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ARTICLE

Fluorescent nanoparticles present in Coca-Cola and Pepsi-Cola: physiochemical properties, cytotoxicity, biodistribution and digestion studies

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ABSTRACT

Foodborne nanoparticles (NPs) have drawn great attention due to human health concerns. This study reports the detection of the presence of fluorescent NPs, about 5 nm, in two of the most popular beverages, Coca-Cola (Coke) and Pepsi-Cola (Pepsi). The NPs contain H, C and O, three elements with a tunable emission and with a quantum yield of 3.3 and 4.3% for Coke and Pepsi, respectively. The presence of sp^3-hybridized carbon atoms of alcohols and ethers bonds was confirmed by NMR analysis. The NPs can be taken up by living cells and accumulate within cell membrane and cytoplasm. Evaluation of the acute toxicity of the NPs revealed that the BALB/c mice appeared healthy after administration of a single dose of 2 g kg^{-1} body weight. Analysis of glutamate pyruvate transaminase (GPT), glutamic oxaloacetic transaminase (GOT), urea and creatinine showed that there were statistically, but not biologically, significant differences in some of these biochemical parameters between the test and control groups. No obvious organ damage or apparent histopathological abnormality was observed in the tested mice. The biodistribution study in major organs indicated that the NPs were easily accumulated in the digestive tract, and they were able to cross the blood–brain barrier and dispersed in the brain. *In vitro* digestion of the NPs showed a significant fluorescence quenching of the NPs. This work represents the first report of foodborne fluorescent NPs present in Coke and Pepsi, and provides valuable insights into physicochemical properties of these NPs and their toxicity characteristics both *in vitro* and *in vivo*.

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Foodborne nanoparticles; Coca-Cola; Pepsi-Cola; cytotoxicity; biodistribution; digestion

Introduction

Foodborne nanoparticles (NPs) have drawn great attention due to human health concerns (Das et al. 2011; Li et al. 2010; Bouwmeester and Sips 2007; Das, Saxena, and Dwivedi 2009). Some foods sold in supermarkets worldwide contain NPs, that either were produced during the manufacturing process or added artificially to enhance the flavors, facilitate nutrient absorption, extend shelf life or provide brighter colors (Sekhon 2010). With the lack of mandatory product labeling anywhere in the world, it is hard for the public to obtain the information on how many commercial food products contain NPs. On the one hand, food manufacturers may not be interested in providing the information about their food containing NPs, possibly due to their ambiguous effects. On the other hand, the food suppliers may not know about the presence of NPs in the food products they are selling. For example, Sk et al. (2012) have reported that bread, biscuits, corn flakes and sugar caramel contain carbon-rich NPs. Also, our group have reported the presence of fluorescent NPs in Nescafe® original instant coffee (Jiang et al. 2014), Tsingtao®-beer (Wang et al. 2015), fermented beverages and sport beverages (Liao et al. 2015). Currently, the presence of NPs in food products is still poorly studied, and researchers are interested in ascertaining how the broad range of NPs present in food is.

Most human foods are made of carbohydrates which consist of carbon, hydrogen, oxygen, nitrogen, etc. During the manufacturing process, these elements may be involved in complex physicochemical reactions to form endogenous NPs. It is well-known that the carbon dots (CDs) can be easily produced via hydrothermal reactions using small

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Supplemental data for this article can be accessed here.

organic molecules (Tan et al. 2015) or biomass (Zhou et al. 2012) as carbon sources. However, little is actually known about the origin and biological effects of NPs present in food. The extent to which NPs are present in food is very important and needs to be thoroughly studied. Accordingly, the finding of nanostructures in human food can help us to understand the types of NPs present in food we are already regularly consuming. In addition, it is necessary to clearly determine the physicochemical properties, toxicity and biodistribution of these NPs present in the ordinary food in order to help people gain an understanding of their toxicity (Sk et al. 2012).

Our hypothesis is that foodborne NPs are produced during the food manufacturing process. As two of the most popular and widely consumed carbonated cola beverages, Coca-Cola (Coke) and Pepsi-Cola (Pepsi) have been consumed worldwide more than 100 years. Occasionally, these two beverages were found to show fluorescence under the illumination of ultraviolet light when they spurted out of the cans, thus we speculated that they may contain fluorescent NPs. Herein, we report the finding of CD-like fluorescent NPs in these carbonated soft drinks. The fluorescent NPs were successfully isolated from these beverages by an effective purification strategy. The obtained NPs were characterized and evaluated for cytotoxicity, acute toxicity and biodistribution in small experimental animal. *In vitro* digestion was conducted using the artificial saliva, gastric and intestine juice. The discovery of NPs in the Coke and Pepsi beverages may be a contentious area of research, as it challenges and puts in doubt the prevalent dogma that exposure to nanomaterials is both new and potentially dangerous.

Experimental

Extraction and characterization of fluorescent NPs

Coke (330 mL in aluminum cans, produced by Coca-Cola Liaoning (south) Beverages Ltd., Dalian, China) or Pepsi (330 mL in aluminum cans, produced by Pepsico Changchun Beverages Ltd., Dalian, China) was added into a 500-mL glass beaker and stirred at room temperature for 4 h to remove the bubbling gas (CO_2). After filtration through a 0.22-μm filter to remove agglomerated particles, the samples were concentrated by the freeze-drying method, and purified using a Sephadex G-25 column. The fractions with the stronger fluorescence intensity were collected and were further lyophilized. The Coke or Pepsi powder (yield about ~3%) was stored in the dark at 4 °C until use for further characterization. Glucose CDs were artificially synthesized by hydrothermal treatment of 0.5 g glucose in 15 mL water at 180 °C for 4 h. The purification procedure of the glucose CDs was same as Cola NPs.

Fluorescence spectral analysis of the NPs was conducted using a fluorescence spectrometer (LS55, Perkin Elmer, Norwalk, CT, USA). The fluorescence quantum yield of the Coke and Pepsi NPs were measured according to a reported method using quinine sulfate as a reference (Liao et al. 2015). A Horiba Jobin Yvon FluoroMax-4 spectrofluorometer was used for time-resolved fluorescence decay measurements (Horiba Scientific, Chilly Mazarin, France) at the excitation wavelength of 376 nm. The fluorescence lifetime measurements were performed using the commercial software of the Horiba Instruments Inc. (Horiba Scientific) (Jiang et al. 2014). X-ray diffraction (XRD) analysis was conducted on an XRD-6100XRD instrument (Shimadzu, Kyoto, Japan) using Cu-Kα as radiation ($\lambda = 0.15418$ nm). A JEM-2000EX transmission electron microscope (JEOL Ltd., Tokyo Japan) was used to examine the morphology of the NPs by adding them on a 400-mesh carbon-coating Cu grid. Absorption spectral analysis was performed with a UV-vis spectrophotometer (UV-2550, Shimadzu) in the range of 200–600 nm. The chemical composition of the NPs was analyzed with a Escalab 250 Xi X-ray photoelectron spectrometer (Thermo Scientific, Waltham, MA). Fourier-transform infrared spectroscopy (FTIR) spectra were recorded using the KBr pressed pellet method with a VECTOR 22 FTIR spectrometer (Bruker Optik, Ettlingen, Germany). Confocal micrographs were taken with an FV1000 inverted fluorescent microscope (Olympus, Tokyo, Japan) equipped with a 40× objective lens in XY mode with a 600 × 600-pixel resolution.

To examine the effect of pH on the fluorescence intensity of NPs in Coke and Pepsi, 200 μL of NPs solution (1 mg mL^{-1}) was added to 1800 μL of Britton–Robinson solution consisting of 0.04 M H_3BO_3, 0.04 M H_3PO_4, and 0.04 M CH_3COOH with pH ranging from 2 to 11. The fluorescence intensity

was recorded with an LS55 fluorescence spectrometer at the excitation wavelength of 300 nm. All the measurements were performed in triplicate and the error bars represent the standard error of the mean.

In vitro cytotoxicity of Coke and Pepsi NPs was evaluated in Chinese hamster ovary (CHO) cells using the Cell Counting Kit-8 (CCK-8, Donjindo Molecular Technologies, Rockville, MD) assay using the highly water-soluble 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium salt. CHO cells were seeded in 96-well plates at a density of 5×10^3 cells/well, and were incubated with different concentrations (0, 0.31, 0.63, 1.25, 2.5, 5.0, 10.0 and 20.0 mg mL^{-1}) of NPs at 37 °C with 5% CO_2 atmosphere for 24 h. Then, after incubation in RPMI-1640 for 3 h, 10 μL of CCK-8 solution was added to each well to evaluate cell viability after incubation in RPMI-1640 for 3 h. A Wellscan Mk3 microplate reader (Labsystems Dragon Ltd., Helsinki, Finland) was used to record the absorbance value of each well at 450 nm. The cell viability was calculated by the equation of Cell viability $(\%) = (A_S - A_B) \times 100\%/(A_C - A_B)$, where A_S is the absorbance of the cells, CCK-8 solution and NP solution; A_B is the absorbance of the CCK-8 solution without cells; and A_C is the absorbance of the cells and CCK-8 solution without NPs. The percentage of viable cells was expressed relative to the survival of a control group (control group cells were considered to have 100% viability).

Biodistribution study

Malignant melanoma cells (B16-F10) were incubated in RPMI-1640 medium with 100 units/mL penicillin, 100 mg mL^{-1} streptomycin and 10% FBS in 5% CO_2 at 37 °C. After growing for about 48 h, the B16-F10 cells in 2 mL of medium were seeded in six-well culture plates (6×10^4 cell mL^{-1}) and maintained at 37 °C in a 5% CO_2 atmosphere for an additional 24 h. Then, 10 mg mL^{-1} of Coke or Pepsi NPs were added and incubated for another 24 h. After washing three times with medium, the B16-F10 cells were imaged with a laser confocal fluorescent microscope (Olympus FV1000). The excitation wavelengths of NPs were 405 and 488 nm with emission wavelength within 425–475 and 500–600 nm, respectively.

Acute oral toxicity evaluations

Approved protocols from the Institutional Animal Care and Use Committee at Dalian Medical University were followed for all animal procedures. Three groups ($n = 10$, five males and five females) of BALB/c mice (18–22 g) were orally administrated NPs obtained from Coke and Pepsi to investigate their acute oral toxicity. The mice were orally exposed to Coke or Pepsi NPs through a single administration of 2 g kg^{-1} body weight. The control group mice were orally administrated 400 μL of a 0.9% NaCl aqueous solution and glucose CDs of 2 g kg^{-1} body weight. After 24 h of the administration, the mice were sacrificed. The body weight of the mice was measured and blood samples were collected to measure the levels of glutamate pyruvate transaminase (GPT), glutamic oxaloacetic transaminase (GOT), blood urea nitrogen (BUN), urea and creatinine using a ADVIA 2400 Chemistry System (Siemens, Munich, Germany). The measured parameters from the 10 mice in each group were statistically analyzed and their biochemical analysis values were expressed as the mean ± standard deviation with $p < 0.05$ meaning significant difference [one-way analysis of variance (ANOVA)]. The major organs/tissue (liver, spleen, kidney, thymus, ovarian/testis) were collected and their weight was measured. The specimens were then fixed in 10% formalin buffer for 48 h, and the fixed tissue was embedded in paraffin. The embedded organs/tissue blocks were cut into 6-μm sections and stained with hematoxylin–eosin (HE) for microscopic observation using an FV1000 laser confocal fluorescent microscope (Olympus).

In vivo *biodistribution in mice*

Twenty-seven female BALB/c mice (18–22 g) were equally divided into nine groups ($n = 3$). Eight groups were orally administrated a single dose of 2 g kg^{-1} body weight (400 μL) to evaluate the biodistribution of Coke and Pepsi NPs, while the control group mice were orally administrated a 0.9% NaCl solution. The mice that had been administrated the NPs were sacrificed at 1/4, 2, 6, and 24 h after the NPs administration, while the control group mice were sacrificed immediately after oral administration of a 0.9% NaCl aqueous solution. The major organs/tissue including small intestine,

colorectum, stomach, vermiform appendix, brain, lung, liver, kidneys, heart, spleen and muscle were collected and imaged on a Maestro EX *in vivo* imaging system (Cambridge Research & Instrumentation, Inc., Woburn, MA). Spectral fluorescent images were recorded with the appropriate filter for the NPs (excitation: 455 nm; acquisition settings: 470 – 700 nm in 10 nm steps, long-pass filter). Exposure time was 3000 ms for all the measurements. The liver, brain, heart, etc. were collected after sacrificing the mice that had been orally administrated the Coke and Pepsi NPs, separately. Then these organ's tissues were sectioned into slices for histological analysis.

In vitro *digestion*

Artificial digestive juices for *in vitro* digestion experiments were prepared according to the literature (Peters et al. 2012). The saliva (pH = 6.8) contained 896 mg KCl, 200 mg KSCN, 1021 mg $NaH_2PO_4{\cdot}H_2O$, 570 mg Na_2SO_4, 298 mg NaCl, 1694 mg $NaHCO_3$, 200 mg urea, 290 mg amylase, 15 mg uric acid, 25 mg mucin and 1000 mL distilled water. The gastric juice (pH = 1.3) consisted of 2752 mg NaCl, 306 mg $NaH_2PO_4{\cdot}3H_2O$, 824 mg KCl, 302 mg $CaCl_2$, 6.5 mL glucose, 20 mg glucuronic acid, 85 mg urea, 330 mg glucosamine hydrochloride, 1 g BSA, 2.5 g pepsin, 3 g mucin and 1000 mL distilled water. The duodenal juice (pH = 8.1) was composed of 7012 mg NaCl, 3388 mg $NaHCO_3$, 80 mg KH_2PO_4, 564 mg KCl, 50 mg $MgCl_2{\cdot}6H_2O$, 180 μL HCl (37%), 100 mg urea, 151 mg $CaCl_2$, 1 g BSA, 9 g pancreatin, 1.5 g lipase and 100 mL distilled water. The bile juice (pH = 8.2) contained 5259 mg NaCl, 5785 mg $NaHCO_3$, 376 mg KCl, 150 μL HCl (37%), 250 mg urea, 167.5 mg $CaCl_2$, 1.8 g BSA, 30 g bile and 100 mL distilled water. The $NaHCO_3$ solution was prepared by adding 84.0 g $NaHCO_3$ in to 1000 mL distilled water. HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 1 M) buffer was prepared by adding 119.15 g HEPES to 500 mL distilled water, and adjusting with 1 M NaOH solution to pH 7.0.

The digestion was started by adding 2 mL of artificial saliva to 10 μL of NPs HEPES solution (1 mg mL^{-1}). The mixture was stirred for 5 min at 60 rpm at 37 °C followed by measurement of the fluorescence intensity. Subsequently, 2 mL of gastric juice was added and the pH was adjusted to 2.0. The mixture was then stirred at 37 °C for 2 h. After measuring the fluorescence intensity, 1 mL of bile juice, 2 mL of duodenal juice, and 333 μL of sodium bicarbonate solution (1 M) were subsequently added. The pH of the resulting mixture was adjusted to 6.5 and stirred at 37 °C for 2 h. Ultimately, the fluorescence intensity was measured again. The fluorescence quenching after each step was calculated by calibrating the emission area to estimate the *in vitro* digestion status.

Results and discussion

The public perception of human food containing NPs is usually negative, and the occurrence, fate and toxicity of NPs in food products remain poorly studied (Tiede et al. 2008). Scientific studies on the presence of NPs in food can provide useful information to assess the risks and benefits of emerging nanomaterial-containing food. Also, the NPs in traditional food products might not be safe even though they have been consumed for many years. In this study, fluorescent NPs were found for the first time in commercial Coke and Pepsi after purification of NPs by column chromatography with Sephadex gel. The isolation of NPs by freeze-drying and size-exclusion liquid chromatography separation produced pure NPs with a yield of ~3% based on the weight of Coke or Pepsi. The presence of fluorescent NPs in Coke and Pepsi was subsequently confirmed by transmission electron microscopy (TEM). The images shown in Figure 1(a,b) reveal that the NPs from Coke and Pepsi are uniformly dispersed without apparent aggregation, with an average size of 5.0 ± 0.5 and 4.7 ± 0.8 nm (Figure 1(c,d)), respectively. Accordingly, the Coke and Pepsi NPs are smaller than those of NPs found in bread and jiggery, >20 nm (Sk et al. 2012). The XDR patterns show broad peaks centered at 17.13° and 18.24° for Coke or Pepsi NPs, respectively. Broad shoulder at high angles around 40° was also observed which may be attributed to (10) and (101) graphitic planes (Vassilakopoulou et al. 2017). Although in bulk form these NPs appear to be amorphous according to the XRD characterization, (Supplementary Figure S1), the single particle is crystalline, as indicated by the high resolution TEM (HRTEM) image (Inset of Figure 1(a,b)) and the corresponding selected-area

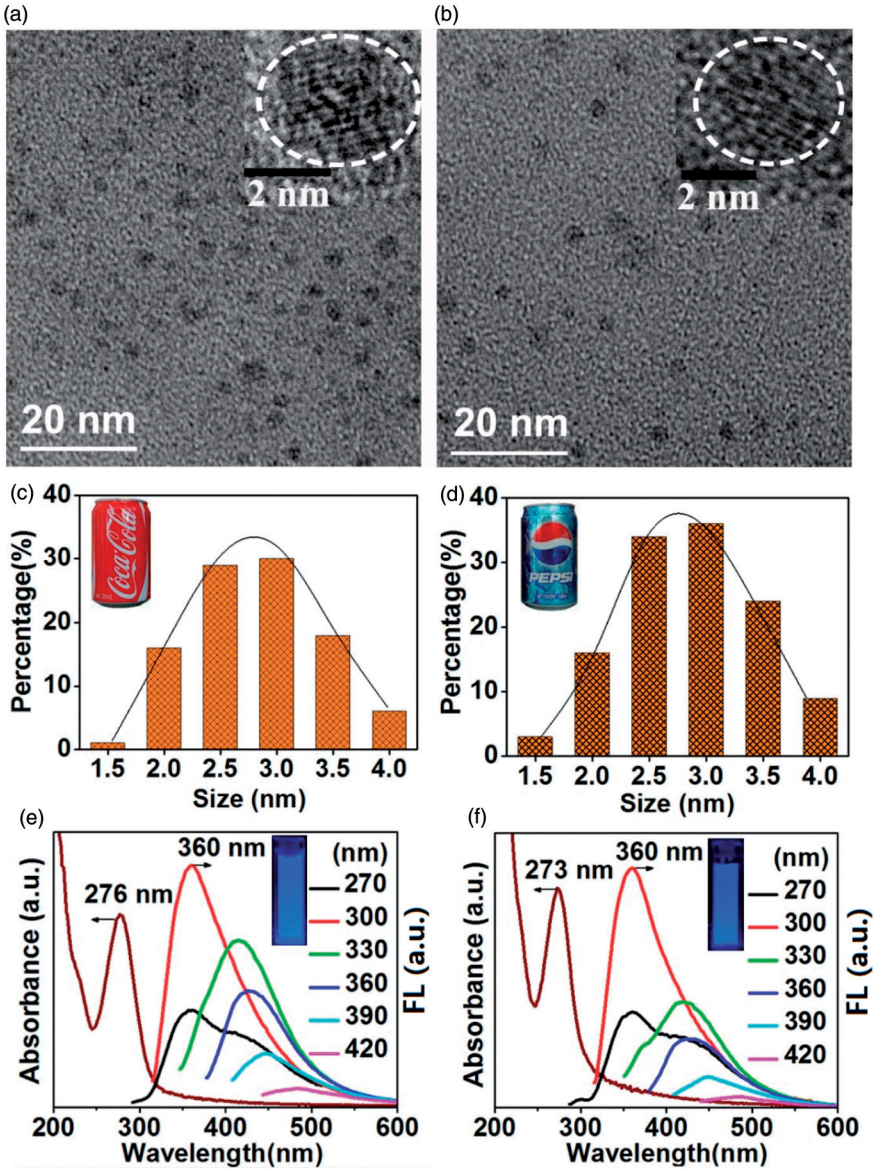

Figure 1. TEM images of NPs from (a) Coke and (b) Pepsi (scale bars 20 nm); insets are HRTEM images (scale bars 2 nm). Histograms of the size distributions of NPs from (c) Coke and (d) Pepsi. Insets are the photographs of Coke and Pepsi cans. UV–Vis absorption and fluorescent (FL) emission spectra of NPs in aqueous solution (0.05 mg mL^{-1}) from (e) Coke and (f) Pepsi. Insets show the photographs of NPs in aqueous solution under UV light.

electron diffraction (SAED) pattern (Inset of Supplementary Figure S1(a,b)). The spacing between lattice planes was 0.208 nm for the Coke NPs, which may be attributed to the (103) diffraction plane of the sp^3 carbon analogous to SiC (JCPDS cards 26-1081) and to the (102) diffraction plane of the sp^2 graphitic carbon (JCPDS 26-1076). The lattice space of 0.246 nm of the Pepsi NPs is consistent with the (213) lattice of the diffraction plane of graphitic carbon (JCPDS 20-0258). The NPs from Coke and Pepsi composed of other elements, such as oxygen and hydrogen, which contain impurities, defects or very small NPs causing the breakdown of symmetry.

The UV-Vis spectra of the NPs from the beverages exhibited very similar profile with a peak at 276 and 273 nm for Coke and Pepsi, respectively (Figure 1(e,f)). In the fluorescence spectra, fluorescence emission was observed when the NPs were excitated at an excitation wavelength between 270 and 420 nm. The blue color emission can be clearly seen with the naked eye under the illumination of a hand-held 365 nm UV lamp (Insets of Figure 1(e,f)). The dispersion of both, the Coke and Pepsi, NPs show excitation-dependent fluorescence behavior, which is a common characteristic in fluorescent CDs (Jariwala et al. 2013, Feng et al. 2013). This behavior

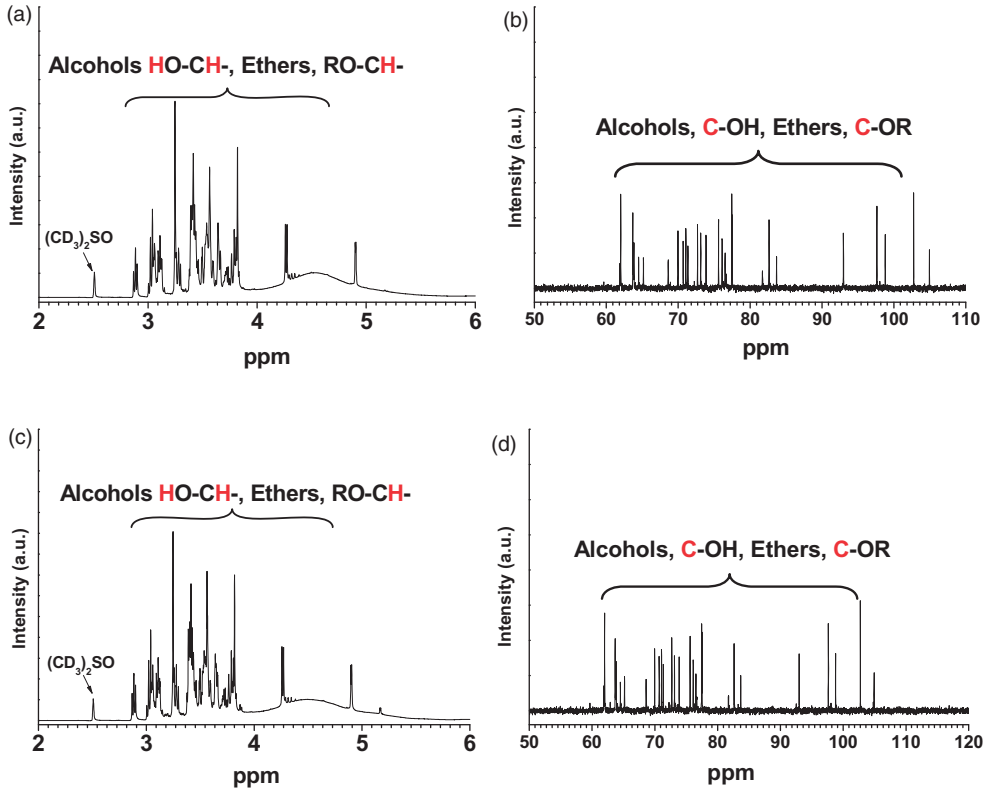

Figure 2. (a) ^{1}H-NMR, (b) ^{13}C-NMR spectra of fluorescent NPs extracted from Coke, and (c) ^{1}H-NMR, (d) ^{13}C-NMR spectra of NPs extracted from Pepsi.

could be attributed to either the complexity of the excited states of the NPs, whose surface state may affect the band gap (Shang et al. 2012, Zhu et al. 2013), or to the presence of various emitting centers on the NP surface (Li et al. 2010). In support of this expanation, the decay of the fluorescence emission were found to be nonmonoexponential for the Coke and Pepsi NPs, with an average lifetime of several nanoseconds, specifically 4.32 and 4.26 ns, repectively (Supplementary Figure S2, Supplementary Table S1). By calibrating against quinine sulfate, the quantum yield of NPs from the Coke and Pepsi was found to be 3.3% and 4.3%, respectively. The Zeta potential of the Coke and Pepsi NPs determined to be −3.07 and −1.12 mV, respectively (Supplementary Table S1). The UV-Vis and fluorescence characteristics were similar to those of the chemically synthesized CDs (Qiao et al. 2010, Wang et al. 2011, Tan et al. 2013), and this findings strongly suggest the presence of CD-like NPs in these commercial beverages.

X-ray photoelectron spectroscopy (XPS) elemental analysis of the NPs from Coke and Pepsi revealed two peaks at 285 and 532 eV associated with elemental C and O, respectively. (Supplementary Figure S3(a,c)). The high-resolution XPS spectra of C_{1s} (Supplementary Figure S3(b,d)) showed that the NPs contain three types of carbon atoms, namely C=C (284.5 eV), C–O (286.05 eV) and C=O (288.05 eV). NMR spectroscopy (^{1}H and ^{13}C) analysis was used to investigate the detailed information of the fluorescent NPs. In the ^{1}H-NMR spectra (Figure 2(a,c)), signals from 2.9–6.0 ppm correspond to alcohols and ethers bonds. In the ^{13}C-NMR spectra (Figure 2(b,d)), signals in the range of 60–110 ppm are attributed to the presence of the sp^3 hybridized carbon atoms of alcohols and ethers bonds. In the FTIR spectra of the NPs (Supplementary Figure S4), the stretching vibrations of C–OH at 3376 cm^{-1} and C–H at 2927 cm^{-1}, stretching vibrations of double bond C=C at 1650 cm^{-1}, bending vibration of O–H bond at 1408 cm^{-1} and C–O–C stretching vibrations at 1059 cm^{-1} were observed, which strongly suggest the presence of ether bonds and hydroxyl groups. The small peak at 1730 cm^{-1} is attributed to the presence of C=O vibrations, which means the formation of ketones and carboxylates after thermal treatment (Vassilakopoulou et al. 2017). The presence of hydroxyl groups was further supported

by the Zeta potential analysis results in Supplementary Table S1. All these results indicated that the fluorescent NPs contained C, O and H (from ^{1}H-NMR) elements and there are ether linkage structures and hydroxyl groups in the nanostructure. Moreover, like the CDs prepared from activated carbon (Qiao et al. 2010), the pH value of the NPs dispersion affects the fluorescence emission. The data presented in Supplementary Figure S5 shows that the fluorescence intensity was significantly decreased upon changing the pH value of the solution from acidic to basic, which is quite consistent with the behavior of multicolor CDs derived from activated carbon by chemical oxidation (Qiao et al. 2010). This result may be a consequence of the radiative recombination of the energy-trapping sites which is significantly affected during the protonation process. The photostability of the Coke and Pepsi NPs were also investigated using the organic dye fluorescein as a control. The results shown in Supplementary Figure S6 revealed that the fluorescence intensity of the Coke and Pepsi NPs decreased 15 and 23%, respectively, the fluorescence intensity of fluorescein decreased 49% at the same illumination time, suggesting that the Coke and Pepsi NPs were more stable than those of the organic dye.

To assess the bio-safety of the NPs, the cytotoxicity analysis was performed using the CCk-8 assay after incubating the CHO cells with varying concentrations of the fluorescent NPs (Jiao et al. 2015). The data of the CHO cell viability analysis at different concentration of NPs are shown in histogram form in Supplementary Figure S7. The data reveal that when the concentration of NPs was 20 mg mL^{-1}, the cell viability with the Coke NPs was about 90% (Supplementary Figure S7(a)), whereas no obvious cytotoxicity was observed with the Pepsi NPs (Supplementary Figure S7(b)). However, significant decrease of the cell viability was found for the glucose CDs at 0.63 mg mL^{-1} (Supplementary Figure S7(c)). This result indicated the Cola NPs showed lower cytotoxicity. The evaluation of the uptake of the NPs by hepato cellular carcinoma cells incubated with Coke NPs and Pepsi NPs showed a robust uptake in living cells, indicating that the NPs had penetrated into the living cells, and were able to stain both the cell membrane and cytoplasm (Figure 3). No fluorescence was observed within the nucleus. The excitation-dependent fluorescence emission resulted in blue and green signal output under the excitation wavelength of 405 and 488 nm, respectively.

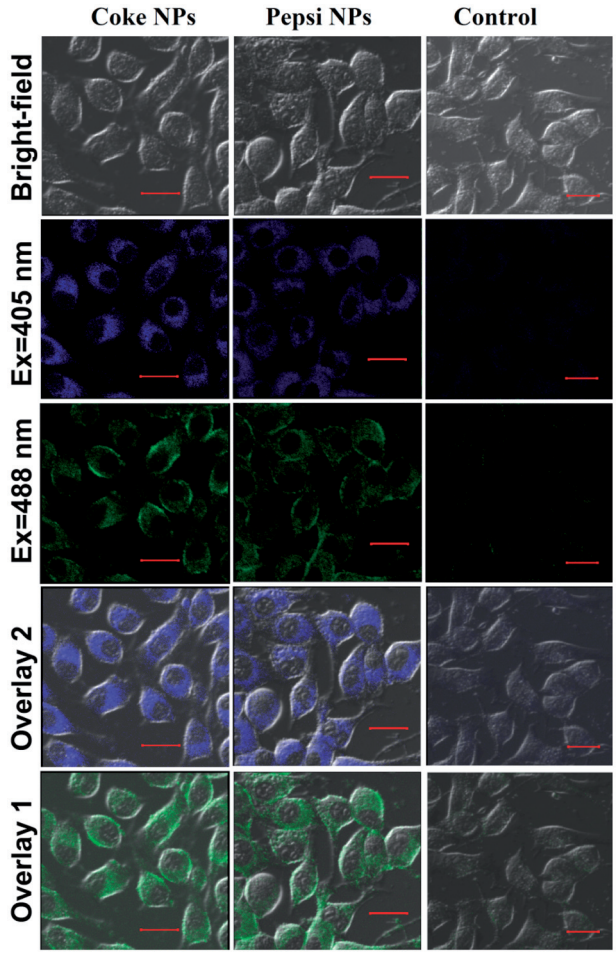

Figure 3. Bright field and fluorescence microscope images of malignant melanoma cells incubated with Coke NPs and Pepsi NPs for 24 h. Exposure time was 400 ms. Control cells were incubated without NPs. Scale bar = 20 μm.

Acute toxicity evaluation

Although carbon is considered as a nontoxic element to human health, a major concern regarding NPs in beverages is their potential toxic effect (Wang et al. 2013). We evaluated the acute toxicity of the Coke and Pepsi NPs in mice by chemical and hematological analysis. Within 24 h of evaluation, the BALB/c mice exposed to the tested concentration of NPs appeared healthy, and the body weight as well as major parenchymatous organs/tissue (liver, spleen, kidney, thymus, heart, brain, lung, ovarian/testis) were similar in appearance to those of the control animals administrated 0.9% NaCl and

Table 1. Body and major organ weight of mice after oral administration of NPs, glucose CDs and 0.9% NaCl.

	Wet weight (g)				Percentage of body (%)			
Items mice	0.9% NaCl	Glucose CDs	Coke NPs	Pepsi NPs	0.9% NaCl	Glucose CDs	Coke NPs	Pepsi NPs
Whole body								
Female	20.54 ± 0.19	20.48 ± 1.16	21.08 ± 0.29	20.7 ± 0.31	100%	100%	100%	100%
Male	20.48 ± 0.16	20.04 ± 0.54	20.97 ± 0.27	20.82 ± 0.17	100%	100%	100%	100%
Liver								
Female	1.02 ± 0.02	1.01 ± 0.08	1.10 ± 0.04	1.02 ± 0.03	4.98 ± 0.21	4.92 ± 0.31	5.21 ± 0.49	4.94 ± 0.25
Male	1.02 ± 0.03	1.00 ± 0.07	0.97 ± 0.02	1.00 ± 0.04	4.99 ± 0.16	5.01 ± 0.21	4.64 ± 0.06	4.80 ± 0.16
Kidney								
Female	0.27 ± 0.02	0.26 ± 0.02	0.29 ± 0.01	0.23 ± 0.03	1.30 ± 0.07	1.27 ± 0.12	1.37 ± 0.04	1.11 ± 0.11
Male	0.25 ± 0.00	0.26 ± 0.01	0.25 ± 0.01	0.27 ± 0.01	1.23 ± 0.02	1.28 ± 0.05	1.22 ± 0.03	1.27 ± 0.04
Thymus								
Female	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.14 ± 0.03	0.40 ± 0.09	0.38 ± 0.05	0.37 ± 0.07	0.68 ± 0.15
Male	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.40 ± 0.05	0.41 ± 0.05	0.40 ± 0.04	0.34 ± 0.06
Spleen								
Female	0.09 ± 0.00	0.08 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.42 ± 0.02	0.39 ± 0.05	0.40 ± 0.03	0.47 ± 0.07
Male	0.09 ± 0.00	0.08 ± 0.01	0.09 ± 0.00	0.09 ± 0.01	0.42 ± 0.02	0.40 ± 0.53	0.41 ± 0.01	0.44 ± 0.03
Heart								
Female	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.73 ± 0.09	0.73 ± 0.08	0.76 ± 0.07	0.75 ± 0.06
Male	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.78 ± 0.09	0.77 ± 0.06	0.77 ± 0.11	0.79 ± 0.11
Brain								
Female	0.55 ± 0.04	0.57 ± 0.05	0.57 ± 0.06	0.54 ± 0.05	2.73 ± 0.35	2.78 ± 0.29	2.88 ± 0.24	2.81 ± 0.27
Male	0.56 ± 0.05	0.59 ± 0.04	0.55 ± 0.06	0.55 ± 0.04	2.90 ± 0.350	2.94 ± 0.23	2.72 ± 0.50	2.82 ± 0.30
Lung								
Female	0.14 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.76 ± 0.09	0.72 ± 0.05	0.78 ± 0.08	0.8 ± 0.06
Male	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.80 ± 0.14	0.76 ± 0.07	0.76 ± 0.07	0.81 ± 0.11
Ovarian/testis								
Female	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.11 ± 0.00	0.10 ± 0.02	0.11 ± 0.01	0.11 ± 0.00
Male	0.15 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.74 ± 0.02	0.76 ± 0.07	0.64 ± 0.05	0.65 ± 0.04

The number of animals in each group is five ($n = 5$).

Table 2. Comparison of serum GPT, GOT, urea and creatinine of mice orally administrated NPs from Coke and Pepsi with control mice with glucose CDs and 0.9% NaCl.

Gender	Biochemical index	0.9% NaCl ($n = 5$)	Glucose CDs ($n = 5$)	Coke NPs ($n = 5$)	Pepsi NPs ($n = 5$)	F-value	p value
Female	GPT	31.80 ± 7.12	36.00 ± 6.75	31.00 ± 6.56	32.20 ± 5.97	0.57	.66
	GOT (U L^{-1})	101.40 ± 10.92	108.00 ± 21.24	99.40 ± 12.44	98.00 ± 18.40	0.37	.78
	Urea (mg dL^{-1})	8.25 ± 1.64	7.91 ± 1.06	7.50 ± 1.14	8.24 ± 1.05	0.40	.76
	Creatinine (mg dL^{-1})	45.60 ± 5.81	52.80 ± 11.82	49.80 ± 9.20	52.80 ± 11.37	0.60	.62
Male	GPT (U L^{-1})	36.80 ± 3.27	31.00 ± 6.78	35.00 ± 6.12	34.80 ± 5.02	1.00	.42
	GOT (U L^{-1})	122.40 ± 12.34	102.60 ± 20.53	107.20 ± 20.69	99.20 ± 12.52	1.81	.19
	Urea (mg dL^{-1})	7.84 ± 0.70	7.48 ± 1.18	8.07 ± 0.93	8.00 ± 1.18	0.33	.80
	Creatinine (mg dL^{-1})	57.00 ± 18.48	49.40 ± 9.37	53.00 ± 6.75	53.20 ± 7.79	0.36	.78

Values in table are mean ± standard deviation.
Values with the p value >.05 are not significantly different.

glucose CDs (Table 1). Neither death nor obvious clinical toxicity sign was observed at 24 h post-NPs administration.

Acute oral poising with NPs can cause changes in the levels of GPT, GOT, urea and creatinine. After 24 h of NPs administration, the GPT, GOT and urea levels in the female group showed a decreasing tendency compared with their levels in the control group, except that the GPT level in the mice administrated the Coke NPs was higher than that in the control mice (Table 2). Although these changes do not show physiological significance, the trend caught our attention. A significant reduction was observed in the GOT level after oral uptake of Pepsi NPs compared with that of the control. In contrast, the biochemical indices in the male group were clearly higher than those of the control, while the urea level in mice administrated the Pepsi NPs was lower than that in the control. The GOT level in mice administrated the Coke and Pepsi NPs did not changed significantly. The unchanged GOT levels may indicate there was no hepatic cell injury. The creatinine level in both tested groups was ~50 mg dL^{-1} and no visible difference was observed compared with the control group.

To examine the effects of NPs on major organs after oral administration, the organs/tissue of heart, liver, spleen, lung, kidney, brain, thymus, stomach, small intestine, colorectum, ovary and testis of mice administrated the NPs at 2 g kg^{-1} body weight were

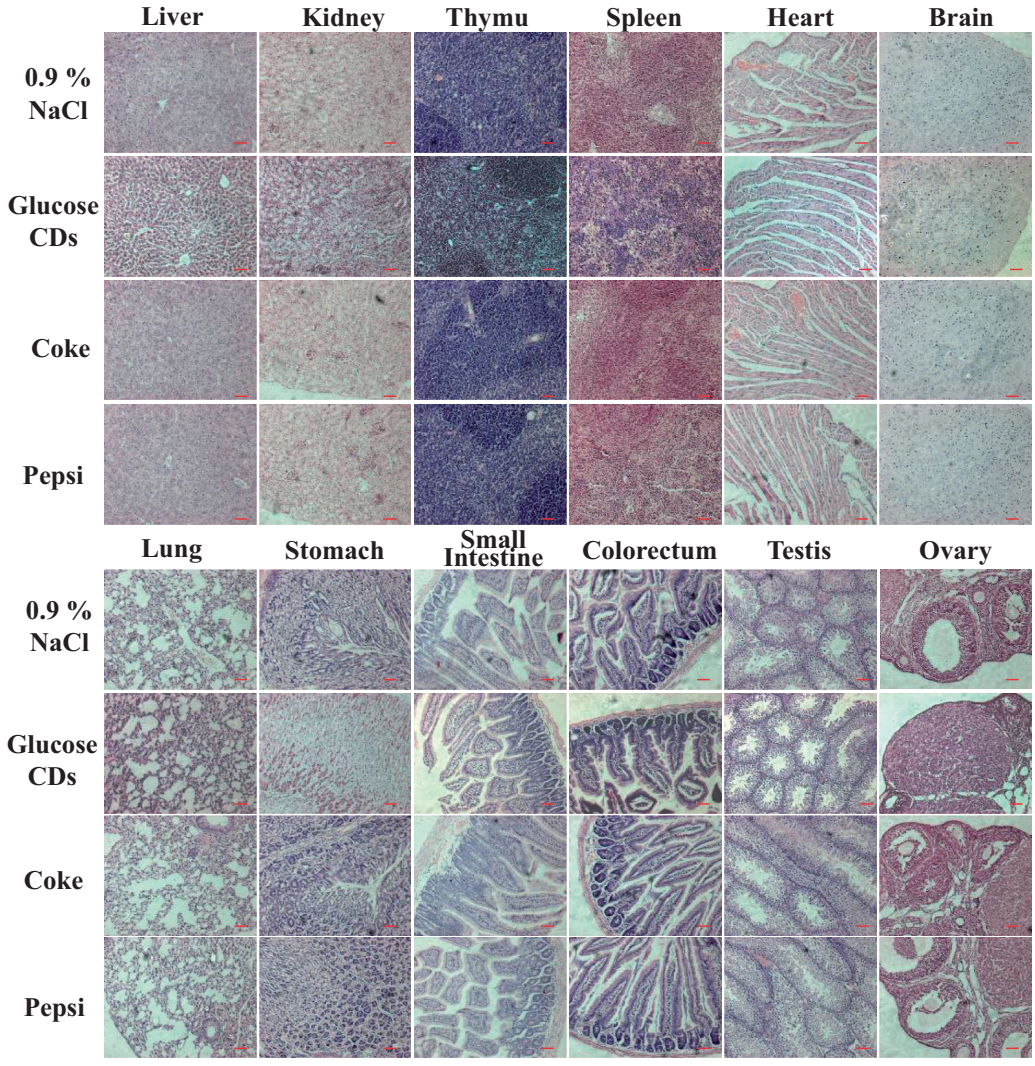

Figure 4. Representative H&E stained images of major organs, including liver, kidney, thymus, spleen, heart, brain, lung, stomach, small intestine, large intestine, testis and ovary for mice treated with NPs from Coke and Pepsi at a dose of 2 g kg^{-1} body weight at 24 h. The control group mice were orally administrated 400 μL of a 0.9% NaCl and artificially synthesized glucose CDs (2 g kg^{-1} body weight) aqueous solution. Scale bar =100 μm.

histopathologically analyzed. No obvious organ damage or apparent histopathological abnormality was observed in the examined mice after oral administration of NPs for 24 h, as shown in Figure 4. The structure and morphology of the cardiomyocytes in the mice were found to be normal and uniform, and there was no cytolysis, necrocytosis, or cell degeneration in the heart tissue sections. In addition, no steatosis, necrosis, or hydropic degeneration was observed in the hepatic sections, indicating the normal structure of the liver. The splenic capsule was complete without any rupture, and the red and white pulps were clear. The lung structure was normal and no bleeding, necrocytosis or inflammation was found. The glomerular structure was clear and easily distinguished in the kidney sections. The neurons and glial cells showed no damage as compared with the control. The thymocytes were clear and no thymus involution was observed. In the sections of the stomach, small intestine and colorectum, there were no ulceration, inflammation and bleeding phenomena. The structure and morphology of the follicle were normal, and there were no blocked lymph nodes. The testicular tubule was easily distinguished, and no abnormal structure was observed in the testis sections. These results were similar to the effects of the CDs derived from multiwalled carbon nanotubes (Tao et al. 2012), and indicated that the NPs extracted from Coke and Pepsi had no obvious acute toxicity in mice major

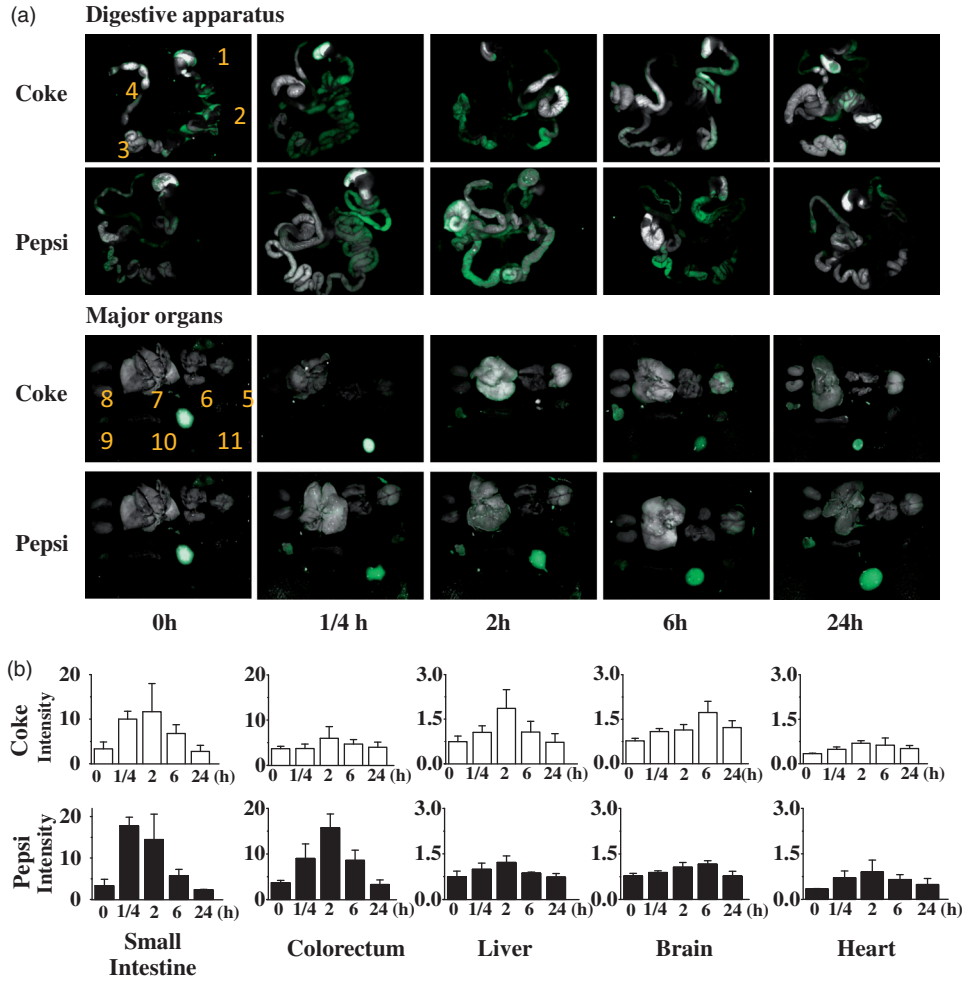

Figure 5. Biodistribution of the NPs from Coke and Pepsi in major organs. (a) *Ex vivo* imaging of digestive apparatus and major organs of the Balb/c mice orally administrated with NPs from Coke and Pepsi at a dose of 2 g kg^{-1} body weight at 0, 1/4, 2, 6 and 24 h. 1, stomach; 2, small intestine; 3, vermiform appendix; 4, colorectum; 5 brain, 6 lung; 7 liver; 8 kidneys; 9 heart; 10 spleen; 11, muscle. (b) Relative fluorescence intensity of major organs of the mice treated with NPs from Coke and Pepsi at a dose of 2 g kg^{-1} body weight at 0, 1/4, 2, 6 and 24 h. The control mice were sacrificed after oral administration of 0.9% NaCl aqueous solution.

organs at the tested dosage. There was also no abnormality for the control animal administrated glucose CDs.

The biodistribution of the Coke and Pepsi NPs in major organs, including the stomach, small intestine, vermiform appendix, colorectum, brain, lung, liver, kidneys, heart, spleen and muscle was evaluated to determine their fate after oral administration. As shown in Figure 5(a), after administration of the NPs, accumulation of both Coke and Pepsi NPs occurred in various sections of the digestive tract, such as small intestine and colorectum, compared with the fluorescence intensities observed in the control. The fluorescence signal detected in the digestive tract in control mice probably comes from mouse feed. We did observe an increased fluorescence signal in the mice administered either Coke or Pepsi NPs, and the fluorescence intensity reached maximum values 2 h after administration of the NPs, except in the case of Pepsi NPs accumulated in the small intestine that only took 15 min to reach the maximum value (Figure 5(b)). These results strongly suggest that the clearance of the NPs from the mouse body possibly occurs through fecal excretions. Similarly, the examination of the NPs distribution in other organs including liver, brain, heart, lung, kidneys, spleen and muscle showed that significant accumulation occurred in the liver, brain and heart 2–6 h after oral administration of the Coke and Pepsi NPs (Figure 5(b)). Remarkably, increased fluorescence was detected in the brain 6 h after administration of the Coke and

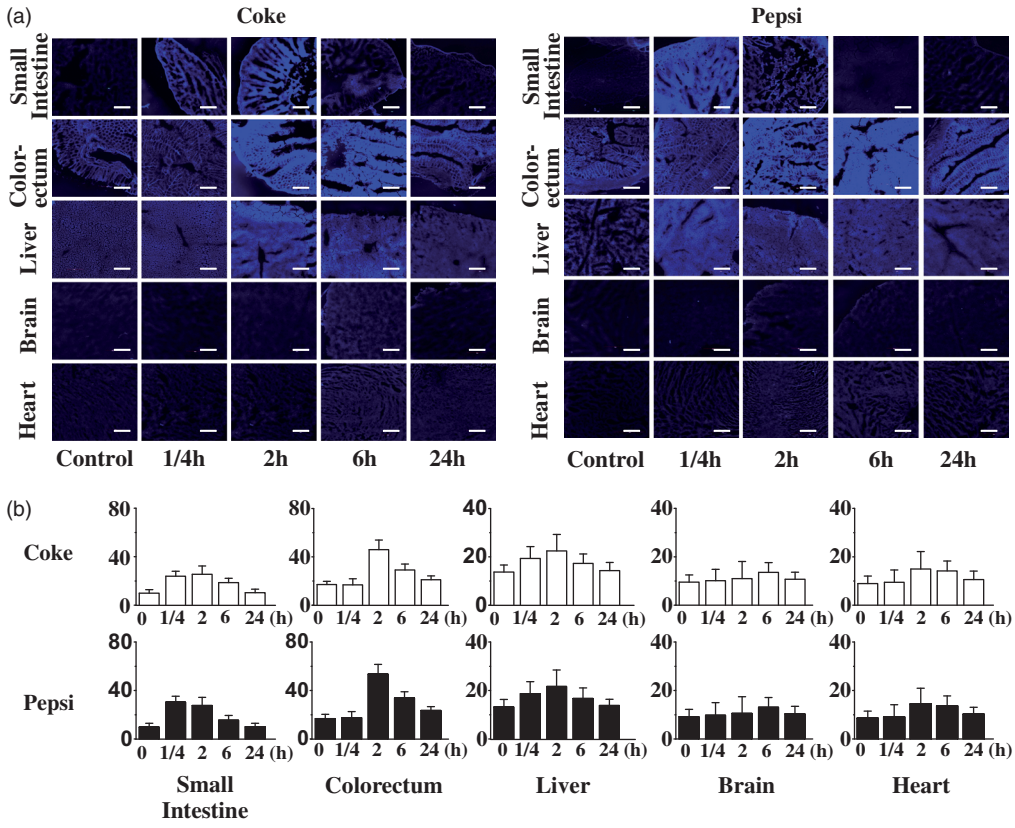

Figure 6. (a) Fluorescence histological analysis of major organs for the mice treated with NPs from Coke and Pepsi at a dose of 2 g kg^{-1} body weight at 1/4, 2, 6 and 24 h. (b) Relative fluorescence intensity of major organs of the mice treated with NPs from Coke and Pepsi at a dose of 2 g kg^{-1} body weight at 1/4, 2, 6 and 24 h. Scale bar =200 μm.

Pepsi NPs, suggesting that the ultra-small (~5 nm) NPs could cross the blood-brain barrier and accumulate in the brain (Supplementary Figure S8). A slight trend of increasing fluorescence intensity in the heart was also observed. There was no obvious fluorescence increase in other organs compared with the control groups. Also, the fluorescence intensity in the major organs and tissues examined 24 h after oral administration decreased to the level observed before administration, indicating that the NPs were excreted from the mouse body.

Also, the small intestine, colorectum, liver, brain and heart of mice that were orally administrated the Coke and Pepsi NPs were collected, and were histologically analyzed. The results presented in Figure 6 reveal that the tissues exposed to NPs display enhanced fluorescence signal compared with the control. Significant accumulation in the small intestine, colorectum and liver was found after oral administration of the NPs, and the fluorescence of the accumulated Coke and Pepsi NPs reached the maximum value 2 h after oral administration, except in the case of the Pepsi NPs in the small intestine, which reached the maximum value after only 15 min. Regarding the brain and heart tissues, the fluorescence of the accumulated NPs reached the maximum value 2–6 h after oral administration (Supplementary Figure S8(b)). The fluorescence histological analysis further confirms the results of the *ex vivo* fluorescence imaging described above. No significant signal was observed in the kidney tissues by the histological analysis, indicating that the NPs quickly excluded from kidneys without noticeable tissue accumulation.

The complete *in vitro* digestion of the Coke and Pepsi NPs was studied using artificial saliva juice, gastric juice and duodenal-bile juice to simulate the digestion process in the mouth, stomach and intestine, respectively (Figure 7(a)). The digestion of the NPs is expressed as the decrease of the fluorescence intensity and all measurements were performed in triplicate. As shown in Figure 7(b), with the artificial digestion mixture of saliva juice, about 69.97 and 64.05% of the fluorescence signal of the Coke NPs, and Pepsi NPs was quenched, respectively. The fluorescence quenching might indicate the

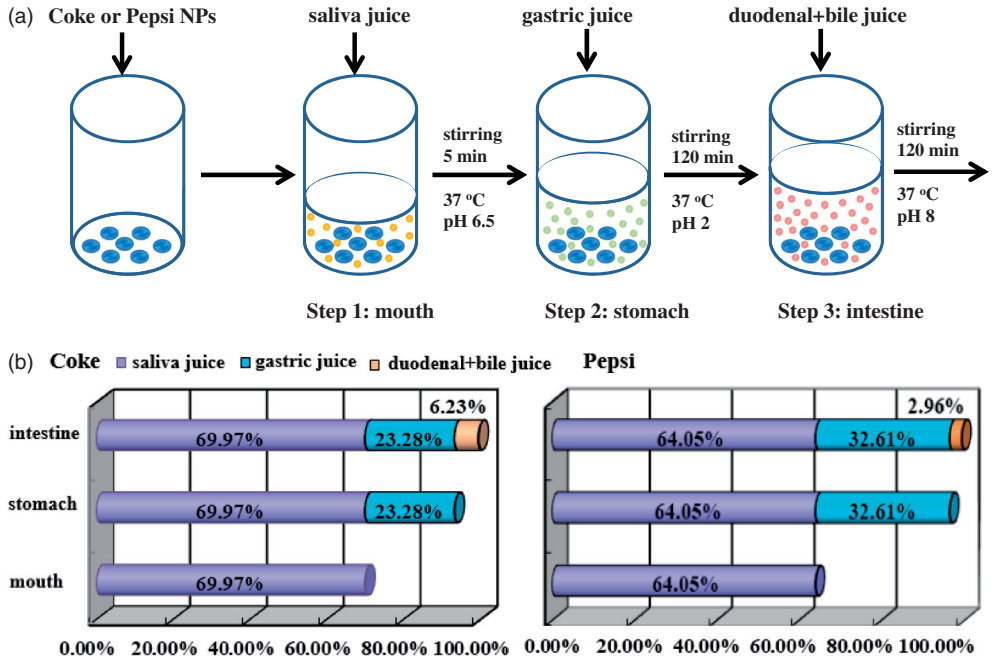

Figure 7. (a) Schematic presentation of the *in vitro* digestion procedure of NPs. (b) Fluorescence quenching of Coke and Pepsi NPs when they are added into saliva juice, gastric juice and duodenal bile juice to simulate the mouth, stomach and intestine digestion.

decomposition or the inhibition by the saliva juice. Moreover, when the gastric juice was added for *in vitro* digestion experiment 23.28 and 32.61% of the fluorescence of the Coke and Pepsi NPs was quenched, respectively. Finally, when the duodenal bile juice was added and stirred for an additional 2 h, more than 99% of the fluorescence of the Coke and Pepsi NPs was quenched. The experiment was conducted under the condition that the digestion was sufficient for the complete uptake of the NPs. In the case when excessive Coke or Pepsi NPs were consumed and were not digested completely, their fluorescence remained visible in the small intestine. This was consistent with the result mentioned above indicating that the NPs accumulated in the digestive tract of mice.

Conclusion

In this study, we reported the finding of the presence of fluorescent NPs in two of the most popular beverages worldwide, namely Coke and Pepsi. The excitation-dependent fluorescent emission behavior of the NPs isolated from Coke and Pepsi was similar to those chemically sysnthsized from carbohydrate like glycerol, glycol, glucose, and sucrose (Wang et al. 2011). Analysis by XPS, NMR and FTIR spectroscopy revealed that the NPs contained C, O and H, and were coated with ether bonds and hydroxyl groups on their surface. The uptake of NPs from Coke and Pepsi by cells demonstrated that the NPs can penetrate into the living cells and accumulate in the membrane and cytoplasm, but not in the nucleus. The cytotoxicity analysis results revealed that no significant reduction in cell viability was caused by NP concentration lower than 20 mg mL^{-1}. Acute toxicity evaluations after a single oral administration of NP suggested that the NPs from Coke and Pepsi do not cause obvious acute toxicity in mice even at the concentration of 2 g kg^{-1} of body weight. The evaluation of the metabolism and biodistribution of the NPs revealed that the NPs were digested and absorbed in the small intestine and colorectum, and were cleared mainly through the liver, and not the kidneys. Significant accumulation of NPs in the brain and heart were also observed, suggesting that the NPs could cross the blood–brain barrier.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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