, **69**, 8784.
2 J. Wang, G. Zhou, C. Chen, H. Yu, T. Wang, Y. Ma, G. Jia, Y. Gao, B. Li and J. Sun, *Toxicol. Lett.,* 2007, **168**, 176.
3 D. Macwan, P. N. Dave and S. Chaturvedi, *J. Mater. Sci.,* 2011, **46**, 3669.
4 R. H. Crabtree, *Science,* 1998, **282**, 2000.
5 Y. J. Hwang, C. Hahn, B. Liu and P. Yang, *Acs Nano,* 2012, **6**, 5060.
6 H. Tang, K. Prasad, R. Sanjines, P. Schmid and F. Levy, *J. Appl. Phys.,* 1994, **75**, 2042.
7 L. Zeng, W. Ren, L. Xiang, J. Zheng, B. Chen and A. Wu, *Nanoscale,* 2013, **5**, 2107.
8 K. Sunada, T. Watanabe and K. Hashimoto, *Environ. Sci. Technol.,* 2003, **37**, 4785.
9 B. D. Johnston, T. M. Scown, J. Moger, S. A. Cumberland, M. Baalousha, K. Linge, R. van Aerle, K. Jarvis, J. R. Lead and C. R. Tyler, *Environ. Sci. Technol.,* 2010, **44**, 1144.
10 Z. Liu, W. Cai, L. He, N. Nakayama, K. Chen, X. Sun, X. Chen and H. Dai, *Nat. Nanotechnol.,* 2007, **2**, 47.
11 X. D. Zhang, J. Chen, Z. Luo, D. Wu, X. Shen, S. S. Song, Y. M. Sun, P. X. Liu, J. Zhao and S. Huo, *Adv. Healthcare Mater.,* 2014, **3**, 133.
12 X. D. Zhang, Z. Luo, J. Chen, X. Shen, S. Song, Y. Sun, S. Fan, F. Fan, D. T. Leong and J. Xie, *Adv. Mater.,* 2014, **26**, 4565.
13 B. D. Chithrani, A. A. Ghazani and W. C. Chan, *Nano Lett.,* 2006, **6**, 662.
14 A. Nel, T. Xia, L. Mädler and N. Li, *Science*, 2006, **311**, 622.
15 W. S. Cho, M. Cho, J. Jeong, M. Choi, H. Y. Cho, B. S. Han, S. H. Kim, H. O. Kim, Y. T. Lim and B. H. Chung, *Toxicol. Appl. Pharmacol.,* 2009, **236**, 16.
16 M. V. Park, A. M. Neigh, J. P. Vermeulen, L. J. de la Fonteyne, H. W. Verharen, J. J. Briedé, H. van Loveren and W. H. de Jong, *Biomaterials,* 2011, **32**, 9810.
17 K. Suttiponparnit, J. Jiang, M. Sahu, S. Suvachittanont, T. Charinpanitkul and P. Biswas, *Nanoscale Res. Lett.,* 2011, **6**, 1.
18 L. Cheng, K. Yang, Y. Li, X. Zeng, M. Shao, S.-T. Lee and Z. Liu, *Biomaterials,* 2012, **33**, 2215.
19 X. Huang, I. H. El-Sayed, W. Qian and M. A. El-Sayed, *J. Am. Chem. Soc.,* 2006, **128**, 2115.
20 C. Ispas, D. Andreescu, A. Patel, D. V. Goia, S. Andreescu and K. N. Wallace, *Environ. Sci. Technol.,* 2009, **43**, 6349.
21 E. J. Park, H. W. Shim, G. H. Lee, J. H. Kim and D. W. Kim, *Arch. Toxicol.,* 2013, **87**, 1219.
22 D. B. Warheit, T. R. Webb, C. M. Sayes, V. L. Colvin and K. L. Reed, *Toxicol. Sci.,* 2006, **91**, 227.
23 C. Jin, Y. Tang, F. G. Yang, X. L. Li, S. Xu, X. Y. Fan, Y. Y. Huang and Y. J. Yang, *Biol. Trace Elem. Res.,* 2011, **141**, 3.
24 E. Fabian, R. Landsiedel, L. Ma-Hock, K. Wiench, W. Wohlleben and B. Van Ravenzwaay, *Arch. Toxicol.,* 2008, **82**, 151.
25 H. Liu, L. Ma, J. Zhao, J. Liu, J. Yan, J. Ruan and F. Hong, *Biol. Trace Elem. Res.,* 2009, **129**, 170.
26 J. Chen, X. Dong, J. Zhao and G. Tang, *J. Appl. Toxicol.,* 2009, **29**, 330.
27 S. Hussain, K. Hess, J. Gearhart, K. Geiss and J. Schlager, *Toxicol. in Vitro,* 2005, **19**, 975.
28 K. S. Novoselov, A. K. Geim, S. Morozov, D. Jiang, Y. Zhang, S. Dubonos, I. Grigorieva and A. Firsov, *Science,* 2004, **306**, 666.
29 X. Li, X. Wang, L. Zhang, S. Lee and H. Dai, *Science,* 2008, **319**, 1229.
30 B. Y. Xia, B. Wang, H. B. Wu, Z. Liu, X. Wang and X. W. D. Lou, *J. Mater. Chem.,* 2012, **22**, 16499.
31 B. Y. Xia, H. B. Wu, J. S. Chen, Z. Wang, X. Wang and X. W. D. Lou, *Phys. Chem. Chem. Phys.,* 2012, **14**, 473.
32 B. Y. Xia, S. Ding, H. B. Wu, X. Wang and X. Wen, *RSC Adv.,* 2012, **2**, 792.
33 Z. Liu, S. M. Tabakman, Z. Chen and H. Dai, *Nat. Protoc.,* 2009, **4**, 1372.
34 J. Chen, H. Wang, W. Long, X. Shen, D. Wu, S. S. Song, Y. M. Sun, P. X. Liu, S. Fan and F. Fan, *Int. J. Nanomed.,* 2013, **8**, 2409.
35 X. D. Zhang, D. Wu, X. Shen, P. X. Liu, N. Yang, B. Zhao, H. Zhang, Y. M. Sun, L. A. Zhang and F. Y. Fan, *Int. J. Nanomed.,* 2011, **6**, 2071.
36 G. S. Hong, Y. P. Zou, A. L. Antaris, S. Diao, D. Wu, K. Cheng, X. D. Zhang, C. X. Chen, B. Liu, Y. H. He, J. Z. Wu, J. Yuan, B Zhang, Z. M. Tao, C. Fukunaga and H. J. Dai, *Nat. Commun.*, 2014 (doi:10.1038/ncomms5206).
37 G. S. Hong, J. C. Lee, J. T. Robinson, U. Raaz, L. M . Xie, N. F. Huang, J. P. Cooke and H. J. Dai, *Nat. Med.*, 2012, **18**, 1841.
38 Z. Liu, S. M. Tabakman, Z. Chen and H. J. Dai, *Nat. Protoc.*, 2009, **4**, 1372.
39 H. G. Yang, C. H. Sun, S. Z. Qiao, J. Zou, G. Liu, S. C. Smith, H.M. Cheng and G. Q. Lu, *Nature*, 2008, **453**, 638.
40 J. Xie, Y. Zheng and J. Y. Ying, *J. Am. Chem. Soc.,* 2009, **131**, 888.
41 D. B. Chithrani, S. Jelveh, F. Jalali, M. van Prooijen, C. Allen, R. G. Bristow, R. P. Hill and D. A. Jaffray, *Radia. Res.,* 2010, **173**, 719.
42 X. D. Zhang, J. Chen, Y. Min, G. B. Park, X. Shen, S. S. Song, Y. M. Sun, H. Wang, W. Long and J. Xie, *Adv. Fun. Mater.,* 2014, **26**, 1718.
43 X. D. Zhang, D. Wu, X. Shen, J. Chen, Y. M. Sun, P. X. Liu and X. J. Liang, *Biomaterials,* 2012, **33**, 6408.
44 X. D. Zhang, D. Wu, X. Shen, P.-X. Liu, F. Y. Fan and S. J. Fan, *Biomaterials,* 2012, **33**, 4628.
45 S. Gui, B. Li, X. Zhao, L. Sheng, J. Hong, X. Yu, X. Sang, Q. Sun, Y. Ze and L. Wang, *J. Agr. Food Chem.,* 2013, **61**, 8959.
46 M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall and K. A. Dawson, *Proc. Natl. Acad. Sci. U S A*, 2008, **105**, 14265.
47 C. Michiels, M. Raes, O. Toussaint and J. Remacle, *Free. Radic. Biol. Med.,* 1994, **17**, 235.