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Single and repeated dose toxicity of citric acid-based carbon dots and a derivative in mice

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Carbon dots(CDs) have recently emerged as a new class of fluorescent nanomaterials and are already competitive in many respects to conventional semiconductor quantum dots(QDs). Despite CDs remarkable advantages in bioimaging, biosensing and other biomedical applications, their biosafety is still unclear. In the present study, the systematic single and repeated dose toxicity and biodistribution of two citric acid-based probes(CDs and a derivative, Et-IPCA) were investigated in vivo. For the single dose toxicity studies, the lethal dose 50 (LD_{50}) of CDs was 391.615 mg/kg and 357.771 mg/kg for female and male mice, respectively, while Et-IPCA reached its saturation point in solution at 25 mg/kg and did not lead to the death of any mice at that concentration. For the repeated dose toxicity studies, although there were temporary changes in hematological parameters suggesting an acute inflammatory response after seven doses, the data on the body weight, organ coefficients, hematological parameters, blood biochemistry, and organ histopathology at the end of the 90-day recovery period suggested that both the CDs and Et-IPCA had low toxicity over the 90-day period. These findings will be useful for the future development of CDs-based drug delivery systems and for the development of clinical applications of these systems.

1. Introduction

The importance of fluorescent probes in biomedical research and practice is rapidly increasing with the rapid developments in fluorescence microscopy, laser technologies, and nanotechnology. Since the discovery of fluorescent fragments in a batch of single-wall nanotubes in 2004,[1] carbon dots (CDs)[2, 3] of different intrinsic structures, including graphene quantum dots (GQDs),[4-6] carbon nanodots (CNDs)[7-9] and polymer dots (PDs),[10,11] have been prepared and characterized. These fluorescent carbon-based materials have drawn increasing attention for their exceptional advantages, such as high optical absorptivity, chemical stability, biocompatibility, and low toxicity, which distinguish them from traditional fluorescent materials and make them promising candidates for numerous applications. Compared to traditional quantum dots (QDs), CDs have shown greater potential for biological applications because they are heavy-metal-free. Hence, much research using CDs for optical bioimaging[12, 13] and drug delivery[14] has been carried out. On the other hand, the biocompatibility of CDs remains a key reason for restricting their further use *in vivo*.[15]

The toxicity of CDs has already been reported, and the results showed that it would be safe to use these kinds of CDs as biological probes for optical bioimaging.[16-18] Zhang's group has studied the synthesis, characterization, *in vitro* toxicity and *in vivo* biodistribution and toxicity of a CDs sample obtained from graphene oxide(GO).[17] They found that the CDs showed no obvious influence on mice owing to their small size, while GO appeared toxic, and even caused death in mice. CDs possess no obvious *in vitro* or *in vivo* toxicity, even after multiple doses. Apart from that study, Cui's group has also successfully prepared CDs with good stability, high dispersibility, and water solubility.[18] They have demonstrated that no significant toxic effects, and no abnormalities or lesions were observed in the organs of the animals at all studied CDs doses. However, more efforts are still required to fully assess the toxicity of CDs considering that there exist so many carbon-based materials which possess the potential to be prepared into CDs. Accordingly, an improved understanding of the toxicity of various CDs is essential, and long-term toxicological and pharmacokinetic investigations involving the degradation, excretion,

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persistence and immune response associated with CDs will ultimately be needed to design carbon-dot-based probes for clinical imaging and therapy.

We have previously established a facile and high-output strategy for the fabrication of CDs using citric acid and ethylenediamine using a hydrothermal method[9] , and obtained polymer-like CDs. On the basis of this strategy, a distinct bright blue fluorophore (imidazo[1,2-a]pyridine-7-carboxylicacid, 1,2,3,5-tetrahydro-5-oxo-, IPCA) was extracted.[19] It was proven to be an important component in CDs and the photoluminescence center for the molecular state. However, pure IPCA is not stable in diluted aqueous solutions, in which light and/or oxygen can affect its photoluminescence (PL) behavior. We thus improved the starting materials using N-ethylethane-1,2-diamine (Et-EDA) instead of EDA and obtained 1-ethyl-5-oxo-1,2,3,5-tetrahydroimidazo-[1,2-a]pyridine-7-carboxylic acid (Et-IPCA).[19] CDs are nano-material, while Et-IPCA is more like an organic dye. However, both of them are citric acid-based probes and share the advantages of facile synthesis, water solubility and a high quantum yield. Compared with CDs, Et-IPCA seems to be advantageous because of its fully resolved chemical structure, resistance to photo-bleaching and higher quantum yield. Although these two fluorescent probes have shown great potential in bioimaging, assessments of their biosafety have not been carried out.

Herein, we systematically studied the single- and repeated-dose toxicity of these citric acid-base probes (CDs and Et-IPCA) in ICR mice. The CDs and Et-IPCA were synthesized via a simple, scalable method, then their cytotoxicity to four different cell lines (HepG2, L02, MCF-7 and HUVEC) was determined by the SRB assay, and hemolysis was also assessed. To mimic clinical drug administration and to more practically study the effects of carbon-based probes in mice, both single- and repeated-dose toxicity testing were carried out using studies designed according to State Food and Drug Administration's (SFDA) acute and chronic toxicity test technical guidelines for chemical drugs (guidelines). In the repeated-dose toxicity test, the body weight, hematological parameters, blood biochemistry, and organ histology were determined at 1, 7, 30, and 90 days after beginning administration. Moreover, the biodistribution of these two probes was examined as well. The results suggest that both CDs and Et-IPCA can serve as optical probes for specific clinical applications.

2. Experimental section

2.1 Materials

Citric acid(CA) was purchased from Aladdin (citric acid anhydrous, 99.5%), ethylenediamine (EDA) and N-ethylethane-1,2-diamine (Et-EDA) were purchased from Alfa Aesar (99%). Sulforhodamine B was purchased from Sigma-Aldrich. RPMI-1640 medium was obtained from GIBCO. Fetal bovine serum (FBS), penicillin and streptomycin were from the Beyotime Institute of Biotechnology (Jiangsu, China). All reagents were commercially available products with analytical grade purity and were used without further purification.

2.2 Synthesis of CDs and Et-IPCA

CDs were prepared by a bottom-up carbonization method as reported previously[9]. Typically, 5 mmol CA (0.9605 g) and 5 mmol EDA (335 μL) were dissolved in ultrapure water (10 mL). Then, the solution was transferred to a poly(tetrafluoroethylene) (Teflon)-lined autoclave (25 mL) and heated at 200℃ for 10 h. After the reaction, the reactors were allowed to cool naturally to room temperature.The CDs solution was then evaporated to obtain the CDs powder. Et-IPCA was synthesized by hydrothermal treatment and was purified by column chromatography as reported previously[19]. A total of 1 mmol CA (0.1921 g) and 1 mmol Et-EDA (67 μL) were dissolved in ultrapure water (10 mL), then, the solution was transferred to a poly(tetrafluoroethylene) (Teflon)-lined autoclave (25 mL) and heated at 140℃ for 10 h. After reaction, the reactors were cooled to room temperature by water or were allowed to cool naturally to obtain a fluorescent Et-CDs solution. Et-IPCA was extracted from the solution by column chromatography using methanol as the developing solvent.

2.3 Characterization of the CDs and Et-IPCA

Fluorescence spectroscopy was performed using a Shimadzu RF-5301 PC spectrophotometer. UV-vis absorption spectra were obtained using a Shimadzu 3100 UV-vis spectrophotometer. Nanosecond fluorescence lifetime experiments were performed on a fluorescence spectrometer. (Edinburgh Instrument, FLS 980). High-resolution transmission electron microscopy (HRTEM) was performed using a JEM-2100F instrument. NMR spectra were obtained on an AVANCEIII500 (500 MHz) from Bruker. Dimethyl sulfoxide-d6 (with 0.03% TMS) was chosen as the solvent for the samples.

2.4 Cell culture and cytotoxicity assessment

The human hepatocellular carcinoma cell line (HepG2), human hepatic embryo cell line (HL-7702; L02), human breast cancer cell line(MCF-7), and human umbilical vein endothelial cells(HUVEC) were maintained at 37°C under 5% CO_2 in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The *in vitro* cytotoxicity of CDs and Et-IPCA in the L02, HepG2, MCF-7 and HUVEC cells were assessed using traditional SRB assays. The sulforhodamine B (SRB) assay is routinely used for cytotoxicity determination, based on the measurement of the live cell protein content. In general, cells were seeded into 96-well plates and then treated with the indicated concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100, 250, and 500 μg/mL) of CDs or Et-IPCA for 72 h. Afterwards, 100μL of 20% trichloroacetic acid (TCA) was added to the culture medium in each well, and the plates were stored at 4℃ for 1 h. After that, the plates were washed three times with a

gentle stream of deionized water, and the excess water was tapped out onto paper towels. A blow dryer was used to completely dry the plates. Then, 100 μL of 0.4% SRB, which included 1% acetic acid, was added to each well, and the plates were incubated for 30 minutes at room temperature. After flicking the SRB off and washing the plates with 1% acetic acid, the plates were dried and 150 μL of 10 mM Tris base solution was added to each well. The samples were shaken for 5 minutes. The absorbance was read by a microplate reader at 570 nm. The survival rate of the cells was expressed as the A/B 100%, where A was the absorbance value from the experimental cells and B was that from the control cells.

2.5 Hemolytic testing

To study the hemolytic characteristics of CDs and Et-IPCA, a classic hemolytic test was conducted. First, 50 mL of rabbit blood in a glass flask was stirred with a cotton swab to remove fibrous proteins. Then, 100 mL of saline was added. The mixture was centrifuged at 1,500 r/min for 15 min, and the sediment was reserved. This procedure was repeated twice until the supernatant was clear. After that, we generated 2 mL of red blood cell (RBC) sediment, which was red blood cells (RBCs) mixed with 100 mL of saline to obtain a 2% RBC suspension. The CDs and Et-IPCA were dissolved to different concentrations(12.5, 25, 50, 100, 250, and 500 μg/mL) with saline. Then, 2.5 mL of the above solutions and 2.5 mL of RBC suspension were mixed in different glass test tubes. We used 2.5 mL of saline and deionized water with 2.5 mL of RBC suspension as negative and positive controls, respectively. The mixtures were gently shaken up and then allowed to settle for 3 or 24 hours at room temperature. Finally, the mixtures were centrifuged for 2 min at 4,000 r/min, and the absorbances of the upper clear solutions were recorded by amicroplate reader at 540 nm. The percentage of hemolysis in each of the samples was calculated by dividing the difference in absorbance between the sample and the negative control by the difference in absorbance between the positive and negative controls.

2.6 Animals and single-dose toxicity testing

All animal experimental protocols were approved by the Ethics Committee for the Use of Experimental Animals of Jilin University. Both sexes of ICR mice (aged 6-8 weeks) were used in the experiments. Ten of each sex of mice were housed in stainless steel cages containing sterile paddy husks as bedding in ventilated animal rooms. The mice were acclimated to the controlled environment (temperature: 25°C; humidity: 60% and light: 12 h light/dark cycle) with free access to water and a commercial laboratory complete chow.

The single-dose toxicity testing was performed before the repeated-dose toxicity testing to investigate the preliminary toxicity profiles of CDs and Et-IPCA, as well as to determine the doses that would be used in the latter studies. In general, the median lethal dose (LD_{50}) was calculated by an improved Bliss method. Several groups of ICR mice were used in a pre-test to determine the minimum dose (Dn) and maximal dose (Dm) of CDs and Et-IPCA. Intravenous injections of CDs and Et-IPCA solutions, which were prepared in physiological saline, were conducted through a mouse tail vein. The Dn and Dm for CDs were 250 mg/kg and 500 mg/kg, respectively. With regard to Et-IPCA, it reached its saturation for solubility in saline at 25 mg/kg, and all mice survived after treatment at that dose. Based on these findings, combined with those of previous results, we concluded that Et-IPCA was safe to use for single-dose treatment, and the single dose toxicity of CDs was further investigated.

One hundred ICR mice were divided randomly into five groups, a control group and four experimental groups, with ten male and ten female mice in each group. The doses used in the LD_{50} experiment were 250 mg/kg, 320 mg/kg, 400 mg/kg and 500 mg/kg. All mice were fed adaptively for seven days, then were treated with different concentrations of CDs saline solutions via a tail vein and were observed for 14 days. Mortality was recorded, and all survival mice were sacrificed. The value of the LD_{50} was calculated using the Origin 6.0 software program.

2.7 Repeated-dose toxicity testing

For the subsequent repeated-dose toxicity study, 160 male ICR mice were randomly divided into groups that received CDs (60 mice), Et-IPCA (60 mice) or the control (40 mice). The mice were injected with CDs (100 mg/kg, by tail vein), Et-IPCA (25 mg/kg, by intraperitoneal injection) or saline once a day for one week. Then, all mice were weighed once a week, and 10 mice from each group were sacrificed on days 1, 7, 30 and 90 days after injection. The symptoms and mortality were observed and recorded carefully throughout the entire study. At the end of the study, all animals were sacrificed.

2.8 Hematological analysis and blood biochemical assay

Blood drawn from the eyeball was collected for blood tests. To reveal any potential toxic effects of CDs and Et-IPCA on the treated mice, blood biochemistry and hematological analyses were carried out. Important markers, including the numbers of white blood cells and red blood cells, the hemoglobin level, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet countfor the hematological assessment, and the alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), albumin (ALB), blood urea nitrogen (BUN), serum creatinine (CRE), total cholesterol (TC) and total triglycerides (TG) for the blood biochemistry studies were selected.

2.9 Organ coefficients and histopathological examinations

The tissues and organs, such as the liver, spleen, kidney, heart, lung, and brain were excised and accurately weighed. The coefficients of the liver, spleen, kidneys, heart, lungs,

and brain to body weight were calculated as the ratio of the tissues (wet weight, mg) to the body weight(g). Then, these tissues recovered from the necropsy were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin(H&E) and examined under a digital microscope.

2.10 Biodistribution of the CDs and Et-IPCA

For the *in vivo* biodistribution studies, 30 male ICR mice were randomly into a CDs group, Et-IPCA group and control group and were injected with CDs (25 mg/kg), Et-IPCA (25 mg/kg) or saline through a tail vein. Then, five mice from each group were sacrificed at three hours and 24 hours after injection. Approximately 1.0 mL of blood from each mouse was collected and centrifuged at 3,500r/min for 10 minutes to collect serum. Then, the serum was diluted four-fold with saline. Major organs, including the liver, spleen, lungs, heart, kidneys and brain, from these mice were harvested and stored in 3 mL of saline. These organs were homogenized, and the tissue homogenates were centrifuged at 3,500 r/min for 10 minutes. The supernatants were collected and examined on a spectrofluorophotometer with a 360 nm excitation wavelength, then the photoluminescence (PL) intensity of the emission spectra at 450 nm were recorded.

2.11 Statistical analysis

The data were expressed as the means ± SD. The statistical significance of the data was compared by Student's *t*-test. An analysis of variance (ANOVA) was used to analyze the differences among the different groups. The level of statistical significance for all tests was set at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of CDs and Et-IPCA

Our group previously reported a facile and high-output strategy for the fabrication of high-quantum-yield CDs from citric acid(CA) and ethylenediamine (EDA), as well the discovery ofa type of bright blue fluorophore(Et-IPCA) derived from CDs.[9,19] These highly photoluminescent probes have drawn increasing attention for their potential applications in multicolor patterning, biosensors, and cell imaging due to their advantages over other fluorophores. As shown in Fig. 1a, the Et-IPCA showed a strong blue PL in an aqueous solution, and its absorption peaks were at 242 nm and 360 nm, which is similar to that of CDs. However, the Et-IPCA exhibited a stronger fluorescence intensity and better photostability than CDs under excitation at 360 nm, indicating that this molecule has stable optical properties that may be useful in bioimaging applications. The appearance of CDs can be confirmed by TEM, and Fig. 1b showed that the CDs had a uniform dispersion without any aggregation, with the diameter of particles being between 1-5 nm. The structure of Et-IPCA can be confirmed from the ^{1}H-NMR spectra(Fig. 1c). Notably, both the CDs and Et-IPCA solutions could be stored at room temperature for as long as 90 days without the formation of any precipitates or a loss of fluorescence(Fig. S1, ESI†). Collectively, these findings indicate that the prepared CDs and Et-IPCA have good biocompatibility and photostability.

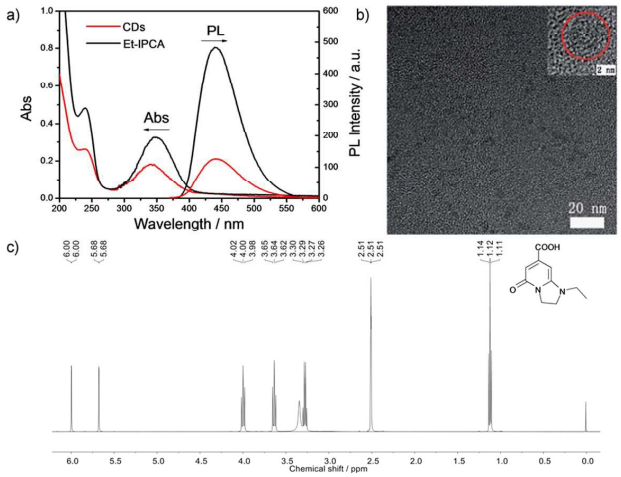


Fig. 1 Characterization of the CDs and Et-IPCA. (a) The UV-vis absorption spectra and fluorescence emission spectra of CDs and Et-IPCA. (b) High-resolution transmission electron microscope (HRTEM) images of the CDs and Et-IPCA. Scale bars are 20 nm. (c) ^{1}H-NMR of Et-IPCA.

3.2 Cellular toxicity and hemolytic testing of CDs and Et-IPCA

The first set of experiments in this study compared the toxicity of CDs and Et-IPCA *in vitro*. The human hepatocellular carcinoma cell line (HepG2), human hepatic embryo cell line (HL-7702), human breast cancer cell line(MCF-7), and human umbilical vein endothelial cells(HUVEC) were chosen for these investigations and were cultivated in the presence of increasing concentrations of CDs and Et-IPCA (1.5625 μg/mL to 500 μg/mL). The cell viability was measured after a 72-h incubation using the sulforhodamine B(SRB) assay. As shown in Fig. 2, no significant reduction in cell viability was observed after the 72-h incubation with either CDs or Et-IPCA, even when the concentration of the CDs was 500 μg/mL, demonstrating that these citric acid-based probes were not obviously toxic *in vitro*. Similar phenomena were found in the case of PC12 and RSC96 cells in our previous study[20], indicating that these citric acid-based probes are not obviously toxic to cultured cells.

The hemolytic test is considered to be a supplementary test for the assessment of cytotoxicity. It is used to evaluate whether erythrocytes will dissolve and release hemoglobin after direct contact with the nanomaterials in the blood. It also can provide a sensitive measure of the influence of a nanomaterial on erythrocytes, and plays an important role in evaluations of biosafety[21]. In the present study, the sedimentation of blood cells was observed for both of the fluorescent probes and for the normal saline group (Fig. S2, ESI†). However, the final hemolysis rate of both the CDs and Et-IPCA leaching solutions was less than 5%, even when the concentration of Et-IPCA reached 500 μg/mL, as shown in

Fig. 2e. The high content of Et-IPCA in solution may cause a change in the osmotic pressure, which might be the reason for the hemolysis. However, this was not a concern for further animal experiments because the final concentrations of both the CDs and Et-IPCA would be relatively low *in vivo*. Taken together, these results further demonstrated the good biocompatibility of these citric acid-based probes.

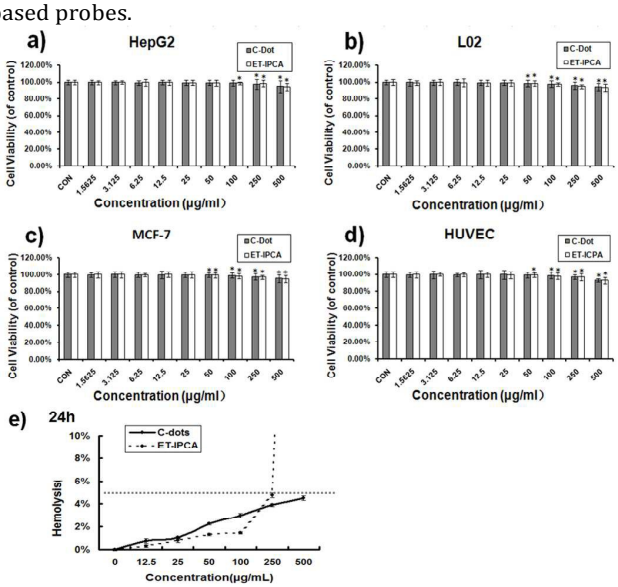


Fig. 2 The biocompatibilities of CDs and Et-IPCA. a-d) The relative viabilities of HepG2 (a), L02 (b), MCF-7 (c) and HUVEC (d) cells after incubation with CDs and Et-IPCA at various concentrations for 72 h. Each bar represents the mean ±SD of three independent experiments. (e) The results of the hemolysis assay of CDs and Et-IPCA.

3.3 Single-dose toxicity of CDs and Et-IPCA

Because the results of the SRB assay and hemolytic test *in vitro* showed that the CDs and Et-IPCA had acceptable biocompatibility, toxicity testing was carried out. No further experiments were performed using Et-IPCA because neither death nor abnormal behaviors were observed at its saturated concentration in saline (25 mg/kg). As shown in Tables 1, the mortality rates following exposure to 320 mg/kg and 400 mg/kg were different between female and male mice, indicating that the single-dose toxicity of CDs is gender-related. The surviving mice returned to normal within six hours, and their behaviors showed no abnormalities compared with the control group during the next 14 days, suggesting that CDs could be rapidly eliminated *in vivo*. The LD_{50} of CDs calculated by linear interpolation were 391.62 mg/kg and 357.77 mg/kg for female and male mice, respectively. It was concluded that male mice are more sensitive to the higher concentrations of the CDs solution than female mice. Based on this conclusion, we believed that male mice would be a more suitable model for the subsequent repeated-dose toxicity test. Single-dose toxicity testing is always considered the first step in toxicological safety evaluations, and the LD_{50} serves as a key indicator of the toxicity of an agent[22]. Moreover, it is an important reference value to use when designing repeated-dose toxicity tests. However, important as it is, there has been little systematic research performed with regard to the single-dose toxicity of CDs, and the results of these studies have been conflicting. In our study, the single-dose toxicity test was designed according to the SFDA's guidelines. Although some criteria were changed to address the unique properties of CDs and Et-IPCA, this experimental design can still be considered superior due to its use of a standard procedure and the discovery of the LD_{50}.

As was mentioned before, the results of the single-dose toxicity testing of CDs differed between female and male mice. Similar phenomena have been reported for many chemicals and traditional QDs. The reasons for this difference might be rather complicated, including physical factors, differences in enzyme systems and hormones. After considering this result, together with the results regarding the biodistribution and Liu's[16] and Maysinger's[23] reports, we assumed that physical factors were likely play an important role because nanoparticles of such a small diameter can be quickly excreted from the body, which means that their half-life *in vivo* is relatively short, indicating that they are unlikely to interact much with organs. However, further testing is required to verify this assumption.

Gender	Dosage(mg/kg)	Death	Mortality(%)	LD50(mg/kg)
Female	250	0	0	
	320	1	10	391.615
	400	5	50	
	500	10	100	
Male	250	0	0	
	320	3	30	357.771
	400	7	70	
	500	10	100	

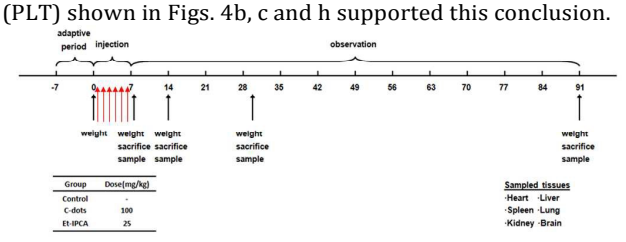
Table 1 The mortalities in the single-dose toxicity test of the different concentrations of CDs and the LD_{50} values in female and male ICR mice.

3.4 Repeated-dose toxicity of CDs and Et-IPCA

To further analyze the toxicity of repeated administration, mice were dosed at different levels every day for seven days and were observed for up to 90 days (Table 2a). During the entire experimental period, neither death nor abnormal behavior was observed. To study the *in vivo* toxicity of CDs and Et-IPCA, a series of experiments were conducted. First, to assess their overall effects, the mice were weighed and then sacrificed. Their major organs, including the brain, liver, spleen, kidneys, heart and lungs were collected and weighed. An organ coefficient, which was the ratio of the organ weight to the body weight, was calculated. No remarkable changes were found in body weight in any of the groups, as shown in Fig. 3. Nevertheless, as is shown in Table 2b, significant changes could be found in certain organs in the experimental groups at each time point, indicating the possibility that there were injuries.

The immune system might have been stimulated by the treatment because the size of CDs *in vivo* is similar to that of viruses and large proteins, and this may have led to changes in hematological factors[24]. Accordingly, blood biochemistry

and hematological analyses were performed. As shown in Figs. 4 and 5, almost all of the parameters examined in the probe-treated groups appear to be normal compared with the control group and were within the normal ranges. It is notable that the white blood cell count (WBC) (shown in Fig. 4a) suggested that both CDs and Et-IPCA could induce inflammatory responses, while the effect of CDs lasted longer than that of Et-IPCA, for more than 30 days. The changes in the red blood cell count (RBC) and platelet count (PLT) shown in Figs. 4b, c and h supported this conclusion.



Days after treatment	Group	Body weight(g)	Liver Coefficient(%)	Spleen coefficient(%)	Kidney coefficient(%)	Heart coefficient(%)	Lung coefficient(%)	Brain coefficient(%)
1	Control	27.50±4.22	6.24±0.65	0.58±0.07	0.96±0.09	0.71±0.08	0.86±0.12	1.53±0.22
	C-dots	27.97±3.13	6.43±0.68	0.67±0.09*	0.87±0.10	0.60±0.06	0.90±0.14	1.44±0.18
	Et-IPCA	27.99±3.88	6.03±0.63	0.69±0.08*	0.95±0.07	0.63±0.06	0.94±0.11	1.50±0.21
7	Control	30.03±2.25	5.18±0.58	0.53±0.08	0.72±0.08	0.54±0.05	0.96±0.16	1.45±0.21
	C-dots	28.82±1.83	6.19±0.69*	0.56±0.11	0.91±0.11*	0.66±0.06*	1.09±0.13	1.50±0.24
	Et-IPCA	30.73±2.33	5.30±0.61	0.53±0.07	0.74±0.07	0.62±0.06*	0.85±0.15	1.49±0.19
30	Control	32.13±2.66	5.10±0.60	0.45±0.06	0.78±0.10	0.50±0.06	0.76±0.14	1.34±0.18
	C-dots	31.71±2.17	6.02±0.65	0.54±0.07	0.85±0.11	0.63±0.06	0.85±0.12*	1.38±0.20
	Et-IPCA	32.18±2.25	5.26±0.63	0.51±0.05	0.68±0.08*	0.61±0.07	0.78±0.13	1.48±0.22*
90	Control	38.67±2.87	5.15±0.50	0.36±0.04	0.80±0.10	0.47±0.04	0.66±0.09	1.22±0.16
	C-dots	38.72±3.82	5.83±056*	0.52±0.04	0.79±0.09	0.60±0.06	0.78±0.08*	1.20±0.16
	Et-IPCA	38.64±3.15	5.24±0.53	0.50±0.05	0.61±0.05*	0.61±0.06	0.73±0.10	1.47±0.19*

Table 2 (a) The experimental design of the repeated-dose toxicity study. (b) The organ coefficients of male ICR mice at 1, 7, 30 and 90 days. Each bar represents the mean ±SD, n=10. P* < 0.05 vs. control group.

The levels of alkaline phosphatase (ALP), a blood biochemistry factor reflecting the health of the liver and bone, showed significant differences in the CDs and Et-IPCA groups compared to the control in first seven days after treatment. However, these differences were randomly distributed and do not appear to be evidence of pathological changes in these tissues. The tissues were sliced and stained, then observed under a microscope to determine in detail whether there were any pathological changes in the CDs and Et-IPCA groups. It can be inferred from Figs. 6 and 7 that there were no remarkable differences in any of the major organs between the control and experimental groups. Overall, the present data demonstrated that the CDs and Et-IPCA at the concentrations used in this study did not lead to significant pathological changes based on the organ coefficient, H/E staining and hematology analysis.

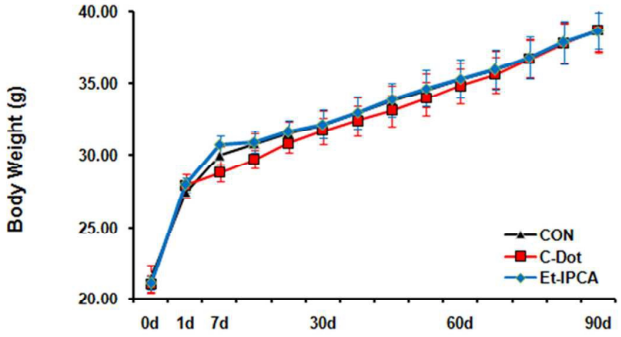


Fig. 3 The body weights of male ICR mice in the control, CDs and Et-IPCA groups at 90 days. Each bar represents the mean ±SD, n=10.

It should be noted that different methods of administration were adopted in our repeated dose test, where the CDs were injected via the tail vein while Et-IPCA was injected into the peritoneal cavity. The reason we chose intraperitoneal administration for Et-PICA is that we concerned that there might be a risk of sudden death if the Et-IPCA solution were injected intravenously due to the volume of the Et-IPCA solution because its solubility is much lower than that of CDs in saline(the approximate injection volumes were 0.5 mL for Et-IPCA and 0.2 mL for CDs). Moreover, the difference in the absorption of these materials between intravenous injection and intraperitoneal injection is not that significant. We considered that the method used in the current research on the *in vivo* toxicity of CDs, which can be described as a one-time treatment and a subsequent long observation period, was not sufficient to reveal all of the potential effects of CDs. Furthermore, repeated-dose testing is an essential part of the preclinical studies during drug development[25]. A seven-day repeated treatment in animals is equivalent to a single dose in human beings according to the guideline, which served as the basis of our study design for our repeated dose test. The time points we chose covered the whole convalescent period and helped us to better understand the biosafety of CDs and Et-IPCA.

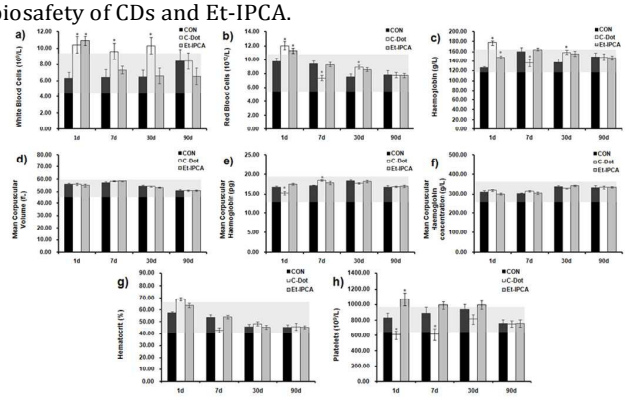


Fig. 4 The hematology data for male ICR mice treated with CDs at 100 mg/kg and Et-IPCA at 25 mg/kg on days 1, 7, 30 and 90. Age-matched untreated mice were also sacrificed on days 1, 7, 30 and 90 as controls. a-h) The time-course of the changes in white blood cells (a), red blood cells (b), hemoglobin (c), mean corpuscular volume (d), mean corpuscular hemoglobin (e), mean corpuscular hemoglobin concentration (f), hematocrit (g), and platelets (h) in control mice, CDs-treated mice and Et-IPCA-treated mice. Each bar represents the mean ±SD, n=10. P* < 0.05 vs. the control group. Gray bars indicate the range of values obtained from healthy ICR mice.

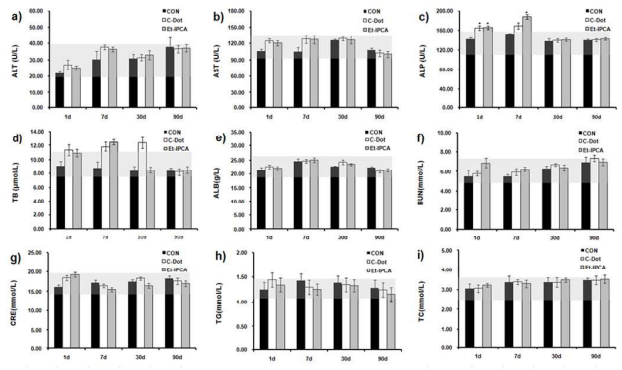


Fig. 5 The blood biochemistry data of male ICR mice treated with CDs at 100 mg/kg and Et-IPCA at 25 mg/kg on days 1, 7, 30 and 90. Age-matched untreated mice were sacrificed on the same days as controls. a-i) The time-course of the changes in the alanine aminotransferase (a), aspartate aminotransferase (b), alkaline phosphatase (c), total bilirubin (d), albumin (e), blood urea nitrogen (f), serum creatinine (g), total cholesterol (h) and total triglycerides (i) in control mice, CDs-treated mice and Et-IPCA treated mice. Each bar represents the mean ±SD, n=10. P* < 0.05 vs. control group. Gray bars indicate the range of values obtained from healthy ICR mice.

As was mentioned above, some factors examined in the biochemistry and hematology analyses changed significantly in the CDs group, and most of which could be still be observed for up to 30 days after injection. Among these changes, the blood cell counts, including the WBC, RBC and PLT, might indicate that there was an inflammatory response. Although the size of our CDs is approximately 3 to 5 nm, the possibility of aggregation *in vivo* should not be ignored. The aggregation of CDs may form a carbon nanocluster whose size would be similar to that of viruses, which would affect the immune system. However, these effects in the Et-IPCA group lasted a shorter time compared to those in the CDs group. Based on these findings, we concluded that the changes in Et-IPCA group might just be a result of the continuous stimulation.

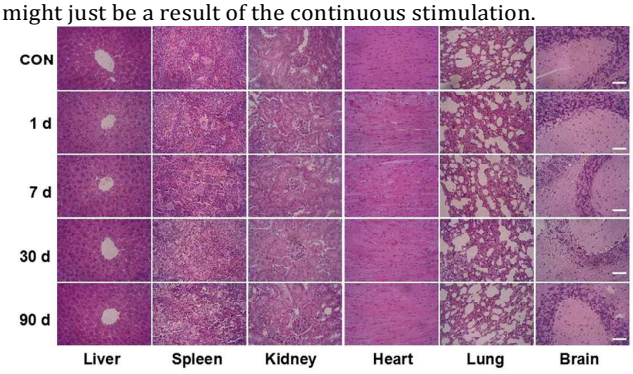


Fig. 6 Representative H&E-stained images of major organs, including the liver, spleen, kidneys, heart, lungs and brain collected from the control untreated mice and CDs-injected (100 mg/kg) mice at various time points post-injection. No noticeable abnormality or lesion was observed in any of the organs from the CDs-injected mice. All scale bars are 100 μm.

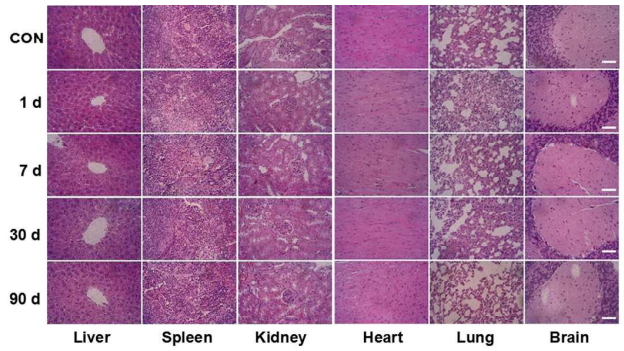


Fig. 7 Representative H&E-stained images of major organs, including the liver, spleen, kidneys, heart, lungs and brain collected from the control untreated mice and Et-IPCA-injected (25 mg/kg) mice at various time points post-injection. No noticeable abnormality or lesion was observed in any of the organs from the Et-IPCA-injected mice. All scale bars are 100 μm.

3.5 Biodistribution of the CDs and Et-IPCA

To study the biodistribution characteristics of the CDs and Et-IPCA *in vivo*, the emission spectra of tissue homogenates were obtained when the excitation wavelength was 360 nm. Then, the PL intensity at 450 nm was recorded because the curve reached its peak at this wavelength. As shown in Fig. 8, it was found that both CDs and Et-IPCA mainly accumulated in reticuloendothelial system (RES) organs, especially in the liver, at three hours after injection, which is similar to the *in vivo* behaviors of many other small nanoparticles. There was also a considerable amount of nanoparticles found in serum and heart, indicating that both of the molecules could be easily transported by blood. We can infer from the data obtained 24 h after injection that the majority of both CDs and Et-IPCA had been excreted. Combining the results of the two time points, we concluded that these two samples share a common *in vivo* distribution and can be quickly eliminated. It is notable that the brain experienced greater exposure to Et-IPCA than CDs, indicating their ability to pass through the blood-brain barrier. Although the reason for this is still unclear, it appears that Et-IPCA may be useful for evaluating brain lesions. On the other hand, our research on the biodistribution of CDs and Et-IPCA is still incomplete. More details on the elimination and pharmacokinetics are therefore required.

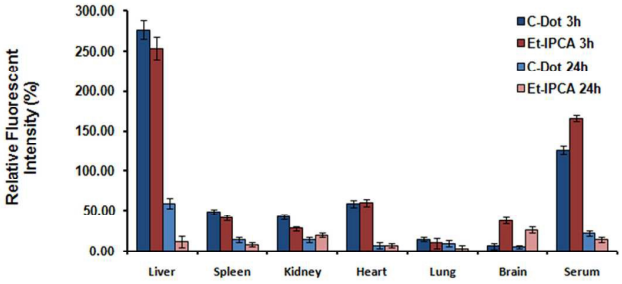


Fig. 8 The biodistribution of the CDs and Et-IPCA at 3 h and 24 h after injection in mice. Each bar represents the mean ±SD, n=10.

Conclusions

In the present study, two strongly fluorescent molecules, CDs and Et-IPCA, with good photostability and biocompatibility were successfully prepared. Systematic single- and repeated-dose toxicity evaluations were carried out for these fluorescent probes in mice. The mortality, body weight, coefficients, hematology, blood biochemistry, and organ histopathology revealed that both CDs and Et-IPCA had low *in vivo* toxicity. Moreover, no abnormality or lesion was observed in the major organs of the mice treated with the CDs or Et-IPCA. Taken together, these results demonstrated that neither CDs nor Et-IPCA exerted any significant toxic effect on mice at the doses used in our experiments. Thus, these citric acid-based probes have good biocompatibility and the potential for use in *in vivo* molecular imaging and biolabeling, and our findings provide important information for the future development of clinical applications using these molecules.

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