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Bottom-Up Synthesis of Carbon Nanoparticles with Higher Doxorubicin Efficacy

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Abstract

Nanomedicine requires intelligent and non-toxic nanomaterials for real clinical applications. Carbon materials possess interesting properties but with some limitations due to toxic effects. Interest in carbon nanoparticles (CNPs) is increasing because they are considered green materials with tunable optical properties, overcoming the problem of toxicity associated with quantum dots or nanocrystals, and can be utilized as smart drug delivery systems. Using black tea as a raw material, we synthesized CNPs with a narrow size distribution, tunable optical properties covering visible to deep red absorption, non-toxicity and easy synthesis for large-scale production. We utilized these CNPs to label subcellular structures such as exosomes. More importantly, these new CNPs can escape lysosomal sequestration and rapidly distribute themselves in the cytoplasm to release doxorubicin (doxo) with better efficacy than the free drug. The release of doxo from CNPs was optimal at low pH, similar to the tumour microenvironment. These CNPs were non-toxic in mice and reduced the tumour burden when loaded with doxo due to an improved pharmacokinetics profile. In summary, we created a new delivery system that is potentially useful for improving cancer treatments and opening a new window for tagging microvesicles utilized in liquid biopsies.

Introduction

Nanoparticle technology is an attractive field at the forefront of research and plays important roles in medicine, agriculture and electronics. Nanoparticles have wide applications in medicinal fields as nanocarriers for drug delivery and agents for multifunctional diagnosis, for example [1,2]. Recently, a new class of carbon nanomaterials, including nanodiamonds [3] and fluorescent carbon nanoparticles (CNPs) [4], have been widely investigated due to their high hydrophilicity, excellent biocompatibility, good cell permeability, high photostability and flexibility in surface modification as a result of the presence of different functional groups (carboxyl, hydroxyl and amino groups), allowing the covalent conjugation of chemotherapeutic and targeting agents [5]. Particularly, fluorescent CNPs have wide applications in areas such as bioimaging, drug delivery [6–10], sensors [11–14], optoelectronics [15] and photocatalysis [16]. CNPs are comparable to quantum dots (QDs) and organic dyes [17]. QDs are semiconductor nanostructures with unique optical and electrical properties and great flexibility in their bright and tunable photoluminescence. The blinking effect is a problem with QDs that can be overcome by surface passivation or core-shell formation [18]. QDs are composed of heavy metal precursors such as selenium (Se) and cadmium (Cd), which are toxic at low concentrations in the human body and environment [17,19]. The use of CNPs in place of QDs might overcome the above mentioned problems. Notably, CNPs have attracted considerable interest, as they offer potential advantages over the other carbon nanomaterials such as carbon nanotubes [20–22] and Halloysite nanotubes [23,24] including their small size, simple and inexpensive synthetic routes, high aqueous solubility, their fluorescence property which make them useful for cell imaging and their high cargo loading.

In recent years, much progress has been made in terms of the synthesis, properties and applications of CNPs [17,25]. The synthesis of CNPs can be classified in two groups: chemical and physical methods. Chemical methods include electrochemical synthesis [26],

acidic oxidation [4,6,27], thermal/hydrothermal synthesis [28–31] and microwave/ultrasonic synthesis [12,17,28,32]. Physical methods include arc discharge [33], laser ablation [34] and plasma treatment [35]. Chemical oxidation was commonly used to prepare fluorescent CNPs, which almost always originate from carbon-based nanomaterials. This method is easier, avoids multi-step synthesis and introduces carboxyl and hydroxyl groups on the CNP surface, making the particles negatively charged and hydrophilic. As a result, a variety of fluorescent CNPs have been prepared using food waste [36], carbon nanotubes [37], candle soot [4], carbohydrates (sucrose, glucose) [30,38], active carbon [32], orange juice, polyphenol [39,40] and honey [41]. Although numerous synthetic approaches have been developed, those that are eco-friendly and inexpensive are in demand. Furthermore, large-scale synthesis and size-controlled CNPs remain unmet technological needs.

In the field of drug delivery, carbon nanomaterials have gained considerable attention as nano-carriers due to their high surface area, enhanced cellular uptake and easy conjugation with therapeutics [42–45]. CNPs are spherical and composed of an sp^2 carbon core, which can be conjugated with chemotherapeutic drugs and biomolecules through covalent or noncovalent interactions (π–π stacking or electrostatic interactions) and used for *in vitro* and *in vivo* drug delivery applications [43,46]. However, most of the published papers to date on this topic have focused on the optical properties and *in vitro* biocompatibility of CNPs [47–50], and few have studied CNPs as delivery agents in depth [9,51,52]. Therefore, clinical application remains a challenge.

In this report, we present a green source, “black tea”, as a suitable precursor for the synthesis of CNPs by nitric acid (HNO_3) oxidation. This synthesis is simple and economical because of the selection of an inexpensive carbon source. These CNPs are non-toxic; easily synthetized in large-scale production with tunable optical properties up to red spectra, which can be utilized for multiplexing applications; and can efficiently deliver doxorubicin (doxo). The

biodistribution, pharmacokinetics (PK) profiles and kinetics of release suggest that CNPs-doxorubicin (Cdoxo) is an optimal drug delivery vector for cancer therapy.

Experimental Section

Materials and Instrumentation

Reagents

Commercially available Brooke Bond Taaza tea was utilized. HNO_3 (70%) and sodium hydroxide (NaOH) were purchased from Sigma Aldrich (St. Louis, Missouri, US), doxo was obtained from Accord Healthcare Ltd. (Durham, NC, US) and daunorubicin was purchased from Teva Pharmaceutical Industries Ltd. (Petah Tikva, Israel). All reagents were used as received without further purification. Minisart® syringe filters with a pore size of 0.2 µm were from Sartorius Stedim Biotech (Concord, CA, US), and a dialysis membrane (MWCO 0.5-1 kDa) was purchased from Spectrum Laboratories (Rancho Dominguez, CA, US) for CNP purification. LysoTracker® deep red probe was purchased from Life Technologies (Carlsbad, CA, US). Exosomes were prepared from exosome-depleted medium conditioned for 48 hours and purified with an AB cell culture-nanovesicle solution according to the instructions (AB ANALITICA, Padova, Italy) [53].

DLD-1 and LoVo (colon) and MDA-MB-231 (breast) and HeLa (cervical) cancer cells were grown as indicated by the supplier (ATCC, Manassas, VA, US). Nude and FVB mice were purchased from Harlan Laboratories (Udine, Italy); the procedures were approved by the Italian Ministry of Health n°788/2015-PR and performed in accordance with the institutional guidelines. Data are reported as the mean and standard error.

Equipment

Water was obtained from a Milli-Q water purification system (18.2 Ω; EMD Millipore, Billerica, MA, US). UV-Vis absorption spectra were collected using a NanoDrop 2000c (Thermo Fischer Scientific, Waltham, MA, US). Fluorescence spectra were collected on an Infinite M1000 PRO and cell viability analyzed using an Infinite 200 PRO (Tecan, Männedorf, Switzerland). X-ray diffraction (XRD) data were collected on a Philips X'Pert

vertical goniometer with Bragg-Brentano geometry. Transmission electron microscopy (TEM) was carried out using a Philips EM 208 microscope (Philips, Amsterdam, Netherlands). Fourier transform infrared (FT-IR) spectra were obtained on a NEXUS FT-IR spectrometer implementing a Nicolet Avatar diffuse reflectance accessory. X-ray photoelectron spectroscopy (XPS) was performed on a PHI Quantera SXM spectrometer using monochromatic Al-Kα X-ray sources at 1486.6 eV and 24.8 W with a beam diameter of 100.0 μm, a 1.2 V and 20.0 μA neutralizer, and FAT analyzer mode. Zeta potential (ζ) measurements were collected on a Zetasizer ZS90 (Malvern Instruments, Malvern, UK) using a 632 nm He-Ne laser as the light source. Fluorescence microscopy was carried out using a Nikon microscope at 20x and 40x magnification (Nikon, Chiyoda, Tokyo, Japan). The PK and biodistribution were evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a 4000 QTRAP MS/MS system equipped with a Turbo ESI source (AB Sciex, MA, USA). The exosome particle size was determined with an L10 NanoSight instrument (Malvern Instruments Ltd, UK).

Preparation of CNPs

CNPs were synthesized from tea in the following steps: (1) carbonization of commercial tea followed by (2) oxidation with HNO_3. The carbonized carbon was prepared by heating the commercial black tea at 200 °C for approximately 3 hours, followed by evaporation of water and heating again at 200 °C for approximately 5 hours. The so-formed carbonized tea powder was cooled to room temperature, dried on rotary evaporator and stored in a glass bottle. Then, 500 mg of the carbonized carbon was dispersed in HNO_3 (0.065 mol, 5 M, 13 ml) and refluxed at 80 °C for 20 hours under vigorous stirring. Then, the orange solution was cooled to room temperature and centrifuged (4300g, 25 min, room temperature) to separate out any unreacted carbon. The orange supernatant was collected, neutralized by 5 M NaOH and filtered through a 0.2 μm Minisart® syringe. To remove salts and impurities, the raw solution was dialyzed against Milli-Q water using a dialysis membrane (MWCO 0.5-1 kDa) for at

least 2 days. Finally, the obtained golden-yellow solution was dried on a rotary evaporator and used for further characterization (yield: 26%).

Fluorescence imaging

A droplet of an aqueous CNP dispersion (25 mg/ml) was imaged on a Nikon fluorescence microscope under different filter sets (nm), Ex 350/Em 460 (blue), Ex 490/Em 520 (green), Ex 550/Em 570 (red) and Ex 630/Em 670 (violet), at 20x magnification.

CNP cellular localization

The CNP cellular internalization was evaluated by plating HeLa cells at a density of 7.5 x 10^4 cells/slide. The next day, the cells were marked with 50 nM LysoTracker® deep red probe (Thermo Fisher, MA, US) for 2 h at 37 ºC. After incubation, the cells were washed twice with 1X PBS and incubated for 24 h with 2 mg/ml CNPs. After incubation, the cells were washed twice with 1X PBS and fixed with 4% PFA for 10 min, and the slides were mounted with Alexa FluorSave solution (Thermo Fisher Scientific, Waltham MA, US). The images were obtained on a Nikon fluorescence microscope at 40x magnification using Ex 630/Em 670 nm filters for the lysosomes and Ex 350/Em 460 nm filters for the CNPs.

Imaging of CNP-loaded exosomes

To load exosomes with CNPs, HeLa cells were grown until 70% confluence, treated with 2 mg/mL CNPs for 2 h, washed and then incubated in exosome-free medium for 24 h. The medium was collected, and the exosomes were extracted using an AB cell culture-nanovesicle solution. The next day, the medium was centrifuged at 103,000g and 4 °C for 80 min, and the pellet was resuspended in 1X PBS. The exosomes were characterized by NTA analysis (nanoparticle tracking analysis, Malvern, UK). For imaging, the exosomes loaded with CNPs were spotted on a slide and analyzed with a Nikon fluorescence microscope at 40x magnification under different filter sets (nm): Ex 350/Em 460 (blue), Ex 490/Em 520 (green), Ex 550/Em 570 (red) and Ex 630/Em 670 (violet).

Doxo loading efficiency and release

CNPs (0.5 mg/ml) were incubated with doxo (0.25 mg/mL) in 1X PBS for 2 h at room temperature. The unbound doxo was eliminated by centrifugation at 13000g for 10 min and washed twice with 1X PBS. The drug loading capacity for doxo was calculated as follows: (weight of loaded doxo)/(weight of CNPs). The weight of free doxo was measured on a UV-Vis spectrophotometer from the absorbance at 450 nm based on a doxo standard curve, and the weight of CNPs was measured from the absorbance at 289 nm based on a CNP standard curve. The release of doxo and Cdoxo (50 µg/500 µL) was evaluated using a dialysis membrane (15,000 MWCO) dipped into 1 L of 1X PBS at pH 7.4 or pH 5.5.

Toxicity, cytotoxicity and apoptosis tests

The toxicity of the CNPs was tested in Hela, MDA-MB-231, LoVo and DLD-1 cancer cell lines. The cytotoxicity of the free doxo, CNPs and Cdoxo was tested in MDA-MB-231, LoVo and DLD-1 cancer cell lines. Toxicity and cytotoxicity were evaluated by the CellTiter-Glo® luminescence assay (Promega, Madison, Wisconsin, US) using an Infinite 200 PRO instrument (Tecan, Switzerland). Cells were seeded in 96-well plates (Falcon BD, San Jose, CA, US) at a density of 10^3 cells/well and incubated for 24 h to allow for cell attachment. The cells were incubated with doxo, CNPs, and Cdoxo at the same drug concentrations for 96 h. The experiments were performed in triplicate. Apoptosis was evaluated after 24 hours by fluorescence-activated cell sorting (FACS; BD Biosciences, San Jose, CA, US) utilizing the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, US).

***In vivo* CNP toxicity and efficacy**

This experiment was carried out using 8 weeks old female nude mice, which were administered by i.v. (intravenous) injection of 4 concentrations of CNPs diluted in PBS 1X

(5, 10, 20 and 40 mg/kg). The body weights of the mice were monitored for more than 45 days.

To evaluate the anti-tumour efficacy of Cdoxo compared to doxo, 3 x 10^6 MDA-MB-231 cells diluted in DMEM w/o phenol red/30% matrigel HC (Corning, New York, US) were inoculated in the mammary fat pad of nude mice.

Histopathology: The organs of the mice were collected and fixed in 10% formalin buffered with PBS, embedded in paraffin, sectioned at a thickness of 3 μm and stained with hematoxylin and eosin (H&E). The tissues were analyzed with light microscopy using different magnifications.

PK and biodistribution

The PK experiments were performed in 8 weeks old FVB mice treated with 3 mg/kg (i.v.) of the drug diluted in PBS 1X, and approximately 100 μl of blood was collected after 0.5, 1, 3, 6, 24, 48, 96 and 192 hours. Blood was collected from each mouse twice: from the mandibular vein (live mouse) and the right ventricle of the heart (sacrificed mouse). A total of 12 mice were utilized. Serum samples were stored at -80 °C. For analysis of the drug tissue distribution, the mice were sacrificed at 3 and 24 hours, and their organs were washed with 10 ml of cold PBS/heparin before collection. The organs were diluted in 500 μl of 4% PBS/BSA and homogenized with a Qiagen TissueRuptor for 20 sec at power 4 in ice (Qiagen, Hilden, Germany).

The doxo concentrations in serum and tissues were measured by LC-MS/MS. The proteins were precipitated with 2 volumes of cold acetonitrile containing 20 ng/ml daunorubicin as an internal standard. After vortexing and spinning at 13000 rpm for 15 min at 4 °C, the cleared supernatant was diluted with 2 volumes of 0.2% formic acid, and 10 μl of the dilution were injected into the LC-MS/MS system. Chromatographic separation was performed on an Accucore 150-C18 column (2.6 μm, 30x2.1 mm; Thermo Scientific, Waltham, MA USA)

equilibrated with 0.2% formic acid/acetonitrile (95:5) at 0.7 ml/min and maintained at 50 °C. An elution gradient B from 5% to 80% acetonitrile was applied over 5 min. A 4000 QTRAP MS/MS system equipped with a Turbo ESI source (AB Sciex, Framingham, MA, USA) was equilibrated for 3 min in positive-ion mode. The transitions of doxo and daunorubicin were monitored in multiple reaction monitoring mode at m/z 544.1→397.2 and 528.2→321.1, respectively. The spray voltage was set at 5000 V, with a source temperature of 400 °C. The curtain gas, nebulizer gas (gas1) and auxiliary gas (gas 2) were set at 20, 50 and 50 arbitrary units, respectively. The declustering potential and collision energy voltages were set at 45 V and 16 V, respectively, for both doxo and daunorubicin.

Results and Discussion

Characterization of CNPs prepared from black tea

The CNPs were prepared from tea by HNO_3 oxidation and characterized by UV-Vis absorption spectroscopy, fluorescence spectroscopy, powder XRD, FT-IR spectroscopy and TEM. The zeta potential of the CNPs was also measured at -16.6 mV, indicating a negative charge on the CNP surface due to the presence of carboxylic groups.

Figure S1 shows the UV-Vis absorption and fluorescence spectra of CNPs excited at 360 nm. The UV-Vis absorption spectrum contained two distinct peaks: one at 300 nm that could be assigned to the n-π^* transition of the C=O groups on the surface of the CNPs and one at 242 nm that could be assigned to π-π^* transitions of the polycyclic aromatic systems (C=C) contained in the polyphenols of the tea [54]. The CNP solution produced a maximum emission peak centered at 470 nm when excited at 360 nm (Figure S1A). To investigate the optical properties of the CNPs, emission spectra were recorded at various excitation wavelengths from 300 to 570 nm; the emission peaks were red-shifted from 390 to 570 nm while the intensities decreased (Figure S1B, S1C). These optical properties mainly result from the different sizes and different distributions of emissive sites, which is generally a characteristic of fluorescent carbon nanomaterials [34]. The fluorescence properties of CNPs are always dependent on the size and the presence of organic functional groups in the carbon source [5].

We applied XRD and FT-IR analyses to identify the functional groups and the phase of the CNPs. The powder XRD spectrum (Figure S2) contained a broader peak at 2θ = 24.8°, revealing an amorphous carbon phase in the CNPs. The FT-IR spectrum (Figure 1A) indicated that the CNPs have many oxygen- and nitrogen-containing functional groups on their surface. The broad peak centered at 3294 cm^{-1} revealed O-H/N-H bonding, and the absorptions at 2937 and 2866 cm^{-1} could be attributed to C-H stretching vibrations.

Moreover, the absorption peaks at 1652 and 1752 cm^{-1} are indicative of C=O bonds. The absorptions at 1110 and 1195 cm^{-1} could be attributed to C-O-C bonds, and the absorptions at 1318 and 1337 cm^{-1} confirm the presence of C-O bonds. Furthermore, the absorption peaks at 1594 cm^{-1} could be attributed to the C=N and C=C groups of aromatic hydrocarbons, indicating the presence of sp^2 hybridization, whereas the absorption peaks at 1406 and 1431 cm^{-1} could be related to C-N bonds. These data suggest that the CNPs were functionalized with hydroxyl, alkyl, carbonyl, carboxylic, and amine groups derived from the organic molecules in the black tea and the use of HNO_3.

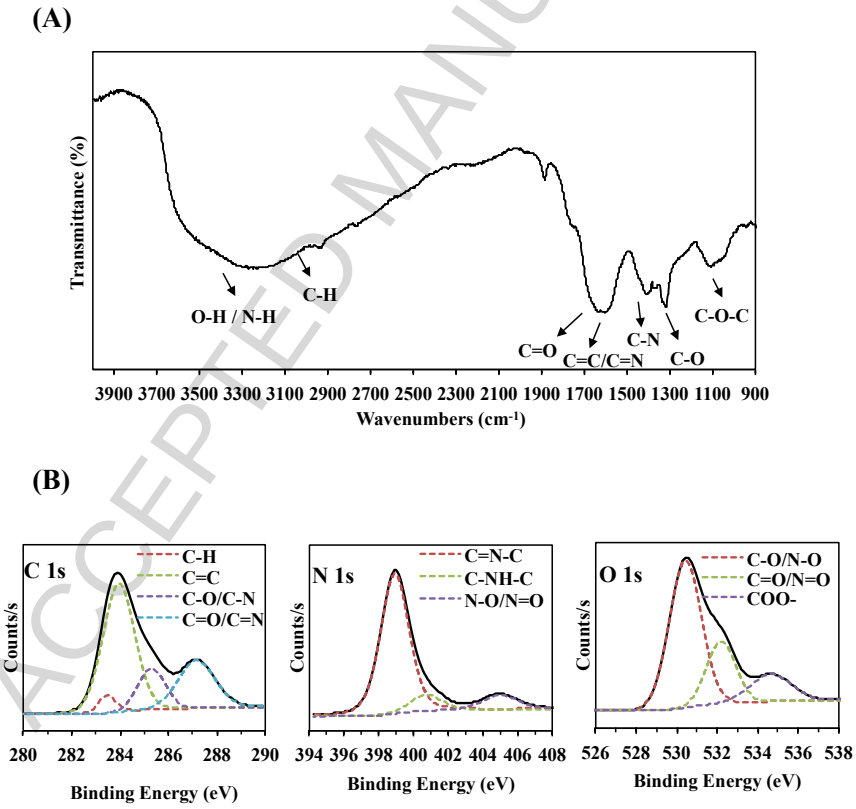

Figure 1. (A) FT-IR spectrum of CNPs; **(B)** C1s, N1s, and O1s XPS spectra.

XPS analysis was used to confirm the functional groups on the CNP surface. From the XPS spectrum (Figure 1B), C, N and O were detected from the peaks at 285 eV (C1s), 400.2 eV (N1s), and 532 eV (O1s), respectively, with 62.56% carbon, 31.43% oxygen, and 6.01% nitrogen. The C1s peaks at 283.5, 284, 285.3, and 287.2 eV could be assigned to carbon in the form of C-H, sp^2 (C=C), C-O/C-N and C=O/C=N, respectively [55–57]. The N1s peaks

consisted of three Gaussian peaks centered at 399, 408.8 and 405 eV, corresponding to C=N-C, C-NH-C, and oxidized N-species such as N-O/N=O, respectively [56,57]. The O1s peaks could be deconvoluted into three Gaussian peaks centered at 530.4, 532.2 and 534.7 eV, corresponding to C-O/N-O, C=O/N=O, and COO^-, respectively [57]. The surface components of the CNPs are in agreement with the FTIR results. It is well known that HNO_3 oxidation produces hydroxyl and carboxylic groups on CNP surfaces, which makes the particles water soluble and negatively charged. In addition, this oxidation can also induce nitration [58]. Our experimental data suggest that refluxing the carbonized carbon derived from tea with HNO_3 induces partial oxidation of the carbons; introduces functional groups, such as OH, COOH, and NO_2; and causes nitrogen doping into the CNPs. The introduction of functional groups imparts water solubility and a surface charge to the CNPs. This oxidation step could also be considered a chemical route to incorporating nitrogen into the CNPs, as observed from the chemical composition analysis.

The morphology and size of the CNPs were investigated by TEM. As shown in Figure 2, the CNPs had a narrow size distribution and were spherical with an average diameter of 17 nm.

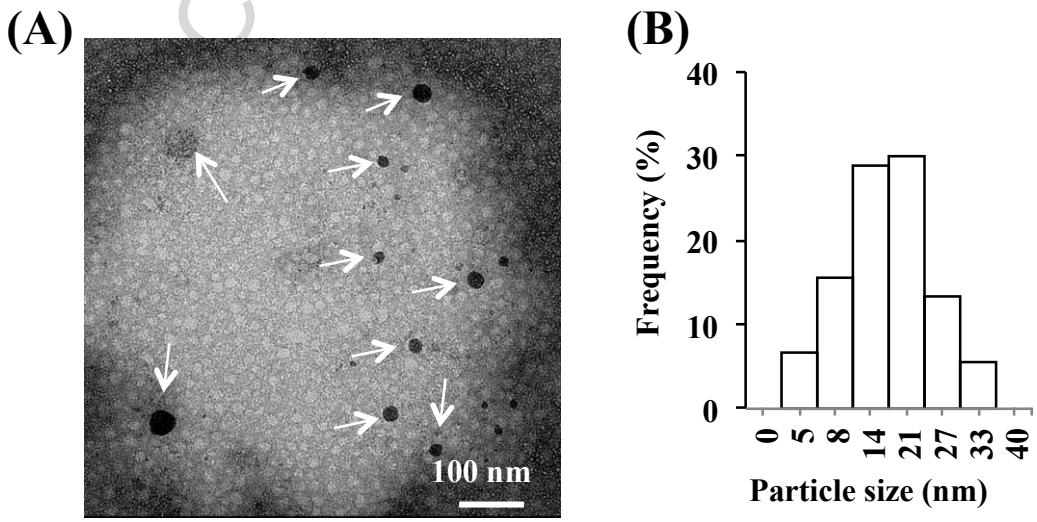

Figure 2. (A) TEM image and **(B)** particle size distribution histogram of CNPs (17.3 ± 7.6 nm). Arrows indicate CNPs.

CNPs were biocompatible and suitable for bioimaging of cellular and subcellular (exosomes) compartments

The CNPs were reported to be not toxic in different experimental set-ups [26,59]. *In vitro* experiments showed that CNPs do not alter cell viability at concentrations up to 200 μg/ml [60]. A toxicity test was performed with HeLa, MDA-MB-231, LoVo and DLD-1 cells. Our CNPs were not toxic at up to 1 mg/ml, illustrating very high biocompatibility (Figure S3), and sustained further testing in *in vivo* experiments. To strengthen these results, an apoptosis test was performed, the results of which are presented in Figure S4. Cells were treated with 1 mg/ml CNPs, and the expression of Annexin V on the surface of the cells was measured by FACS after 24 hours. No change in the percentage of apoptotic cells was observed in the CNP-treated cells over the control.

Although the CNPs were designed and synthetized for drug delivery applications. A droplet of CNPs (25 mg/ml) was deposited on a cover slip under a fluorescent microscope and imaged under different excitation wavelengths commonly utilized for biological experiments. Fluorescence of the CNPs was detected in all the ranges utilized (Figure 3). For biological applications, a wavelength range over 600 nm is more suitable (Figure 3D) and does not overlap with the fluorescence of doxo, which has a maximal excitation/emission of approximately 490/590 nm.

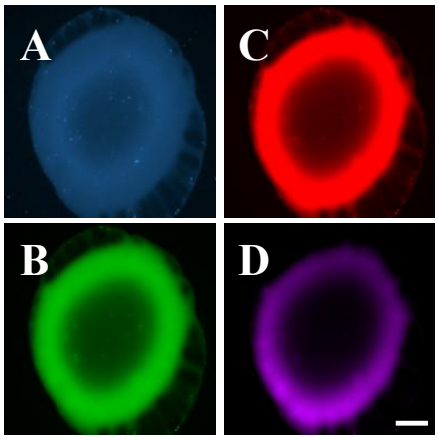

Figure 3. (A-D) Fluorescence microscopy photographs of an aqueous solution of CNPs under different excitation filter sets: **(A)** 350 nm, **(B)** 490 nm, **(C)** 550 nm and **(D)** 630 nm. Scale bar: 200 μm

Due to the increasing number of papers focused on exosome biology and the possibility of utilizing exosomes in liquid biopsies, the CNPs were tested for use as potential fluorescent probes. Exosomes are extracellular vesicles with nanometric dimensions (30-200 nm) and diagnostic [61] and therapeutic potential [62]. Exosomes incubated with CNPs were collected after 24 hours and verified by NTA analysis (Figure S5). Equal quantities of exosomes were evaluated under fluorescence microscopy from CNP-treated and untreated cells. A clearly noticeable dotted appearance of CNP-loaded exosomes can be observed in Figure 4 and data that is not shown here, suggesting that these CNPs can be utilized to probe exosomes for biological applications.

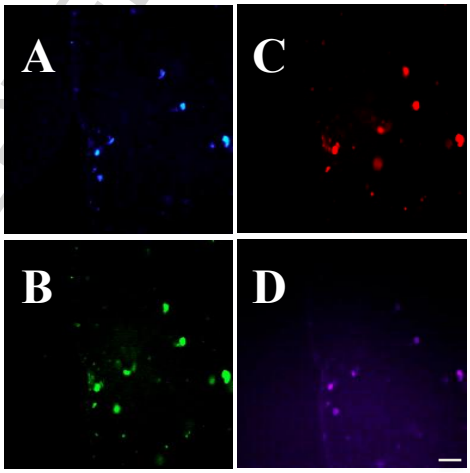

Figure 4. Exosomes isolated from the cell culture medium of MDA-MB-231 cells treated with CNPs (2 mg/ml) for 2 h and collected after 48 h. Images were acquired with different excitation filter sets, as in Figure 3. Scale bar: 20 μm.

CNPs avoided lysosomal entrapment and delivered doxo efficiently in *in vitro* experiments

Lysosomal degradation is a natural process by which cells eliminate unnecessary endogenous and exogenous materials [63]. The failure of many nanomaterials is due to their accumulation inside lysosomes [64]. Escaping lysosomal degradation is a desirable functional property for drug delivery applications. Under this scope, HeLa cells were probed with LysoTracker for 2 hours and incubated with 2 mg/ml CNPs for 24 hours. Under fluorescence microscopy, the CNPs (green) had a clearly uniform distribution in the cytoplasm and nucleus, and the typically punctuated appearance of lysosomal accumulation (red) was not apparently observed (Figure 5).

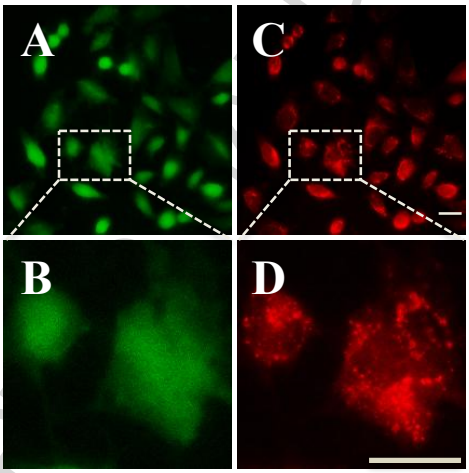

Figure 5. HeLa cells **(A)** treated with 2 mg/ml CNPs after 24 h and marked with **(C)** LysoTracker. **(B) and (D)** Zoom-in of **(A)** and **(C)**. Scale bar: 20 μm.

A desirable property of nanomaterials is an intrinsic ability for loading therapeutic drugs and a controlled release over time under physiological conditions [65,66]. To demonstrate this concept, the CNPs were loaded with doxo, and the kinetics of drug release was calculated from a dialysis experiment in PBS at 37 °C at different pH values. For drug loading, the CNPs were mixed with doxo at room temperature, and the drug loading was calculated to be approximately 60% (Figure 6A). Doxo is a weak amphipathic base with pKa= 8.3. At physiological pH (7.4), the protonated fraction of doxo is still 10-fold that of the free base, while the carboxylic acid moieties on the CNP surface are nearly completely dissociated to their negative carboxylate form (pKa range: 3-5) [67,68]. Thus, the doxo molecules retain

their electrostatic interactions with the CNPs at physiological pH. At pH 4, the carboxylic acid groups on the CNPs are partially dissociated, decreasing the negative charge on the CNPs and reducing the electrostatic interactions of the drug carrier with protonated doxo. To support our conclusion, CNPs were loaded with doxo at different pH levels: 4, 5.5 and 7.4 (Figure S6). The percentage of loading positively correlated with the pH.

The extracellular pH (pHe) of tumour tissues is acidified by the metabolism of tumour cells [69]. Cell survival is conditioned by maintenance of a favourable acid-base balance (pH). Because of cellular metabolism, which produces CO_2 and lactic acid, cancer cells are continuously exposed to large acid-base fluxes, which would disturb the pH. In contrast to normal cells, most tumour cells preferentially convert glucose and other substrates to lactic acid, even under aerobic conditions. This phenomenon, termed "the Warburg effect", was reported by Warburg and co-workers in the 1920s [70–72]. Due to increased glucose metabolism, tumours possess a greater capacity to pump lactic acid and protons out to the extracellular spaces to maintain an appropriate neutral-alkaline intracellular pH (pHi), which is essential for cell vitality. The inefficient removal of protons and lactic acid from extracellular spaces creates a reversed gradient characterized by an acidic pHe and alkaline pHi [73–75]. *In vitro* and *in vivo* studies revealed that tumour cells have a pHi ranging from 7.1 to 7.6 (pHi of normal cells: 7.0 to 7.2) and a pHe of 6.2-6.9 (pHe of normal extracellular space: 7.3-7.4) [76]. The intravesicular pH along the endocytic pathway ranges from pH 6.0–6.5 in early endosomes to pH 4.5–5.5 in late endosomes and lysosomes [77].

A drug delivery system that is able to release its cargo more efficiently around the tumour site at low pH (approximately pH 6) represents an intelligent system to specifically target tumour cells [78]. To study the capacity and release of Cdoxo over different pH gradients, we carried out a release experiment at pH 5.5 and 7.4 to mimic the bloodstream, tumour microenvironments and intracellular endosome/lysosome pathway (Figure 6B) [79,80]. The release of doxo was derived from a log-log plot of the cumulative release versus time.

Noticeably, the CNPs maintained a stable interaction with doxo at alkaline pH (pH of the bloodstream) with a slow release profile (approximately 15 hours), compared to a fast release profile (approximately 1 hour) when the medium was acidified to levels of the extracellular space of the tumour and in subcellular compartments. This pH gradient increases the ratio of the tumoral/non-tumoral drug concentration, thereby elevating the therapeutic index of doxo.

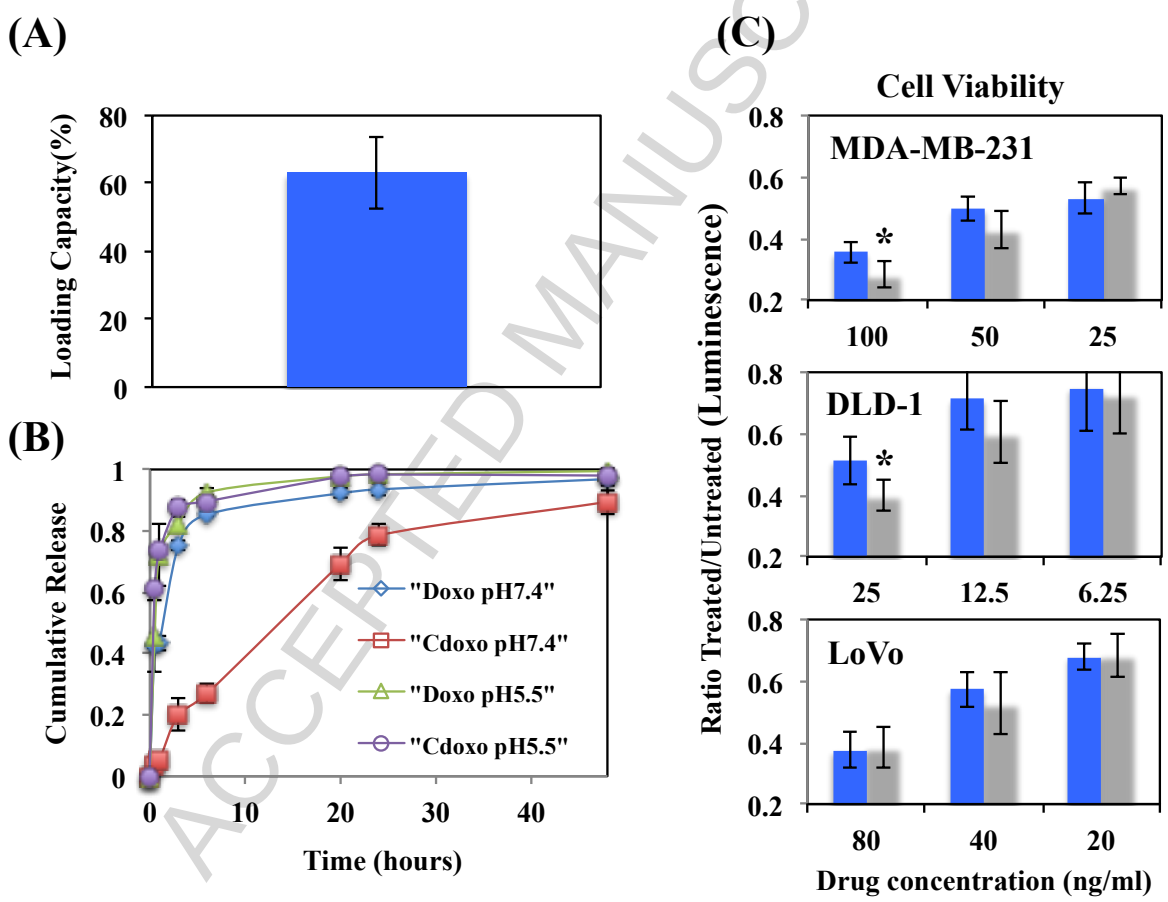

Figure 6. (A) Loading capacity of Cdoxo. The graph displays the percentage of doxo loading (y-axis) **(B)** Release of doxo from CNPs. The cumulative release of doxo was evaluated by measuring the fluorescence of doxo, which resides inside the dialysis membrane at each time point at pH 5.5 and 7.4. **(C)** Cytotoxic effects of Cdoxo on MDA-MB-231, DLD-1 and LoVo cell lines treated with increasing concentrations of doxo (blue) or Cdoxo (red), as indicated on the x-axis (ng/ml). *p value <0.05 (y-axis). The quantity of utilized doxo was based on previously calculated IC50 (middle value).

Subsequently, the cell viability of MDA-MB-231, LoVo and DLD-1 cells treated with Cdoxo was tested. Cells were treated with 3 different concentrations of free doxo or Cdoxo, and the cell viability was assessed after 96 hours (Figure 6C). Cdoxo exhibited better cytotoxicity than free doxo in MDA-MB-231 and DLD-1 cells (p value < 0.05). Based on these results, we further evaluated the CNPs as a drug delivery system in a mouse model of breast cancer.

CNPs were not toxic in mice and increased the efficacy of doxo

In vitro experiments demonstrated that our CNPs were not toxic at concentrations above the necessary dosage for drug delivery applications. To better predict toxicity in humans, nude mice were treated with a single i.v. injection of 5, 10, 20 and 40 mg/kg CNPs. Their body weight was monitored as an objective parameter of mice wellness. The mice were followed over a period of approximately 2 months. We did not observe any symptoms of stress or clinical illness. The body weight of the mice increased during the observational period (Figure 7A). After more than 6 months, the mice were sacrificed, and their tissues were histopathologically analyzed. No obvious signs of toxicity were observed (Figure S7).

Supported by this encouraging data, MDA-MB-231 cells were orthotopically inoculated in the mammary fat pad of nude mice. After the tumours had reached an average volume of 57 ± 8 mm^3, the mice were treated 3 times on a weekly base with 3 mg/kg Cdoxo or free doxo. Figure 7B demonstrates that the tumour volume of the Cdoxo-treated mice was reduced compared to the tumours of mice treated with free doxo (*p* value < 0.05). The body weight of the mice was similar among the groups of mice tested during the experiment (Figure 7C).

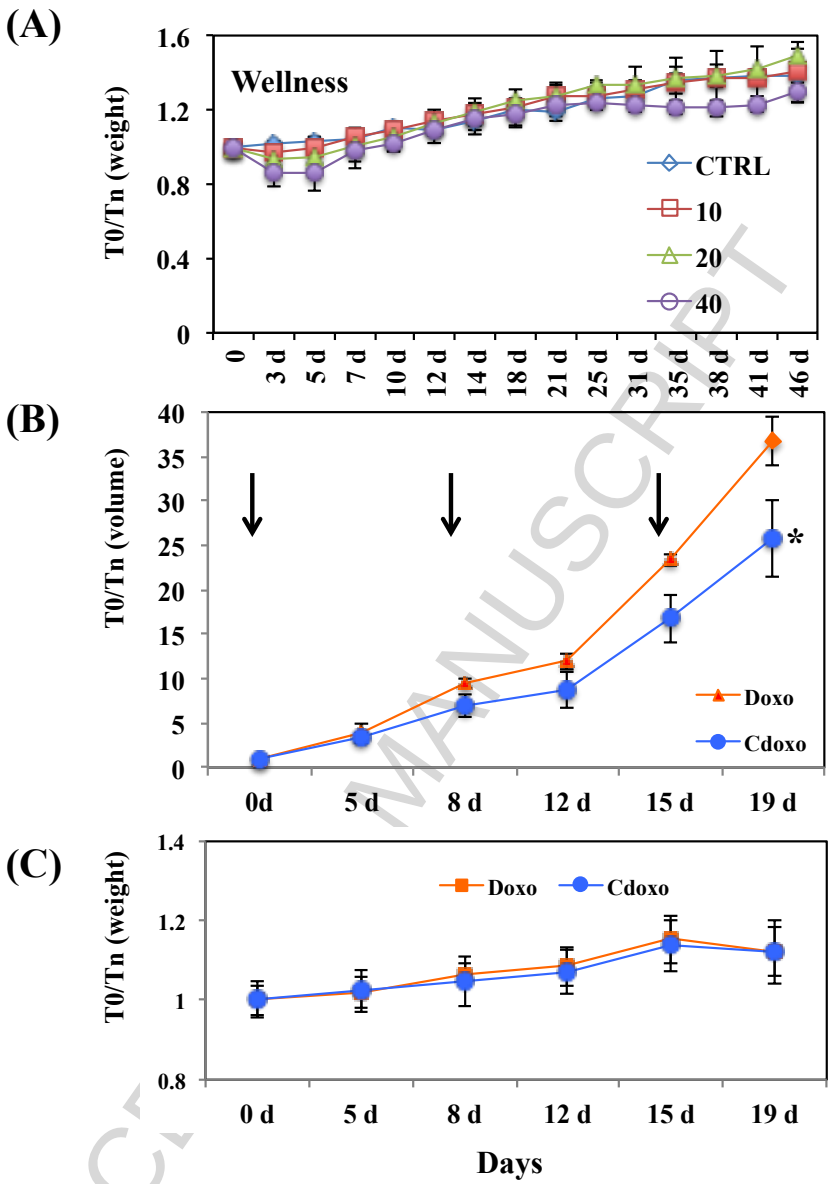

Figure 7. (A) Weight of mice treated with different concentrations (mg/kg) of CNPs as indicated. **(B)** Mice were treated 3 times (arrows) at 3 mg/kg Cdoxo or free doxo, and the tumour volume was measured (y-axis). **p* value <0.05 **(C)** Weight of mice treated as in (B). T_0: time at the beginning of the experiment; T_n: time on day n as indicated. Eight tumours were analyzed per data point.

CNPs altered the biodistribution and prolonged the circulation time of doxo

Data obtained from well-known and successful liposomal formulations revealed that drug efficacy can be increased by a longer circulation time, avoiding rapid clearance [81]. To investigate the potential changes in the PK profile, we administered 3 mg/kg doxo (i.v.) and

Cdoxo to FVB/N mice. The PK profile of doxo in blood and tissues was qualitatively similar when administered as the free drug or Cdoxo. Both PK profiles were characterized by fast first-phase elimination. However, in the late phase of elimination, up to 4 days, the concentration of doxo in blood remained higher when administered as Cdoxo (Figure 8A) compared to free doxo. This result is consistent with the increase in the mean residence time from 14.1 ± 2 to 20.1 ± 0.5 hours (p value < 0.01) and the apparent constant elimination from 0.07 ± 0.01 hour^{-1} to 0.050 ± 0.01 hour^{-1} (p value < 0.05) for free doxo and Cdoxo, respectively.

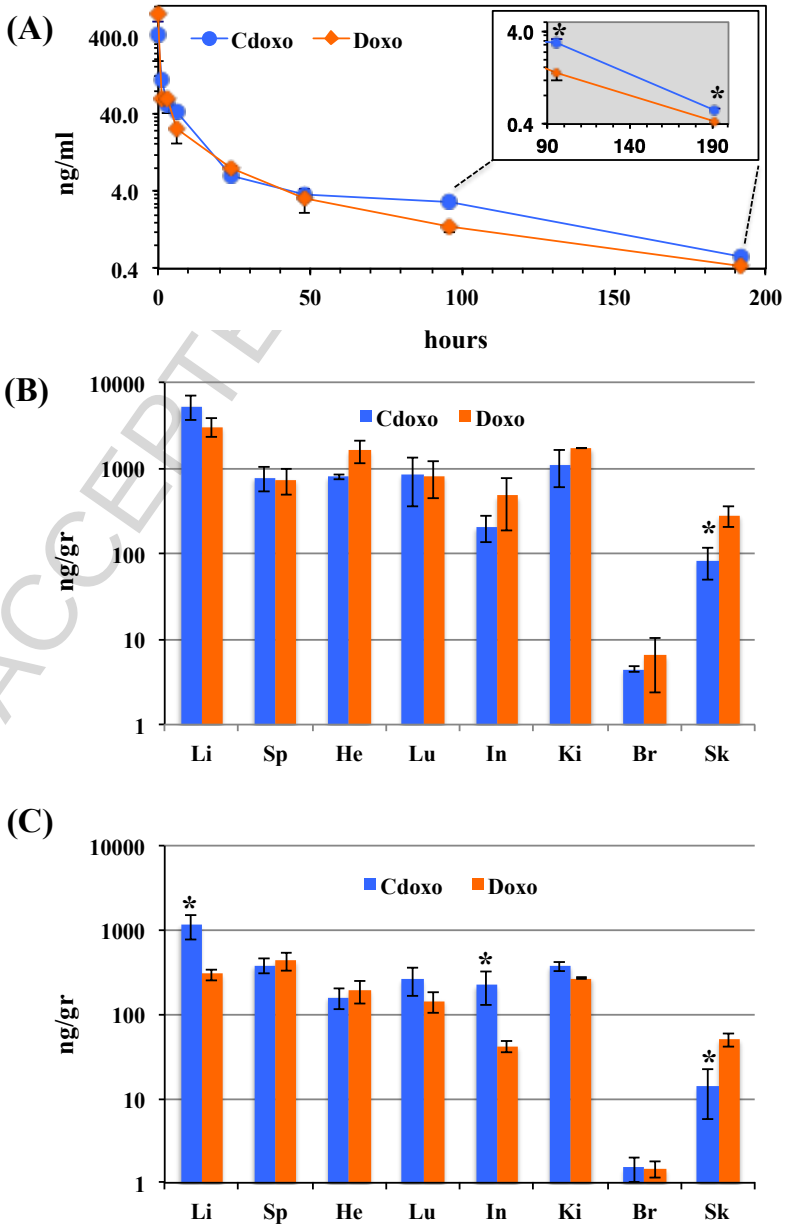

Figure 8. (A) PK profile of free doxo and Cdoxo at 0.5, 1, 3, 6, 24, 48, 96 and 192 hours. In the insert, the time points 96 and 192 hours were zoomed in. *p value <0.05. **(B) and (C)** Biodistribution of free doxo and Cdoxo at 3 (B) and 24 (C) hours. At 3 hours, less Cdoxo was present in the skin. At 24 hours, increased accumulation of Cdoxo in the liver and intestine was observed. The y-axis is in logarithmic scale (ng/gr of drug/tissue). Li: liver, Sp: spleen, He: heart, Lu: lung, In: intestine, Ki: kidney, Br: brain, Sk: skin. Three mice were utilized for each data point.

The tissue distribution of the drug 3 hours post-injection demonstrated a similar profile between Cdoxo and free doxo, except in the skin (Figure 8B). After 24 hours, the distribution of Cdoxo changed, with an accumulation in the liver and intestine and a reduction in the skin (p value < 0.05; Figure 8C). These data suggest that Cdoxo could reduce the skin toxicity associated with liposomal formulations of doxo and be utilized to treat gastro-intestinal cancers.

Conclusions

In this study, we prepared a new nanovector that can be used to image subcellular compartments such as exosomes with excellent properties for drug delivery [82]. These CNPs can be efficiently loaded with doxo, a widely used chemotherapeutic drug, and exhibit controlled release under acidic conditions, as in the tumour microenvironment. Cdoxo was more effective *in vivo* than free doxo due to a different PK profile. Hence, a simple and green synthesis starting from tea could produce a tunable and safe drug delivery nanocarrier with excellent biocompatible properties.

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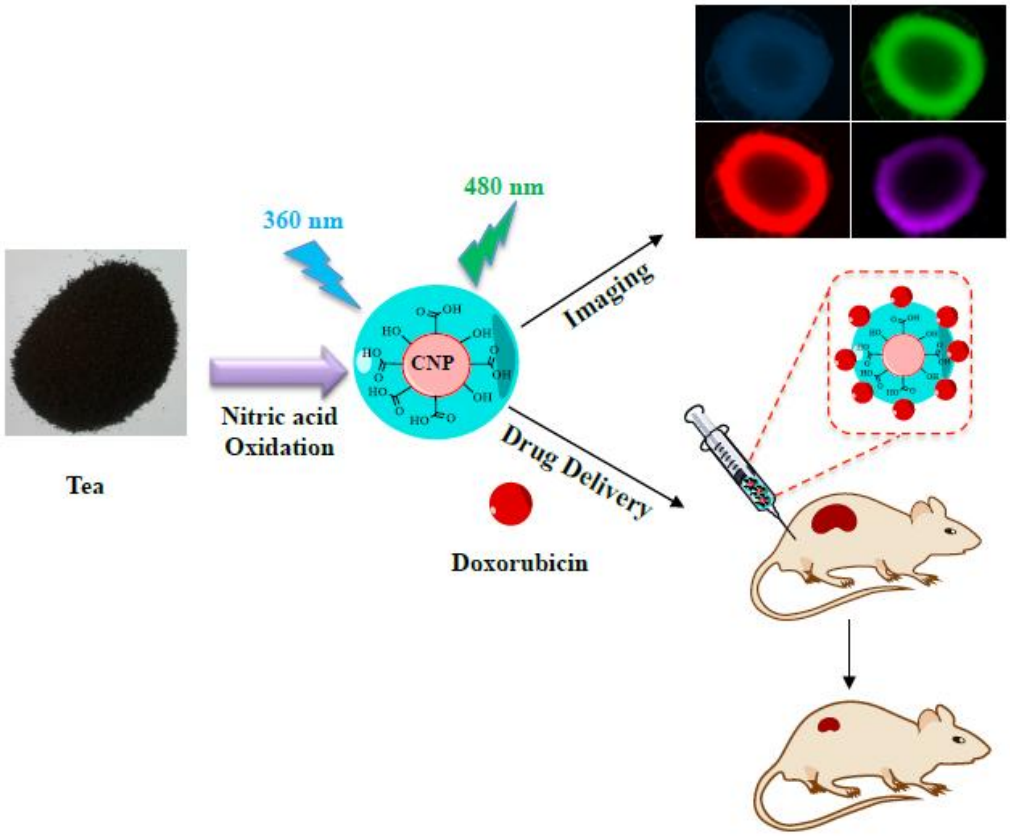
Competing Interests

The authors declare no competing interests.



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