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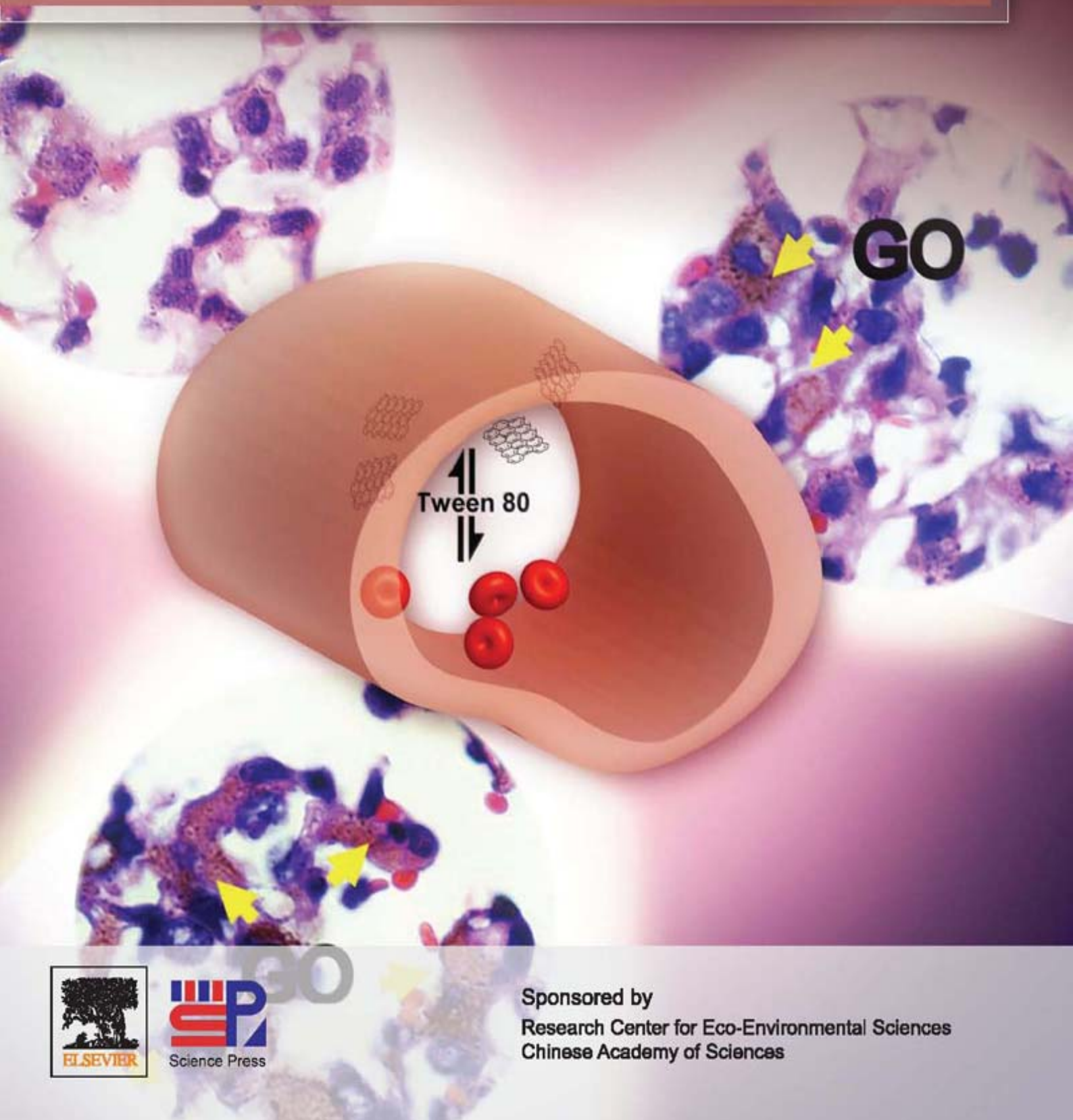

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The *ex vivo* and *in vivo* biological performances of graphene oxide and the impact of surfactant on graphene oxide's biocompatibility

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Abstract

Graphene oxide (GO) displays promising properties for biomedical applications including drug delivery and cancer therapeutics. However, GO exposure also raises safety concerns such as potential side effects on health. Here, the biological effects of GO suspended in phosphate buffered saline (PBS) with or without 1% nonionic surfactant Tween 80 were investigated. Based on the *ex vivo* experiments, Tween 80 significantly affected the interaction between GO and peripheral blood from mice. GO suspension in PBS tended to provoke the aggregation of diluted blood cells, which could be prevented by the addition of Tween 80. After intravenous administration, GO suspension with or without 1% Tween 80 was quickly eliminated by the mononuclear phagocyte system. Nevertheless, GO suspension without Tween 80 showed greater accumulation in lungs than that containing 1% Tween 80. In contrast, less GO was found in livers for GO suspension compared to Tween 80 assisted GO suspension. Organs including hearts, livers, lungs, spleens, kidneys, brains, and testes did not reveal histological alterations. The indexes of peripheral blood showed no change upon GO exposure. Our results together demonstrated that Tween 80 could greatly alter GO's biological performance and determine the pattern of its biodistribution in mice.

Key words: graphene oxide; biological performance; nonionic surfactant Tween 80; intravenous injection
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Introduction

Graphene is a single or a few-layered sheet of sp2-carbon nanomaterial, and it is the building block of fullerene, carbon nanotubes (CNTs), and graphite (Feng and Liu, 2011; Geim, 2009; Wang et al., 2011). Graphene, as well as its oxidized derivative graphene oxide (GO), displays prominent chemical and physical properties with promising applications in diverse fields, such as electronics and biotechnology. Graphene is a versatile building block for designing multifunctional nanoproducts. For instance, graphene has been proved to be an emerging tool to induce differentiation of neural cells (Park et al., 2011). Functionalized GO exhibits great potential in tumor inhibition, drug delivery, and biosensing (Robinson et al., 2011; Wang et al., 2011). Under medical applications, such as drug, biomolecule delivery, and targeted thermotherapy with graphene, these materials might enter the human body and distribute into various organs, posing potential adverse effects on cells. Although a few studies have indicated its biocompatibility towards certain types of cells (Liao et al., 2011; Sasidharan et al., 2012; Singh et al., 2011; Yang et al., 2011; Yue et al., 2012), an increasing number of studies suggest its potential side effects (Li et al., 2012; Sasidharan et al., 2011). The high density of hydroxyl group on its surface endows these materials with distinct biological activities. For example, GO could significantly promote cell death on macrophages and neuronal cells (Li et al., 2012; Zhang et al., 2010). GO was also reported to trigger hemolysis based on an *in vitro* assay (Hao et al., 2011). When intratracheally instilled into lungs, GO caused pronounced interstitial inflammation and epitheloid granuloma (Duch et al., 2011). Under GO exposure through intravenous injection, these materials could accumulate in lungs (Zhang et al., 2011). The intravenously administrated GO could result in remarkable thrombus (Singh et al., 2011), whereas amine-modified graphene could ameliorate the formation of thrombus due to its positive charge (Singh et al., 2012). Therefore, the

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surface properties of GO likely determine its toxicity and its performance under the biological setting.

Surfactant has been widely used in drug delivery design, diagnosis and thermotherapy (Moghimi and Szebeni, 2003), and has also been demonstrated to be one of the most helpful approaches for nanoparticles' escaping from the mononuclear phagocytic system (MPS) reorganization for long circulation (Zhang et al., 2008). Surfactants are of great assistance in obtaining single nanoparticle suspension by hindering agglomeration (Liu et al., 2007; Moghimi et al., 2001). For instance, PEGylation is one of the most successful approaches for controlling nanomaterials' pharmacokinetic behaviors, including circulation time, tissue distribution, and elimination (Hamidi et al., 2006; Liu et al., 2008; Longmire et al., 2008). Synperonic® F108 was recently reported to reduce the toxicity of GO after direct administration in lungs compared with GO without this surfactant assisting (Duch et al., 2011). We previously demonstrated that nonionic surfactant, polyoxyethylene sorbitan monopalmitate Tween 80, can resist carboxylated multiwalled carbon nanotubes (MWCNTs) COOH from aggregation and therefore improve their biocompatibility (Qu et al., 2009). Tween 80 is a commonly used surfactant for drug delivery and nanomaterial administration. However, whether Tween 80 is able to change the surface characteristics of GO and alter its biological activities is elusive.

To investigate the role of Tween 80 in modulating the potential side effects of GO, in the current study, we therefore addressed the accumulation tendency of GO suspension with or without Tween 80 in various organs in mice. Its consequential toxicity was also assessed in these mice.

1 Experimental

1.1 Graphene preparation and characterization

Graphene oxide was synthesized using the Hummers method with minor modifications described in our previous study (Liu et al., 2011). In brief, graphite powder was preoxidized in 12 mL of concentrated sulfuric acid containing 2.5 g $K_2S_2O_8$ and 2.5 g P_2O_5 at 80°C for 4.5 hr. The mixture was then added into 500 mL H_2O, filtered through a 0.20 μm Millipore nylon membrane, washed with H_2O, and air-dried at room temperature. The product was added into 120 mL concentrated sulfuric acid, and 15 g $KMnO_4$ was slowly added into the mixture with stirring in an ice-bath to keep the temperature continuously under 2°C. Afterwards, the product was stirred for 2 hr at 35°C, and diluted with 250 mL H_2O in an ice-bath at a temperature below 50°C. The mixture was stirred for another 2 hr, diluted with 700 mL H_2O and then 20 mL H_2O_2 (30%, *V*/*V*) was added immediately. The mixture was filtered and washed thoroughly with HCl (1:10, *V*/*V*) and H_2O to remove impurity. The enriched portion was finally dialyzed with distilled H_2O for 7 days to remove potential contamination of trace heavy metals, and then air-dried at room temperature. The as-prepared GO was dispersed in H_2O at 1 mg/mL, and ultra-sonicated at 300 W for 1 hr to obtain a clear dispersion. Thereafter, 20 mL dispersion was centrifuged at 12,000 ×*g* for 20 min to separate the small size- and large size-fractions. The supernatant containing the small size-fraction of GO was diluted in the same volume of phosphate buffered saline (PBS) two times, and then centrifuged at 12,000 ×*g* for 10 min. After removal of the supernatant, the pellet was diluted with 2 mg/mL in PBS for further experiments. Purified GO could be well dispersed in PBS for several months.

1.2 Assessment of the interaction between graphene oxide and peripheral blood

Peripheral blood was collected from heart and diluted 50-fold with PBS. Thereafter, 100 μL GO suspension in PBS (called GO1 here) or GO suspension in PBS containing 1% Tween 80 (called GO2 here) was added to the blood dilution in individual tubes. The packing of blood cells was then examined at 0, 15, 30, 60, and 120 min.

1.3 Animal experiments and graphene oxide administration

All animal care and surgical procedures were approved by the Animal Ethics Committee at Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Male BALB/C mice (6–8 weeks old) were purchased from the Vital River laboratories, China and housed under a standard SPF condition. After 1-week acclimation, the mice were randomly divided into three groups, i.e., group A, group B and group C, and each group consisted of 8 mice (n = 8). For group A, the vehicle control group, mice received PBS containing 1% Tween 80 only. For group B and group C, mice intravenously received a single dose of 1000 μg/mL GO1 and GO2 in a volume of 200 μL, respectively. Mice were sacrificed 24 hr post the GO administration.

1.4 Histopathological examination

Organs including brain, livers, lungs, kidneys, hearts, spleens and testes were collected from each animal immediately after sacrifice. Tissues were immersion-fixed in 10% formaldehyde in PBS. Tissue sections were prepared after dehydration and embedding in paraffin. The sections were stained with hematoxylin and eosin, and subsequently processed for histopathological examination under a light microscope.

1.5 Tissue digestion

After perfusion with saline, livers were perfused with 0.05% collagenase, and then minced and re-suspended in 0.05 g/mL collagenase type IV (Sigma-Aldrich) in Hank's Balanced Salt Solution without either cadmium

or magnesium for enzymatic digestion at 37°C for 30 min. The digested samples were passed through 70 μm filters and the single-cell suspension was assayed using flow cytometry (FACS). Spleens were minced and the total splenocytes were processed for FACS analysis.

1.6 FACS analysis

Cells were re-suspended in PBS and then incubated with FITC-conjugated anti-F4/80 mAb (eBioscience) to select the macrophage population. PE-conjugated anti-Ter119 mAb (BD Pharmingen) was applied to cell suspension for erythroid cell selection. After washing, cells were analyzed on a FACS Calibur^{TM}(BD Biosciences).

1.7 Statistical analysis

Statistical analysis of experimental data was performed using Student's *t*-test. Mean values and standard deviations (SD) were calculated, and data were shown in mean ± SD. A value of $p < 0.05$ was considered significant and that of $p < 0.01$ was considered highly significant when compared to the corresponding control.

2 Results

2.1 Graphene oxide characterization

The size of GO used in the present study was approximately 300–1000 nm, and the thickness was ~1 nm (**Fig. 1a**). To evaluate the agglomeration propensity of GO, GO1 and GO2 were compared. As shown in **Fig. 1b**, both GO1 and GO2 samples were stable for at least 1 month. This characteristic was different from that of MWCNT COOH as previously described (Qu et al., 2009). Our results suggested that the same amount of MWCNT COOH could not be suspended well in PBS; however, it was well dispersed in PBS supplemented with 1% Tween 80 (data not shown). Nevertheless, the zeta potential was significantly changed by the addition of 1% Tween 80, as the zeta potential of GO in PBS was approximately –29.1 mV in contrast to a value of –3.9 mV in PBS containing 1% Tween 80. These combined results demonstrated that the addition of Tween 80 greatly changed the aggregative behaviors of GO by abolishing its negative charge.

2.2 Graphene oxide provoked packing of blood cells

To address whether Tween 80 could alter the biological performance of GO under a biological setting, we looked at the packing of blood cells with treatment of GO1 or GO2. As shown in **Fig. 2**, all samples existed in evenly clear blood cell suspension before treatment (0 min). After incubation with GO1 and GO2, the speed of blood cell packing was largely accelerated from 15 to 120 min, especially for the samples treated with GO1, compared to the blank control. After incubation for 30 min, GO1 substantially caused aggregation of blood cells to tube bottom in comparison to the blank control, which was hardly visualized in the tubes with Tween 80, suggesting Tween 80 greatly undermined the ability of GO in promoting blood cell deposition. After 2 hr incubation, most blood cells were packed to tube bottom with exposure to GO1; whereas, the addition of Tween 80 largely reversed the aggregation formation and slowed down the deposition. These results indicated that Tween 80 could greatly blunt the active groups on GO, and thus attenuate the interaction between GO and blood cells.

2.3 Graphene oxide accumulation within lungs in mice challenged by graphene oxide

After GO administration into mice, no mortality in any group was found, and no sign of gross toxic symptoms (such as body weight loss and abnormal activity or diet) was observed. Previous studies suggested that lungs were often the predominant organ for GO accumulation in animals upon exposure to GO (Zhang et al., 2011), coupled to remarkable injuries to lungs, including thrombus formation (Duch et al., 2011; Yang et al., 2011). We thus examined the morphological and histological alterations of lungs from mice administrated with GO1 or GO2. The lungs

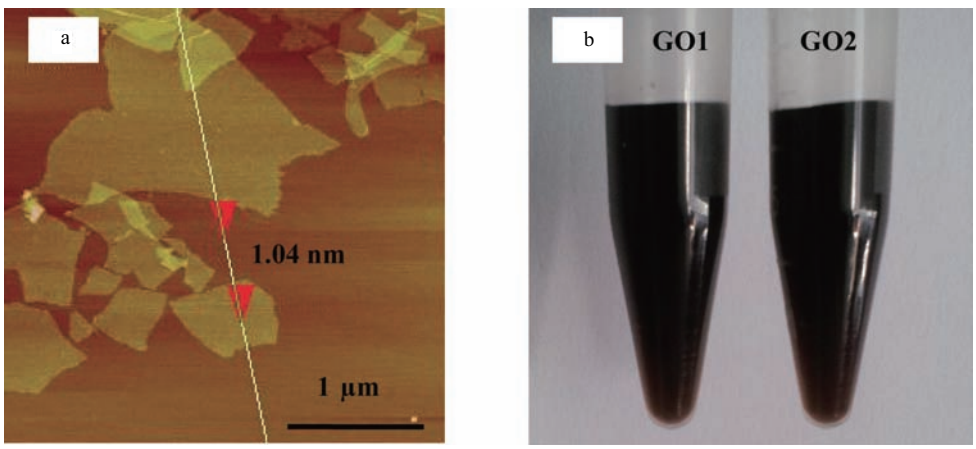

Fig. 1 Characterization of GO. (a) AFM topography image of GO, the thickness of GO was 1.04 nm; (b) the appearance of GO suspension after 1 month at room temperature. GO suspended in PBS (GO1) and GO suspended in PBS containing 1% Tween 80 (GO2).

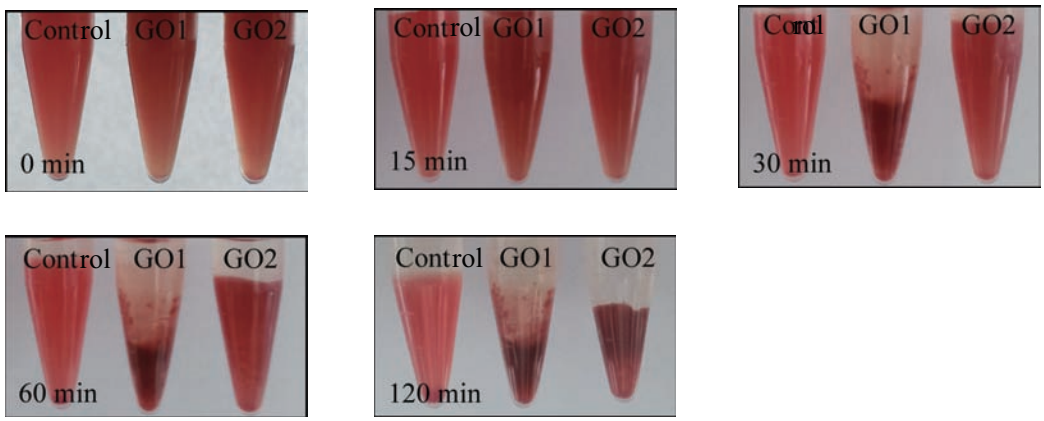

Fig. 2 Representative images describing blood cell packing and the interaction between GO and the diluted blood from mice at different time points.

from the control mice appeared normally pink (**Fig. 3a**); in stark contrast, the lungs from the GO1-treatment mice were heavily dark, suggesting the retention of a large amount of GO in these lungs (**Fig. 3b**). Meanwhile, the

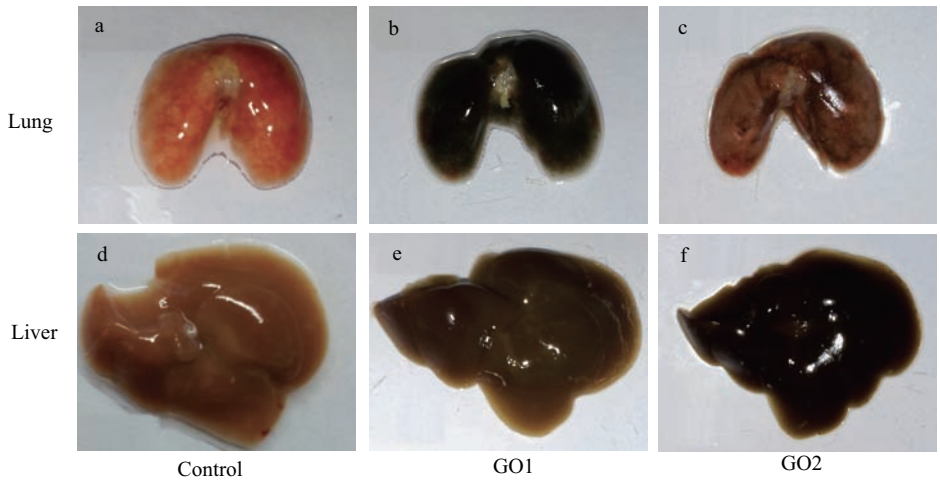

Fig. 3 Graphene oxide's accumulation in lungs and livers. The upper panel (a–c) shows representative photographs of lungs from the 3 groups, and the bottom panel (d–f) presents representative images of livers from each group.

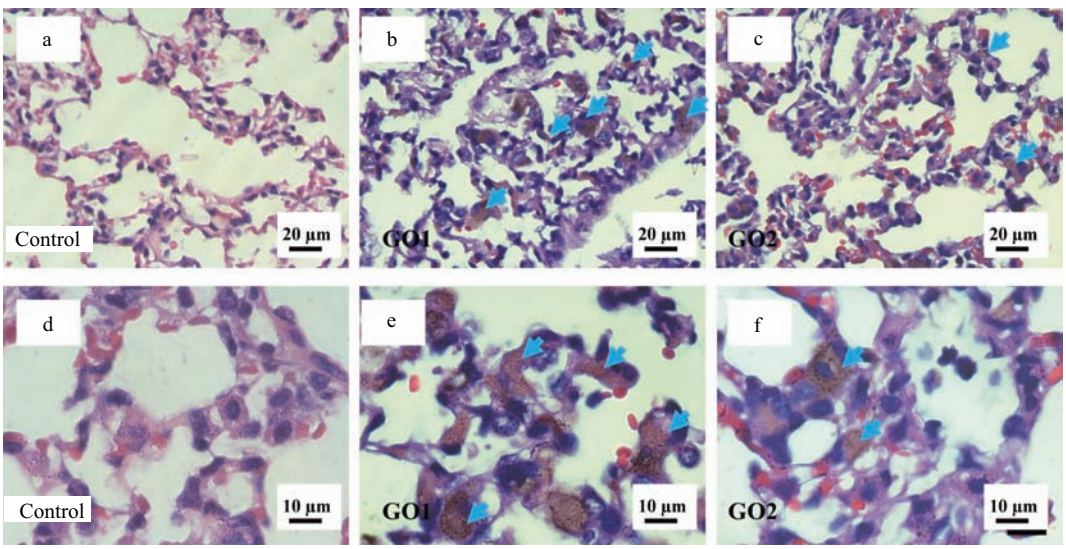

Fig. 4 Representative histological images of lungs from mice challenged by GO1 or GO2. Hematoxylin and eosin stains of lungs tissues of control mice treated with PBS containing 1% Tween 80 (a, d), GO1 (b, e) and GO2 (c, f). The original magnification for the upper panel is 40×, and 100× for the lower panel. Arrows show GO aggregations.

lungs from mice treated with GO2 displayed a slightly dark color, indicating that Tween 80 remarkably reduced GO accumulation in these lungs (**Fig. 3c**). The results demonstrated that GO1 agglomerates were rapidly cleared from the circulation and became accumulated into lungs, likely due to the filtration of the capillary bed; whereas, Tween 80 significantly ameliorated GO's accumulation into lungs through increasing its biocompatibility towards cells.

2.4 Graphene oxide localization in other organs

To determine whether GO was able to accumulate in the MPS in addition to that in lungs, other organs were also collected and observed after perfusion. Similar to the changes for lungs, livers from mice upon GO exposure displayed dark color for both the GO1 and GO2 groups, compared to those in the control group with light red color (**Fig. 3d**, **e** and **f**). However, the livers from the GO1-treated mice appeared light brown (**Fig. 3e**), while the color was much darker in the livers from the GO2-treated mice (**Fig. 3f**). Additionally, other organs, such as brains, kidney, and testes, did not show any gross abnormalities in appearance after GO administration for both the GO1 and GO2 groups, compared to the control group (data not shown). These observations suggested that Tween 80 could greatly reduce GO retention in lungs, and GO2 with the assistance of Tween 80 had greater mobility in the circulating system and tended to accumulate in livers.

2.5 Histological alterations in organs in response to GO treatment

To investigate injuries in organs in response to GO treatment, we carried out a histological examination after staining with hematoxylin and eosin staining with hematoxylin and eosin. As the blue arrows show in **Fig. 4a–c**, GO aggregation was clearly visible in lungs from mice challenged by GO in contrast to lungs from the control mice. The amount of GO in lungs was much greater in the GO1-treated mice compared to those in GO2-treated mice (**Fig. 4b, c**), consistent with the resulting color changes. Moreover, a large number of brown GO agglomerates were located within macrophages in lungs (**Fig. 4e, f**), in comparison to those in the control group (**Fig. 4d**), suggesting a vital role of macrophages in engulfing these invading nanoparticles. It should be noted that no significant histological impairments were observed in lungs in spite of GO accumulation, which might be due to the limited time of exposure to GO.

Although the mass of GO1 agglomerates in lungs was significantly greater than that of GO2 (**Fig. 4**), its distribution in livers was fewer than that of GO2 (**Fig. 5**). The great difference between the GO1 group and the GO2 group confirmed the important role of Tween 80 in endowing GO with a greater ability to pass the capillary bed without massive deposition in lungs. This finding was similar to the cumulative characteristic of Tween 80 assisted MWCNT COOH dispersion as previously reported (Qu et al., 2009). The reason for this difference presumably resided in the first filtration of GO by lungs when circulating in blood. Sequentially, the remaining GO in circulation might be cleared by livers, leading to greater GO deposition in livers for the GO2 group than the GO1 group. Therefore, the accumulation of GO in livers was likely subject to the dynamics of filtration by lungs. In livers, most GO aggregates were localized within kuppfer cells (macrophages) and no GO could be found in hepatocytes (**Fig. 4**), which highlighted the important role of MPS in clearing GO from circulation. These results were consistent with studies on other types of nanomaterials (Liu et al., 2008; Qu et al., 2012; Yang et al., 2007). For example, it was documented

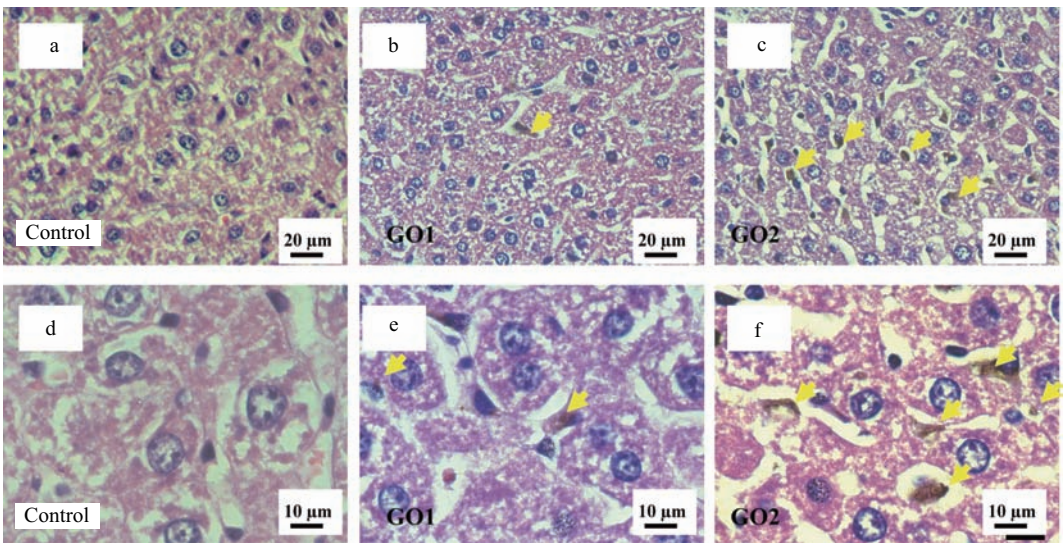

Fig. 5 Representative histological images of livers from mice with exposure to GO1 or GO2. Hematoxylin and eosin stains of lungs tissues of control mice treated with PBS containing 1% Tween 80 (a, d), GO1 (b, e) and GO2 (c, f). Arrows indicate GO aggregations. The original magnification for the upper panel is 40×, and 100× for the lower panel.

that single-walled CNTs were engulfed by kuppfer cells, evidenced by transparent electron microscopy (Qu et al., 2012). We also demonstrated that quantum dots were predominantly phagocytized by macrophages using a co-culture system with both hepatocytes and macrophages (Qu et al., 2012).

No GO localization was found in kidneys from mice administrated with GO for both the GO1 group and the GO2 group, suggesting that GO aggregates and agglomerates were not able to penetrate the glomerular basement membrane when going through kidneys. This was probably attributable to the inability of larger particles, such as GO, to cross the basement membrane. To this end, no histopathological changes were observed in kidneys (data not shown). Previous studies reported that carbon-based nanomaterials might transport through the blood-brain barrier or the blood-testes barrier (Bai et al., 2010; Yang et al., 2007). We thus evaluated whether GO also translocates into brains and testes coupled with histological damages. Based on a pathological examination, we found no GO aggregation in brains or testes, and no significant histopathological changes in these sites from mice upon treatment with GO (data not shown).

2.6 No alterations to blood constituents in mice challenged by GO

The blood circulating system is the vehicle for GO's systemic translocation and tissue distribution. Upon the intravenous administration, the blood circulating system is presumably the first barrier against the invasion of graphene. In other words, blood cells take the primary responsibility in governing graphene's trafficking and systemic translocation. We further investigated whether GO could pose side effects to blood cells after intravenous injection. The CBC analysis indicated that the hematological indexes, including white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume and platelet, were not significantly altered in mice on day 1 post-treatment (**Fig. 6**, $p > 0.05$). These data suggested that GO did not exert acute toxicity on blood cells, which might be partially due to a rapid clearance of GO from circulation with a greatly reduced chance of GO's performance on blood cells (**Fig. 6**).

3 Discussion

Since the zeta potential of nanomaterials largely determines their interaction with cells and proteins (Nel et al., 2009), we addressed the biological performance of GO with distinct zeta potentials. Our results demonstrated that GO suspension could incur a rapid packing of blood cells; however, the addition of Tween 80 essentially changed the zeta potential of GO and greatly slowed down blood cell packing. As foreign particles, the fate of most intravenously injected nanomaterials is to be cleared from circulation by MPS. MPS recognizes nanomaterials through the binding of serum opsonin proteins to nanomaterials (Gaumet et al., 2008). The physical-chemical properties of nano-

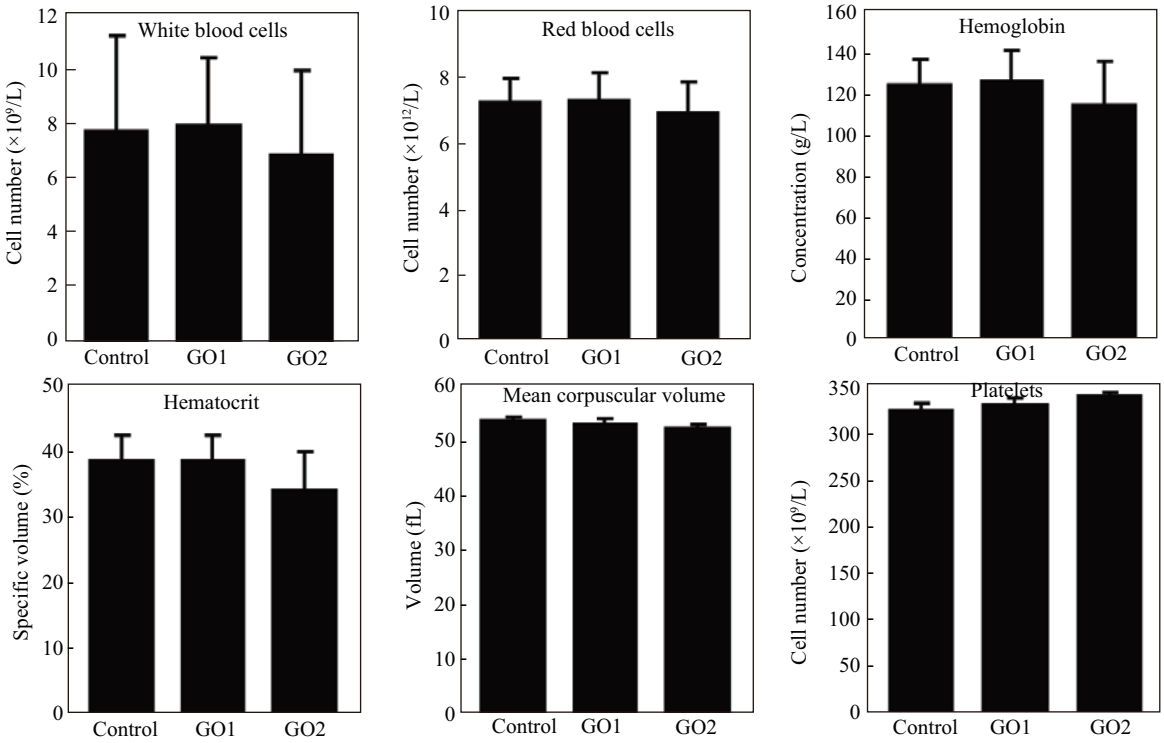

Fig. 6 Results of blood cell indexes. The complete blood count analyses were performed in the 3 groups for white blood cells, red blood cells, hemoglobin concentration, hematocrit, mean corpuscular volume, and platelets.

materials affect their interaction with MPS and eventually decide the course in circulation, tissue biodistribution, and elimination (Gaumet et al., 2008). For example, stronger hydrophilicity might partially prohibit the interaction between nanomaterials and serum proteins, and thus eliminate the possible changes in protein conformational structure, which is contributive to the MPS recognition (Liu et al., 2008). With respect to carbon-based nanomaterials, MPS also takes a leading role in clearing them from the circulation, e.g., engulfment by macrophages. For instance, most types of CNTs were taken up by MPS (Guo et al., 2007; Liu et al., 2007, 2008; McDevitt et al., 2007; Yang et al., 2007), while limited CNTs were eliminated through kidneys without clearance by MPS due to shape and size variation. Regarding fullerene, intravenously-injected hydroxylated fullerene was mainly deposited in livers, spleens and kidneys (Cagle et al., 1999). In terms of graphene, a recent study documented that PEGylated graphene was cleared from the blood pool and retained in lungs, livers and spleens owing to the uptake by MPS, in parallel to the fate of other nanomaterials (Ding et al., 2012). Similar to most carbon-based nanomaterials, in the current study, GO deposition was observed in lungs and livers, indicating that MPS played a principal role in GO's clearance from blood. After exposure, both GO1 and GO2 could be found in lungs, and a greater amount of GO was demonstrated in lungs from mice treated with GO1. The increased accumulation of GO1 in lungs was likely attributable to GO-induced aggregation on blood cells within lungs, which were retained by capillaries in lungs. In contrast, pre-treatment of GO with Tween 80 fundamentally changed the biological actions of GO in circulation, and largely ameliorated blood cell packing and GO's own aggregation within lungs, which significantly decreased GO retention in lungs. These findings further confirmed the notion that the interaction between nanomaterials and blood cells affected their biodistribution and potential toxicity (Albanese et al., 2012; Chambers and Mitragotri, 2007). Previous studies suggested that negative charges on GO surface triggered activation of PLT with a resultant formation of thrombus in lungs, which could be reversed through the modification of GO with amine (Singh et al., 2011; Yang et al., 2011). Our results revealed that the remarkably changed zeta potential of GO reduced the ability of GO to activate PLT and the chance of GO's binding to blood cells as well. Fewer GO aggregations could be found in lungs from the GO2-treated mice compared to those from the GO1-treated mice, demonstrating that Tween 80 is of great assistance in eliminating the potential risks to lungs in response to GO.

The uptake of nanomaterials by macrophages in MPS might impair macrophagic functions. We previously demonstrated that intravenously-injected quantum dots largely undermined the macrophagic capacity in phagocytosis, and thus resulted in pronounced retention of aged RBCs within spleens with causal splenomegaly (Qu et al., 2012). In this study, upon GO exposure, the average weight of spleens did not significantly change in comparison to that in the control group. The portions of erythrocytes and macrophages were similar in spleens from mice treated with GO compared to those from the control mice, suggesting that a single dose injection of GO did not incur impairment of the ability of macrophages to clear aged RBC. A recent study demonstrated that PEGylated GO could deposit in kidneys (Yang et al., 2011). Different from this finding, here we did not observe the occurrence of GO aggregation in kidneys. This result implied that GO aggregates were rapidly eliminated through kidney filtration. Nonetheless, we could not exclude the possibility of trace existence of single dispersed GO in kidneys. It has been documented that certain carbon nanomaterials, such as MWCNTs, could transport through the blood-testes barrier, and consequentially harmed reproduction due to the accumulation of CNT in testes and the resultant oxidative stress in mice (Bai et al., 2010). At a similar dose, we did not find that GO incurred impairments to the reproductive system in male mice in the current study. Additionally, GO was able to cause hemolysis of RBC according to an *in vitro* hemolysis assay reported in a recent study (Liao et al., 2011). In contrast, another study revealed the compatibility of GO towards RBC (Sasidharan et al., 2012). These observations indicated that the size or surface modification largely determined the biocompatibility of GO towards RBC. We demonstrated here that a single dose of intravenously-injected GO posed no toxicity to blood cells upon 1-day exposure, although chronic studies are guaranteed for closer evaluation.

4 Conclusions

We studied here the potential acute toxicity of intravenously-injected GO in mice. GO aggregation was predominantly accumulated in lungs and livers after 1-day exposure. Upon exposure for this short time, GO did not cause damage to blood cells, and GO's accumulation in lungs and livers induced no acute adverse effects to them. The interaction between GO and plasma essentially determined the cumulative tendency of GO in lungs and livers. Tween 80 fundamentally altered the zeta potential of GO and further prevented the interaction of GO with blood cells, with diminished stimulation of blood cell packing. Therefore, the surfactant Tween 80 strikingly changed the pattern of the biodistribution of GO in organs with reduced GO aggregation in lungs and an increased amount in livers.

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