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ORIGINAL ARTICLE

A comparative evaluation of anti-tumor activity following oral and intravenous delivery of doxorubicin in a xenograft model of breast tumor

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Abstract

Purpose Natural materials have been extensively studied for oral drug delivery due to their biodegradability and other unique properties. In the current research, we fabricated sodium caseinate nanomicelles (NaCNs) using casein as a natural polymer to develop a controlled-release oral delivery system that would improve the therapeutic potential of doxorubicin (DOX) and reduce its toxicity.

Methods DOX-loaded NaCNs were synthesized and thoroughly characterized, then subjected to in vivo anti-tumor evaluation and bio-distribution analysis in a 4T1-induced breast cancer model.

Results Our findings indicated that the tumor would shrink by eight-fold in the group orally treated with DOX-NaCNs when compared to free DOX. The tumor accumulated drug 1.27-fold more from the orally administered DOX-NaCNs compared to the intravenously administered DOX-NaCNs, 6.8-fold more compared to free DOX, and 8.34-times more compared to orally administered free DOX. In comparison, the orally administered DOX-NaCNs lead to a significant reduction in tumor size (5.66 ± 4.36 mm^3) compared to intravenously administered DOX-NaCNs (10.29 ± 4.86 mm^3) on day 17 of the experiment. NaCNs were well tolerated at a single dose of 2000 mg/kg in an acute oral toxicity study.

Conclusion The enhanced anti-tumor effects of oral DOX-NaCNs might be related to the controlled release of DOX from the delivery system when compared to free DOX and the intravenous formulation of DOX-NaCNs. Moreover, NaCNs is recognized as a safe and non-toxic delivery system with excellent bio-distribution profile and high anti-tumor effects that has a potential for oral chemotherapy.

Keywords Casein · Oral drug delivery · Breast cancer · Doxorubicin · Biodistribution studies

Introduction

Tumor-targeted delivery of chemotherapeutic agents aimed at enhancing drug concentrations inside the tumor and simultaneously reducing their side-effects while promoting patient compliance with chemotherapy has been an important issue and area of focus in the drug delivery field (Reddy et al. 2004). For effective chemotherapy, the uptake of chemotherapeutic agents by non-target cells and their quick clearance by the kidneys must be minimized, which together contribute to prolonging the lifetime of these agents in the blood circulation and accelerating their accumulation in solid tumors (Kim et al. 2009).

Following Paul Ehrlich´s idea of a "magic bullet", the parenteral route for anti-cancer drug delivery is generally preferred to oral, in order to deliver drugs directly to

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targeted sites rather than to healthy tissues (Bertoni et al. 2020). However, intravenous (IV) administration of various chemotherapeutic agents may cause some distress and discomfort to patients and is confirmed to be an economical burden on lower income people diagnosed with cancer. This is because multiple hospitalisations are required to complete the relatively long IV sessions of a combined chemotherapeutic regimen (Shapira et al. 2010).

Of late, oral chemotherapy is at the forefront of the search space for a method to radically improve the current chemotherapy regimen and the patients quality of life (Mei et al. 2013). Various colloidal drug carriers are being investigated for various routes including the oral method (Bertoni et al. 2020). However, the key challenges of oral delivery include poor drug solubility, drug stability at the various pH levels of the gastrointestinal (GI) tract, the digestive enzymes that can degrade drugs, and the protective mucus layer that can block drug from penetrating across the epithelium (Huang et al. 2015). Another significant factor contributing to the GI barrier is the existence of multidrug efflux proteins, i.e. P-type glycoproteins (P-gp), found mainly in the epithelial membranes of the GI tract. Some researchers employed P-gp inhibitors, such as cyclosporine A to circumvent this problem, although they could suppress the body's immune system and cause further medical complications (Mei et al. 2013). Thus, designing and formulating an oral dosage form for an anticancer agent requires several carefully considered strategies to ensure good bioavailability with reduced side-effects. Inhibiting or minimizing drug release in the stomach and small intestine (Cheewatanakornkool et al. 2017) is also an added advantage.

Recent advancement in nanotechnology has introduced more tools to address the limitations involved in the prevention, earlier detection, and effective treatment of cancer and to significantly improve the comfort of cancer patients (Ferrari 2005; Grobmyer et al. 2010; Kumar and Kumar 2014). In this respect, nanoparticles may be harnessed as drug carriers for improved oral delivery of various drugs by (1) enhancing the solubility of hydrophobic drugs in the aqueous environment of the GI tract (2) preventing premature degradation of unstable drugs, and (3) facilitating sustained drug absorption from the mucus-lined epithelium into the blood via trans- or paracellular routes (Mei et al. 2013; Roger et al. 2010).

Various polymeric nano-carriers and core-forming block co-polymer micelles such as pluronics, poly(esters) like poly(lactic acid) (PLA), hydrophobic poly(amino acids), copolymers of lactic and glycolic acids, and poly(caprolactone) (PCL) have been extensively investigated in the past years for their oral uses (Xu et al. 2013). Some examples include amphiphilic block copolymers consisting of a micellar shell-forming poly(ethylene glycol) (PEG) and a core-forming poly(2-(4-vinylbenzyloxy)-N,N-diethylnicotinamide) (P(VBODENA)) block. Herein, N,N-Diethylnicotinamide (DENA) in the micellar inner core improved Paclitaxel solubilization and stabilization against colon cancer lines with oral bioavailability recorded at 12.4% of that of the intravenous administration (Lee et al. 2007; Mei et al. 2013). Bhatt et al. (2015) also successfully developed novel polyplex-loaded enteric-coated calcium pectinate microbeads for oral gene delivery for effective colorectal cancer therapy, utilizing cationic polymethacrylate polymers (Eudragit® E100 and Eudragit® RLPO). On the other hand, D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) can improve the intestinal absorption of drugs such as teniposide, increasing its concentration over sevenfold in tumor-bearing mice (Zhang et al. 2013). In addition, polymeric micelles based on monomethylether poly(ethyleneglycol) (750)-poly(caprolactone-co-trimethylene carbonate) (mmePEG750P) also showed a very low clearance rate by the reticuloendothelial system (RES) and renal excretion, demonstrating an oral bioavailability of 40% in rats (Mathot et al. 2006). The transport of radio-labelled mmePEG750P(CL-co-TMC) polymeric micelles across Caco-2 cell monolayer was further investigated by Mathot et al. (2007) to confirm that the polymeric micelles used both passive diffusion and fluid-phase endocytosis in order to cross a Caco-2 model of the intestinal barrier.

Natural polymers such as the major milk protein casein have recently attained great attention for drug delivery due to their low cost, easy availability, biodegradability, non-toxicity, unique structural and physicochemical properties, and the ability to form micelles like a di-block copolymer (Głąb and Boratyński 2017; Jain et al. 2016; Liu et al. 2020; Penalva et al. 2015; Rehan et al. 2019). Although casein micelles re-assembled from sodium caseinate can be used as natural casein micelles, the distinguishing feature of re-assembled casein micelles over the natural ones is the tailor-made particle size. Furthermore, drug entrapment is performed before the re-assembling process, which confers higher encapsulation efficiency (Malekhosseini et al. 2019).

In the past, casein-based micelles and nanoparticles were employed to encapsulate folic acid (Penalva et al. 2015), resveratrol (Peñalva et al. 2018), vitamin D (Semo et al. 2007), paclitaxel (Shapira et al. 2012), curcumin (Esmaili et al. 2011; Pan et al. 2013), thymol (Chen et al. 2015), and mequindox (Chen et al. 2020) for oral delivery. The major factor contributing to increased oral bioavailability is the ability of casein to encapsulate drug and isolate it from the acidic gastric juice (Penalva et al. 2015). This resulted in a controlled release of drugs from the casein micelles into the intestinal environment, allowing for sufficient uptake of these agents from thee. There are two scenarios to consider: (1) there might be controlled release of the encapsulated drug from the casein micelle in the small intestine resulting

in the controlled absorption of the free drug through the epithelium into the blood (Peñalva et al. 2018) although this process may not increase drug accumulation in the tumor compared to the orally or intravenously administered free drug, and (2) the drug-loaded micelles might survive the proteolytic and enzymatic degradation in the GI tract and subsequently undergo internalization into the intestinal epithelial cells (through endocytosis), release the drug following lysosomal degradation of the casein and thus facilitate controlled release (absorption) into the blood through the basolateral membrane of the cells. This could prevent the drug from backtracking to GI tract through P-glycoprotein (P-gp) present in the apical surface of the cells. This controlled absorption of the drug in blood could contribute to prolonging its plasma half-life and enhancing passive tumor accumulation. Normally, drugs are encapsulated inside the casein micelles through hydrophobic bonds, electrostatic interactions, or covalent bonding (Liu et al. 2020; Penalva et al. 2015).

Doxorubicin (DOX) belongs to the anthracycline antibiotic family and is considered as one of the most effective chemotherapeutics currently being used to treat various cancers, including leukemia, sarcomas, solid tumor of breast, ovaries, or thyroid, non-Hodgkin's and Hodgkin's lymphomas. DOX exerts its cytotoxic effects mainly through the inhibition of topoisomerase II, DNA double helix intercalation, production of reactive oxygen species (ROS), activation of caspases, mitochondrial dysfunction, and induction of p53. With that said, it does have a shorter half-life, drug resistance development, and severe cardiotoxicity, which particularly limited its clinical use (Hira et al. 2014). The FDA however has approved various DOX-loaded nano-formulations, including Abraxane, DaunoXome, and Doxil/Caelyx, for clinical use in malignant cancer. To date, some nano-formulations of DOX are currently under clinical investigations, including Aurimmune (phase I), Genexol-PM (phase I) and ThermoDox (phase III). Nevertheless, the low stability in physiological conditions and systemic toxicity hinder the wide applications of liposome- or macromolecules-based nano-formulations for cancer treatment (Zhang et al. 2018).

In this study, we report on the fabrication and characterization of DOX-loaded sodium caseinate nano-micelles (DOX-NaCNs) and on the biodistribution profile and therapeutic potential of the resultant complex in a syngeneic mouse model of breast cancer following oral administration, in comparison to the intravenously delivered DOX-NaCNs and the free drug. DOX-NaCNs were characterized through Zetasizer, field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM) and Fourier-transform infrared spectroscopy (FTIR). In vitro and in vivo evaluations were performed to determine the anticancer efficacy of DOX-NaCNs and to specify the unique potential of DOX-NaCNs for oral delivery against breast cancer. To our knowledge, this is the first comprehensive study demonstrating the bio-distribution profiles and therapeutic potential of DOX-NaCNs for both intravenous and oral administrations.

Materials and methods

Materials

Sodium caseinate from bovine milk, DOX (10 mg), and acetone were purchased from Sigma-Aldrich, Germany. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Nacalai Tesque Inc., Japan. Fetal Bovine Serum (FBS) and penicillin–streptomycin (P/S) was purchased from Gibco, Life Technologies, U.K. 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck, Malaysia. Trypsin-ethylene diamine tetra-acetate (trypsin–EDTA) salts were obtained from Sigma-Aldrich. MCF-7 and MDA-MB-231 cell lines were purchased from ATCC (Manassas, VA, USA).

Methods

Preparation and synthesis of DOX-loaded NaCNs

NaCNs were prepared via gentle mixing on a nutating mixer at room temperature, as previously reported (Rehan et al. 2020). DOX was used a model drug in the current research to evaluate the efficacy of a partly hydrophobic drug inside NaCNs. Briefly, DOX in water solution (10 µM) was added (up to 1 mL) to the sodium caseinate powder (1 mg) to form the DOX-NaCNs micelles on a nutating mixer by vortexing for one hour. Since the caseinate has many amphiphilic phosphate subunits, it would reassemble itself into micelles while inadvertently entrapping the DOX in the process (Elbialy and Mohamed 2020). The DOX loaded NaCNs prepared herein was designated DOX-NaCNs. Negative control (containing only DOX in water) and blank NaCNs without DOX were also prepared under similar conditions as described above.

DOX-loading into micelles

Loading efficiency (LE) is an essential physicochemical characteristic to evaluate the entrapment of drug in casein micelles. The drug loading efficiency (DLE) and drug loading content (DLC) of DOX-NaCNs were measured via an indirect centrifugation method (Scheeren et al. 2018). The samples were centrifuged for 20 min at 15,000 rpm at 4 °C (Eppendorf Centrifuge 5424 R, Sigma Aldrich, Germany). The concentration of free drug was

determined by carefully collecting the supernatant from the centrifuge tubes. The supernatant's absorbance was measured using a fluorescence spectrophotometer (Glomax Explorer GM3500, Promega Corporation Australia) at 475 nm (excitation wavelength) and 550 nm (emission wavelength). The DLE and the DLC (w/w) were calculated using Eqs. 1 and 2 as below (Elzoghby et al. 2013a, b) after standardizing the calibration curve for DOX (Figs. S1 and S2).

$$\%\mathrm{DLE} = \frac{\text{Mass of drug entrapped in nanoparticles}}{\text{Mass of drug used in formulation}} \times 100 \quad (1)$$

$$\%\mathrm{DLC} = \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of nanoparticles recovered}} \times 100 \quad (2)$$

Particle size analysis through dynamic light scattering (DLS) and stability test

Particle size, distribution, and zeta-potential were measured based on the DLS technique using Malvern Zetasizer Nano (Malvern, Worcestershire, U.K.) (Rehan et al. 2020). The formulations were diluted with water (1:10) at ambient temperature followed by measurement using a Zetasizer. To evaluate the DOX-NaCNs stability, the samples were stored at 4 °C. Later particle size, distribution, and the zeta potential were measured over a three month period using a Malvern Zetasizer at 25 ± 0.1 °C where each sample was diluted tenfold before zeta measurement. Measurements were taken three times at 12 runs each time and were reported as mean (± SD).

FESEM and HR-TEM imaging of micelles

The shape, surface morphology, and size analyses of the micelles were investigated using FESEM (Hitachi/SU8010, Tokyo, Japan) at 5.0 kV (Rehan et al. 2020). Samples were prepared as previously stated in the methodology "Preparation and synthesis of DOX-loaded NaCNs" section 10 µL of the sample was placed on a glass cover and was left to air-dry at room temperature. The dried sample was placed on a sample holder coated with carbon tape for platinum sputtering with a 30 mA sputter current for 40 s at 2.30 tooling factor. Both blank and drug-loaded samples were observed under FESEM. For morphological analysis via HR-TEM imaging, the samples were prepared by suspending a copper grid (300 mesh size) in the micelle suspension. The grid was then dried at room temperature and was analysed using a HR-TEM (FEI tecnai G2 20S-TWIN, Netherlands) at 200 kV.

Compatibility study of DOX-NaCNs using FTIR

FTIR enables the identification of chemical bonds present in drug molecules, polymers, or proteins used in formulations (Quintás et al. 2004). The IR spectra of sodium caseinate, DOX, NaCNs, DOX-NaCNs and their respective physical mixtures were recorded using a Varian FTIR equipped with a Varian Resolution Pro 640 software (Agilent, Santa Clara, CA, USA). The infrared (IR) spectra were taken over the range of 4000–500 cm^{-1}.

In vitro drug release profile

To assess the pattern of drug release from DOX-NaCNs at different pH levels (7.4/5.0), an in vitro release study was conducted through a dynamic dialysis method modified from one previously reported (Scheeren et al. 2018). DOX-NaCNs were prepared and the sample (1 mg/mL) was sealed inside the dialysis membrane. Prior to use, the membrane was activated and soaked overnight in ultra-pure water. The dialysis bag was tied to the paddle of USP XXIV dissolution apparatus II (Electro lab dissolution Tester USP, TDT-08L, India) and was dialyzed against 250 mL of phosphate-buffered saline (pH 7.4/5.0). The entire system was maintained at 37 ± 2 °C under a continuous magnetic stirring (100 rpm) for 24 h. At the same time, the free drug solution was also dialyzed using a similar dissolution media. The medium was withdrawn (1.5 mL) at specific time intervals and was replaced with a fresh buffer to maintain a constant volume and sink condition. The DOX content in the samples was then measured using a fluorescence spectrophotometer at 475 nm (excitation wavelength) and 550 nm (emission wavelength).

In vitro cell viability and cytotoxicity studies

Cell culture and seeding MCF-7 and MDA-MB-231 are human breast cancer cell lines used in the current research to determine in vitro cell viability and cytotoxicity. Both cell lines were cultured in a 25 cm^3 flask with a complete DMEM containing 10% FBS and 1% P/S antibiotic. The flask was then placed in a humidified incubator at 37 °C. The growth medium was changed every alternate day after an 80% confluency is achieved. Once confluent, the cells were trypsinized with 0.05% of trypsin (Sigma, USA) and then passaged in 75 cm^2 tissue culture flasks. The cells were then allowed to grow in a 96-well plate containing 50,000 cells per mL for 24 h before drug treatment.

Cell viability and cytotoxicity were measured using an MTT assay (El-Far et al. 2018; Fatemian and Chowdhury, 2018). After 24 h of cell seeding, the cells were treated with different concentrations of free DOX (0.0625–1.0000 µM). DOX-loaded NaCNs were prepared in the presence of

similar drug concentrations, along with blank NaCNs and control (only media) for another 48 h. Following treatment, MTT stock solution [5 mg/mL in phosphate buffer solution (PBS)] was added into each well. After 4 h of incubation, 100 µL of dimethyl sulphoxide (DMSO) was added into each well to dissolve the purple formazan crystals. Absorbance was measured at 560 nm against a 600 nm reference wavelength. The measurement was taken using a microplate reader (Glomax Explorer GM3500, Promega Corporation Australia) with a built-in plate shaker to shake the plates for 10 s before measuring the absorbance.

The percentage cell viability (% CV) was calculated by using the formula:

$$\%\,\text{Cell Viability(CV)} = \frac{\text{Absorbance of treated cells} - \text{Absorbance of reference}}{\text{Absorbance of Control} - \text{Absorbance of reference}} \quad (3)$$

where the concentration causing 50% inhibition (IC_{50}) of the free and bound DOX was also calculated using a Graph pad prism (version 8.0). All the experiments were performed in triplicates and the standard deviations (SD) were calculated for mean values.

Cellular uptake

Qualitative analysis Qualitative cellular uptake of DOX, a fluorescent compound, was measured using fluorescence microscopy (Bae et al. 2003; Cui et al. 2013). MCF-7 cells were seeded on a 24-well plate with a density of 50,000 cells per well followed by an incubation step for 24 h. After the incubation, the medium was replaced with 10 µM and 5 µM of free DOX and DOX-NaCNs respectively prepared in the presence of similar drug concentrations (10 and 5 µM). The control was prepared by treating the cells with DMEM. The treated cells were incubated for 4 h and 24 h. After removing the media, the cells were treated with ethylenediaminetetraacetic acid (EDTA) (5 mM) in PBS to remove the extracellular particles followed by visualization under a fluorescence microscope.

Quantitative analysis using a spectrophotometry A quantitative cellular uptake analysis was conducted after the treated cells were washed with PBS several times followed by lysis using a lysis buffer. The cell lysate (200 µL) was collected from the 24-well plate and was subjected to a fluorescence intensity measurement using a fluorescence spectrophotometer (PerkinElmer, USA) coupled with a 2030 manager software attached with 2030 multilabel reader victor (X5) with an excitation at 485 nm and an emission at 535 nm. The fluorescence intensity of the cells was measured to determine the relative amount of the DOX that they have successfully internalized.

In vivo tumor regression study

Animals Female healthy Balb/c mice (5–6 weeks old) were selected and randomized with body weights. The animals were procured from Monash University Malaysia animal facility. The mice were acclimatized for 7 days prior to the study. They were maintained under a standard husbandry, stress-free and non-pathogenic condition with a 12:12 h light and dark cycle, at 25 ± 2 °C and relative humidity of 50 ± 10%. Water and food were given ad libitum. The study was approved by Monash University Malaysia Animal Ethics Committee (Project ID: 2020-19843-39399) following standard protocols for animal handling and care.

Synthesis of DOX-loaded micelles The formulations were also fabricated for comparative analysis with the blank NaCNs, where DOX-loaded NaCNs were prepared by adding an aqueous solution (up to 1 mL) of DOX (at an equivalent dose of 5 mg/kg) to sodium caseinate (1 mg) to prepare the micelles using a nutating mixer.

Induction of murine breast tumor Murine breast cancer cells (derived 4T1 cells) were cultured in the complete media (DMEM) containing 1% P/S antibiotic and 10% FBS in a 25-cm^2 flask and was subsequently placed in a humidified incubator (at 37 °C and 5% carbon dioxide). When the cells reached the exponential growth phase, they were further sub-cultured in a 75-cm^2 flask and then trypsinized. After counting the number of cells using a hemocytometer, the cells were re-suspended in DMEM at 10^6 cells/mL and were later suspended in PBS to yield a concentration of 10^5 cells/100 µL.

On day 1, the cells were subcutaneously injected into the left side of the mice's mammary gland (at 1×10^5 cells/100 µL) using a 27 G needle. The mice which developed tumors were observed at least three times a week until the presence of a palpable tumor nodule, following which daily monitoring was conducted. When the tumors reached an average volume of 13.73 ± 2.51 mm^3 about one to 2 weeks following inoculation, the mice were randomly assigned to the various treatment groups in the study ($n = 5$/group). Following the injection of the treatment formulations, the size of the tumor (by volume) was monitored at regular intervals from day 1 to day 28, with the help of a digital Vernier calliper.

The formula below was used to measure tumor volume (Rehan et al. 2020):

$$\text{Tumour Volume}\,(\text{mm}^3) = \frac{1}{2}\left(\text{length} \times \text{width}^2\right) \quad (4)$$

In-vivo anti-tumor effects To evaluate the anti-tumor effect of the drug, the animals were divided into eight groups ($n = 5$ for each group). Tumor-bearing mice were administered with free DOX solution, DOX-NaCNs formulation (5 mg/kg/day) along with the negative control and blank NaCNs both intravenously and orally. Intravenous administration was done into either the right or left side of the tail vein via a 29 G needle. Oral administration was via the esophagus into the stomach using a steel oral gavage (11G) with a gap of 48 h for comparative analysis between micelles being administered intravenously and orally. Treatment was given on day 14 and day 17. The mice were weighed every 3 days.

At the end of the experiment (day 28), the mice were exposed to 100% carbon dioxide in a chamber for euthanization. Their vital organs including the brain, spleen, lungs, liver, kidney, and heart as well as any visible tumors were removed. The excised tumor and different organs of the mice were washed in cold PBS and weighed. All the measurements were presented as mean ± SD for each group.

Biodistribution of DOX, intravenously and orally administered in free form or in casein micelles, in a xenograft mouse model

The 4T1 cells (1×10^5 cells/100 µL) in PBS were subcutaneously injected into the mammary pads of female balb/c mice (5–6 weeks of age) weighing 17–19 g for breast tumor induction. Tumor-bearing animals were divided into two sets, where one set of mice (three groups; $n = 4$/group) were orally treated with NaCNs, free DOX, and DOX-NaCNs using a steel gavage. In contrast, the other set (three groups; $n = 4$/group) were intravenously treated with NaCNs, free DOX, and DOX-NaCNs via the caudal tail vein. An equivalent dose (5 mg/kg) was used to prepare free DOX and DOX-NaCNs. Blank NaCNs were used as controls in both sets of experiments. 1 day post-administration of the formulations, the mice were anaesthetized before blood collection into heparinized tubes via cardiac puncture. Subsequently, the animals were sacrificed through cervical dislocation before collection of vital organs (brain, heart, spleen, liver, lung, and kidney) and tumor from the treated mice. All the organs were kept in 400 µL of lysis buffer (pH 7.4) after being washed with PBS and were stored at − 150 °C for further analysis.

The organs were mechanically homogenized and centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant (100 µL) was collected and placed in a 96-well plate to determine the fluorescence intensity of DOX at 475 nm (excitation wavelength) and 550 nm (emission wavelength) using a fluorescence spectrophotometer. The data was presented as relative means of the values ± SD (after being blank corrected from the control group values) in fluorescence intensity unit/mg of the tissue mass.

Blood analysis Blood serum analysis was also conducted following blood collection from all groups via a cardiac puncture using 27 G needle. The samples were centrifuged at 10,000 rpm for 30 min at 4 °C to collect the supernatant (blood serum) and the fluorescence intensity of the samples was measured using a fluorescence spectrophotometer (PerkinElmer, USA. 2030 manager software attached with a 2030 multilabel reader victor X5) at 485/535 nm.

Toxicity evaluation of micelles and determination of LD50

Acute oral toxicity study The Organization for Economic Co-operation and Development (OECD) 425 up-and-down procedure (Oecd 1994) is often used to minimize the number of animals required in acute oral toxicity testing. The current study is based on the OECD 425 up-and-down guideline to determine the 50% lethal dose (LD_{50}) of NaCNs. The changes in the mice's behavior were also monitored according to the Irwin test (Roux et al. 2004) to observe their overall health and well-being.

Toxicity profile Balb/C mice were selected for toxicity studies based on the micelles' evaluation against breast cancer cells following loading with anti-cancer drugs. This choice was made due to their long lifespan, resistance against atherosclerosis development, simple dietary needs, and ease of monoclonal antibodies production. The mice were divided into two groups; control (water) and treated (blank NaCNs), with five animals per group.

On the first day, one of the mice was administered with a single dose of casein micelles (2000 mg/kg) through oral gavaging. The short time toxicity profile was observed at different points (0 min, 15 min, 30 min, 1 h, 2 h, 4 h and 24 h). Any clinical sign of toxicity/abnormality such as lower food or water intake or any signs of morbidity and mortality among the treated mice was recorded. Based on the short-term toxicity profile (24 h) of the mouse, a similar dose (2000 mg/kg) was simultaneously administered to another four mice and the short-term toxicity parameters were observed. The mice were kept under observation for 14 days to establish any further toxic signs.

The overnight-fasted mice were administered with a single dose of micelle formulation (2000 mg/kg) via an oral gavage needle (11G). Some of the critical short term toxicity parameters recorded based on Irwin's test include:

(i) Righting reflex (RR)

The ability of the mouse to regain healthy posture within 30 seconds after being placed flat on its back.

(ii) Body weight

Abnormality in body weight was observed where a 15% change in body weight is deemed as a sign of toxicity.

(iii) Sedation

Reduction in the movement of the animal indicates a sedative effect even after manipulation.

(iv) Clinical signs

Abnormalities in the frequency of urination and defecation were monitored.

All the parameters were recorded once before treatment and twice daily subsequently. In addition, body weight, water, and food consumption were measured and recorded every other day throughout the study period before necropsy. After 14 days of observation, the mice were weighed and euthanized (Anadón et al. 2014) before further analysis.

Statistical analysis

Statistical analyses of the in vitro and in vivo studies were performed using Graph pad prism version V8. For the pairwise comparison analysis, one-way ANOVA followed by post-hoc Tukey's multiple comparison test were used. Values with $p < 0.05$ were considered as borderline for significance, with a 95% confidence interval (CI).

Results and discussion

Synthesis and physicochemical characterization of DOX-NaCNs

NaCNs were prepared by allowing casein in aqueous solution to self-assemble into micelles (Casanova 2017; Rehan et al. 2020). An aqueous solution of DOX (1 mg/mL) was added to sodium caseinate powder (1 mg) prepared from raw skim milk (Casanova et al. 2018), followed by the addition of water (q.s. 1 mL). The mixture was placed on a nutating mixer at 24 rpm for one hour at ambient temperature to allow casein to assemble into micelles while encapsulating DOX in the interior. Blank micelles were prepared similarly, only without the addition of the drug (DOX). Both DOX-loaded NaCNs and blank NaCNs were characterized in terms of zeta size, polydispersity index (PDI), and zeta potential (Table 1). In addition, drug loading contents (DLC) and drug loading efficiency (DLE) for DOX-NaCNs were measured (Table 1).

The hydrodynamic size, PDI, and morphology are important parameters since they may influence the properties of the nanocarriers such as assay design, delivery, and migration of NPs as well as their bioconjugates in living tissues and cells. Therefore, characterization of NPs surface charge and hydrodynamic size are required to control the bioconjugation of NPs ligand chemistry and its performance in biological imaging or assays.

Measurement of zeta size through dynamic light scattering (DLS) (Fig. S3) revealed that the blank NaCNs had a particle size of around 470.2 ± 68.12 nm, while the DOX-loaded NaCNs were 270.86 nm in size, indicating the self-assembled nature of casein (Rehan et al. 2020). The interaction of DOX within the micellar interior components of eblocke may reorganize and condense particles into smaller micelles which have a more heterogeneous size distribution and are less electronegative when compared to the blank micelles (Table 1). Additionally, DOX-NaCNs showed robust DLE (78.00%) and DLC of approximately 24.83%, signifying the successful encapsulation of DOX inside the casein. Since casein micelles are natural diblock amphiphilic polymer, they can increase DOX solubility through hydrogen bonds and hydrophobic interactions (Wang et al. 2017).

The biological fate of nanoparticles in the blood depends on their shapes and sizes as the lymphatic system and the vascular systems would filter and eliminate non-spherical foreign particles (Ali et al. 2020). Considering this fact, the fabricated micelles were designed to be spherical as demonstrated in the FESEM (Fig. 1A–C) and HR-TEM (Fig. S4A, B) images. The micelles were smaller when observed through HR-TEM and FESEM compared to as measured by DLS, because the sample preparation step for these analyses dehydrated and shrank the micelles (Ali et al. 2020; Shi and Goh 2011).

FTIR spectroscopy is an important characterization tool that offers both qualitative and quantitative insight into the molecular structure of the subject (Quintás et al. 2004). It can also analyze the absorption bands that are pertinent in determining the conjugation of a drug carrier with the drug (Jain et al. 2020).

Figure S5 (IR spectra of DOX-NaCNs), DOX showed a characteristic peak at 3278.845 cm^{-1}, thus showing only a slight shifting of peak in DOX-NaCNs from the free DOX peak (3329.741 cm^{-1}), justifying an efficient loading of DOX in DOX-NaCNs. Furthermore, the presence of the 3278.854 cm^{-1}

Table 1 Zeta size, PDI and zeta potential of blank micelle (NaCNs) and DOX-NaCNs along with DLE and DLC for DOX-NaCNs

Formulations	Size (nm)	± SD	PDI	± SD	Zeta potential	± SD	DLE (%) v/v	± SD	DLC (%) w/w	± SD
Blank NaCNs	470.20	68.12	0.471	0.009	− 1.93	0.53	–			
DOX-NaCNs	270.860	17.958	0.681	0.069	− 0.054	0.003	78.990	1.590	24.830	1.630

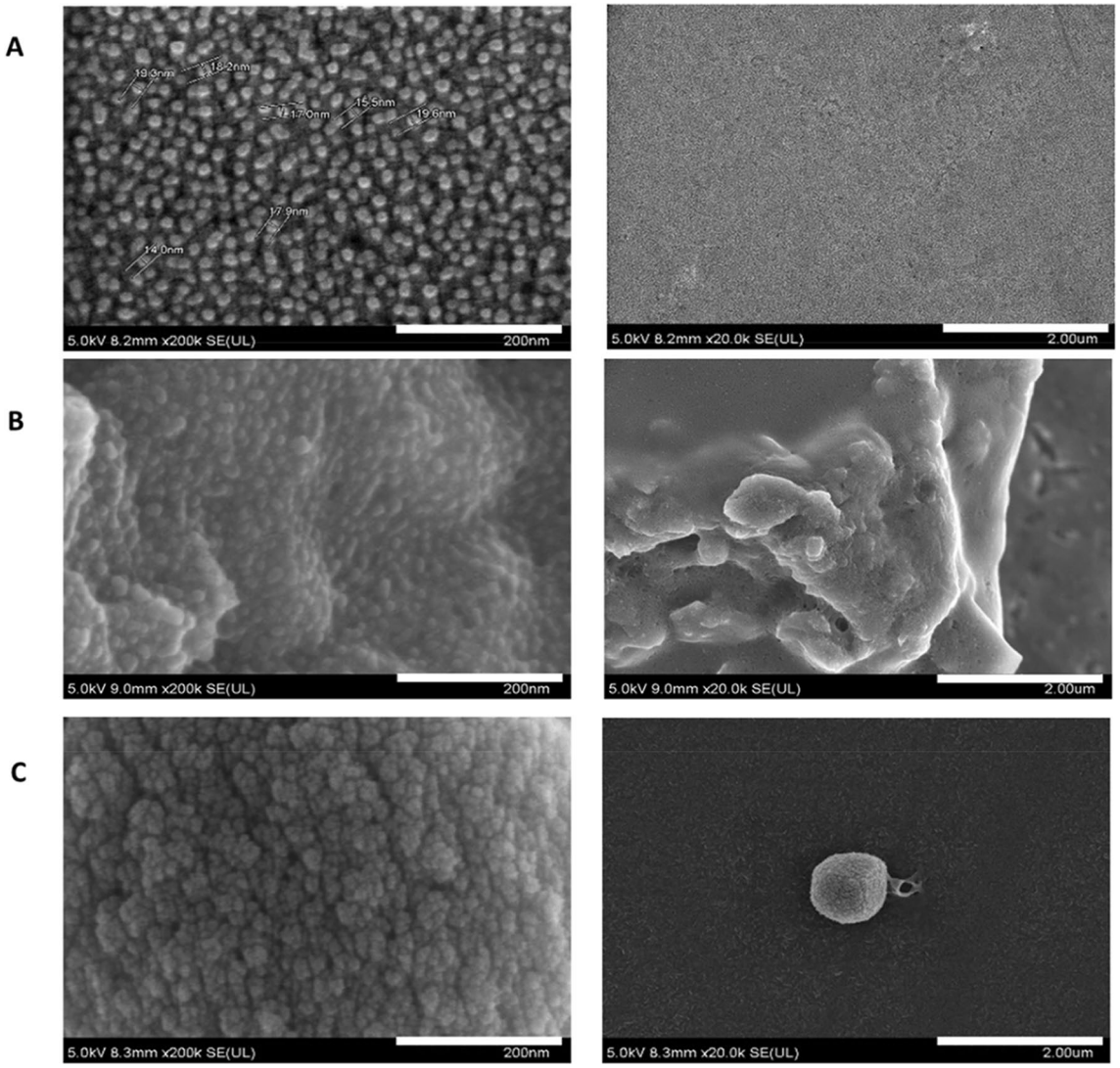

Fig. 1 FESEM micrographs of **A** DOX-NaCNs **B** Blank NaCNs **C** negative control (DOX + water). Scale bar: 200 nm and 2 µM. The samples (10 µL) were placed on glass covers and air-dried at an ambient temperature for FESEM analysis

and 1632.111 cm^{-1} peaks in DOX-NaCNs indicate the presence of -OH and water (H_2O), which are responsible for bonding DOX (Victor et al. 2014). The characteristic peaks of free DOX appeared at 3329.741 cm^{-1} and 1635.703 cm^{-1} (Fig. S5) indicating O–H stretching (Gnapareddy et al. 2015) and C=O (Gandhi and Roy, 2019; Victor et al. 2014) respectively. In contrast, sodium caseinate showed some characteristic peaks at 3278.854 cm^{-1}, 1632.111 cm^{-1}, and 1516.589 cm^{-1} which originate from N–H stretching and amide bending vibrations. Sodium caseinate also exhibited a characteristic peak around 1600 cm^{-1}, indicating the presence of C=O (Elzoghby et al. 2013a, b; Raj and Uppuluri, 2015). There was no significant shifting in C=O of DOX around 1632.11 cm^{-1} in the DOX-NaCNs, indicating the absence of chemical bonding between the C=O group of the casein micelles and that of DOX (Victor et al. 2014), indicating the chemical stability DOX inside the micelles. The slight overlapping and shifting of bands in the IR spectra of the DOX-NaCNs indicated the binding of DOX with the protein (Gandhi and Roy 2019).

Polymeric micelles tend to suffer from a low colloidal stability, especially following drug encapsulation (Abdelmoneem et al. 2018). To assess this characteristic, the micelles were characterized through zeta potential, particle size, and size distribution at 4 °C over a long period, based on the concept that colloidal stability can be evaluated via stability measurements or by measuring the aggregation rate in dynamic experiments (Liu et al. 2013). After 3 months of storage, the DOX-NaCNs particles was stable and remained in a desired size range (Fig. S6A) with a slight size increase after 60 days followed by a decrease that brought it back to the original size at the end of the third month of storage. Moreover, the zeta potential (Fig.

S6C) of DOX-NaCNs remained negative, indicating their stability over the 3 months period with no significant sign of aggregation (Fig. S6B).

In vitro drug release profile

The release of a drug from nanocarriers can be triggered by either a pH swing or by an enzymatic hydrolysis. Therefore, the drug release kinetics of DOX-NaCNs was investigated at pH 5.0 and pH 7.0 to determine the carrier's pH sensitivity (Jain et al. 2020). In this study, both control and DOX-NaCNs exhibited comparatively diverse release patterns at both pH levels (Fig. 2A, B). The free DOX solutions followed the burst and continuous release patterns, whereas the DOX-NaCNs released its payload in a more controlled manner the DOX-NaCNs released approximately 50% of its payload in 24 h at pH 7.4. At pH 5.0, the DOX-NaCNs released its payload in a more controlled pattern compared to the free drug, releasing more than 50% within 3 h. Thus, NaCNs can provide a more sustained release of DOX, thereby lowering the drug's dose-related adverse effect (Tang et al. 2015).

Additionally, casein stability is an added advantage at a lower pH and does not impact the cumulative release of DOX in a complex biological system (Chaudhary et al. 2015). A similar release pattern for casein protein was also reported previously, where drugs were more uniformly dispersed in synthetic block-copolymer micelles before being released out due to diffusion (Amjad et al. 2012; Kataoka et al. 2000; Sun et al. 2011). The pH can also influence the ionization of casein molecules and the integrity of the casein micelle, impacting their drug release profile.

In vitro cell viability and cytotoxicity

The cell viability and toxicity of DOX-NaCNs were analyzed against human breast cancer cells (MCF 7 and MDA-MB 231) after incubating for 48 h by using an MTT assay. DOX-loaded NaCNs showed considerable cytotoxicity against both cell lines when compared with both free DOX and blank micelles in almost all dosages ranging from 0.0625 to 1.0000 µM (Fig. 2C, D).

Following the 48 h incubation, DOX-NaCNs formulation showed the most significant cytotoxicity at 1 µM against MCF-7 (83%) and MDA-MB 231 (91%) cells compared to free DOX. DOX-NaCNs also showed an IC_{50} of approximately 129.3 nm compared to the IC_{50} of free DOX (151 nm) against MCF-7 cells. On the other hand, DOX-NaCNs showed an IC_{50} of approximately 103 nm compared to the IC_{50} of free DOX (116 nm) against MDA-MB 231. DOX-NaCNs displayed a significantly higher cytotoxicity against breast cancer cells compared to the free drug possibly because casein micelles are better able to penetrate cancer cells (El-Far et al. 2018). The process may have occurred via an energy-dependent manner through a more efficient endocytosis which facilitates the accumulation of the drug in the intracellular active site of nuclei, thus enhancing the cytotoxic effect of the drug-loaded NaCNs compared to the free drug (Cui et al. 2013). Furthermore, following cellular internalization, DOX is released from the micelles due to the low pH in the acidic endosomes or lysosomes as indicated in our drug release profile assay where the controlled release of DOX from micelles was observed at an endosomal pH 5.5 (Hossain et al. 2013).

Free DOX faces multidrug resistance (Gandhi and Roy 2019) mainly due to P-gp efflux and also passive diffusion (Chaudhary et al. 2015) which resulted in higher cell viability in both MCF and MDA-MB 231 cell lines compared to the loaded micelles. Another reason for the enhanced cytotoxicity may be attributed to the slow release of entrapped drug from the micelle matrix (as demonstrated in our drug release studies at a lower pH) which facilitates drug diffusion from the tumor extracellular environment into the cells through a concentration gradient (Mohapatra et al. 2019). The in vitro viability of blank micelles was also analyzed by exposing cells to different micelle concentrations (6.25, 12.50, 25.00, 50.00 and 100.00 µg/mL) to evaluate the effect of different micelles concentrations along with the free and loaded micelles. Following a 48 h incubation, the viability of the cells in almost all the blank formulations was close to that of control (media) at a concentration up to 100 µg/mL (Fig. 2C, D), confirming the non-toxic nature of casein (Picchio et al. 2018).

Cellular uptake

The cellular uptake of nano-formulations greatly influences drug delivery and its therapeutic efficacy (Zhang et al. 2018). Therefore, it is important to measure cellular uptake both by qualitative and quantitative analyses. Uptake is highly-dependent on the size and surface charge of particles (Victor et al. 2014). The cellular uptake of DOX-NaCNs was analyzed and compared to the free DOX in water following 4 h and 24 h of incubation using the MCF-7 cells.

Qualitative analysis through confocal microscopy

For the qualitative analysis, the cellular uptake was investigated using confocal fluorescence microscopy. The images showed bright green fluorescence signals in the cytoplasm of the MCF-cells (Fig. 3). The vigorous fluorescence intensity in the cells treated with DOX-loaded micelle, compared to the free DOX formulation, indicated that this formulation conferred significantly higher cellular uptake. Based on the particle size and negatively charged surface of NaCNs, the mode of entry of the micelles into the MCF-7 cells was

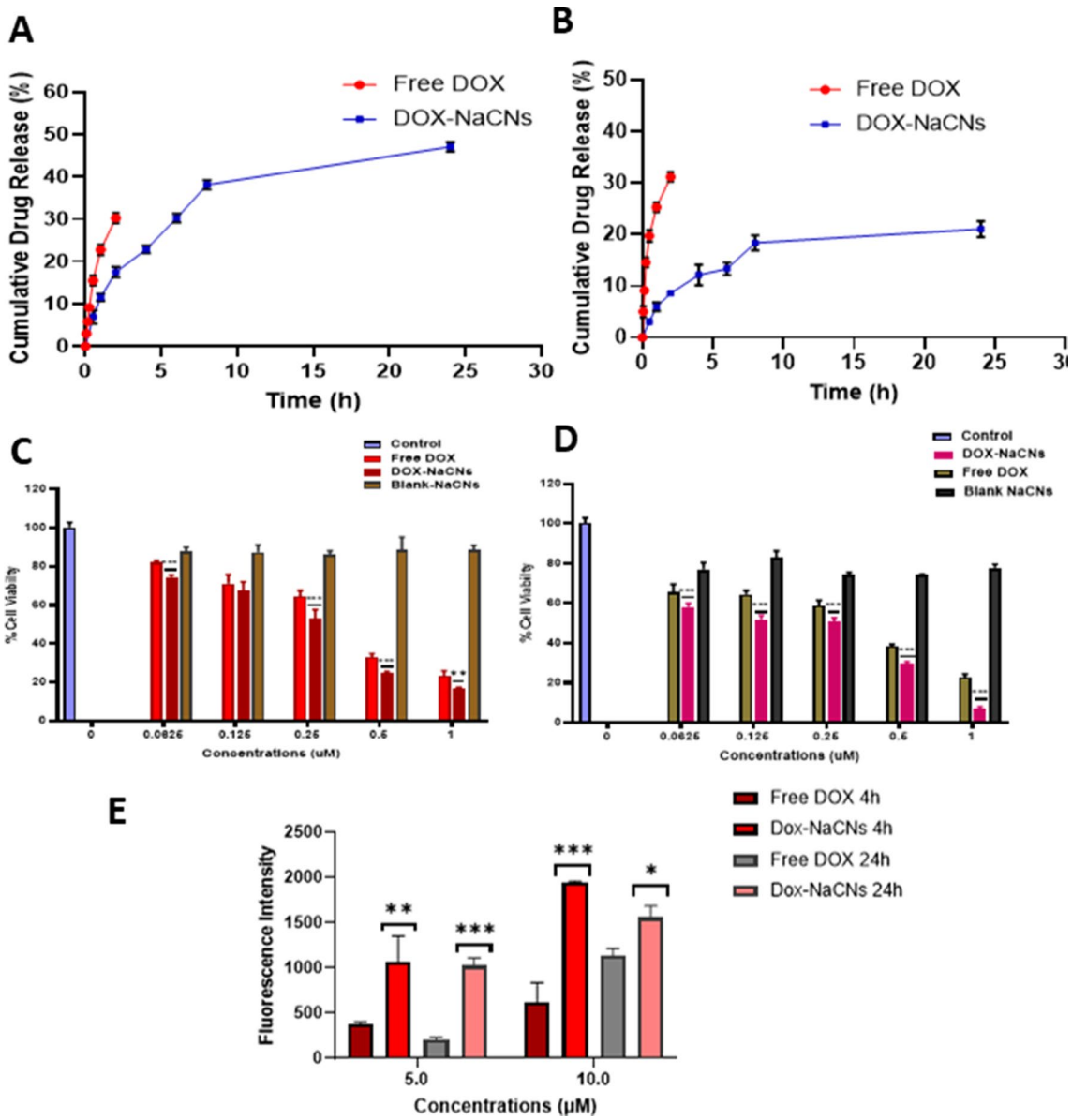

Fig. 2 In vitro release profile of DOX-NaCNs at pH 7.4 and 5.0, cell viability and cellular uptake analysis: *In-vitro* release profile of DOX-NaCNs at pH **A** 7.4 and **B** 5.0. Samples were prepared and sealed inside the dialysis membrane. Later, the dialysis bag was tied to the paddle of USP XXIV dissolution apparatus II and dialyzed against 250 mL of phosphate-buffered saline (pH 7.4/5.0) at 37 ± 2 °C and under continuous magnetic stirring (100 rpm) for 24 h. In vitro cell viability analysis was carried out through an MTT assay of blank NaCNs, free DOX and DOX-NaCNs on **C** MCF-7 and **D** MDA-MB 231 cell lines at 0.0625–1.0000 μM after 48 h of incubation. Data were shown as mean ± SD where $n \geq 3$ and (***) is $p < 0.001$ and (**) is $p < 0.01$ vs free DOX. **E** Cellular uptake of free DOX and DOX-NaCNs at 5 μM and 10 μM observed after 4 h and 24 h of treatment. Data were shown as mean ± SD where $n = 3$ and (***) is $p < 0.001$ and (**) is $p < 0.01$ vs free DOX

through caveolae-mediated endocytosis (Chowdhury 2016; Komuro et al. 2019).

Quantitative analysis

The intracellular uptake of DOX-loaded NaCNs was also quantitatively evaluated using a lysis buffer to get a more objective measurement. MCF-7 cells were treated for 4 h and 24 h with DOX-NaCNs prepared in the presence of 5 μM and 10 μM of the drug. DOX-loaded NaCNs showed both dose- and time-dependent cellular uptake. There was a significantly higher cellular uptake of DOX-NaCNs at both the 5 μM and 10 μM loading level, compared to free DOX after 4 h and 24 h of the treatment (Fig. 2E). Overall, our findings supported the notion that DOX is efficiently internalized inside human breast cancer MCF-7 cells through

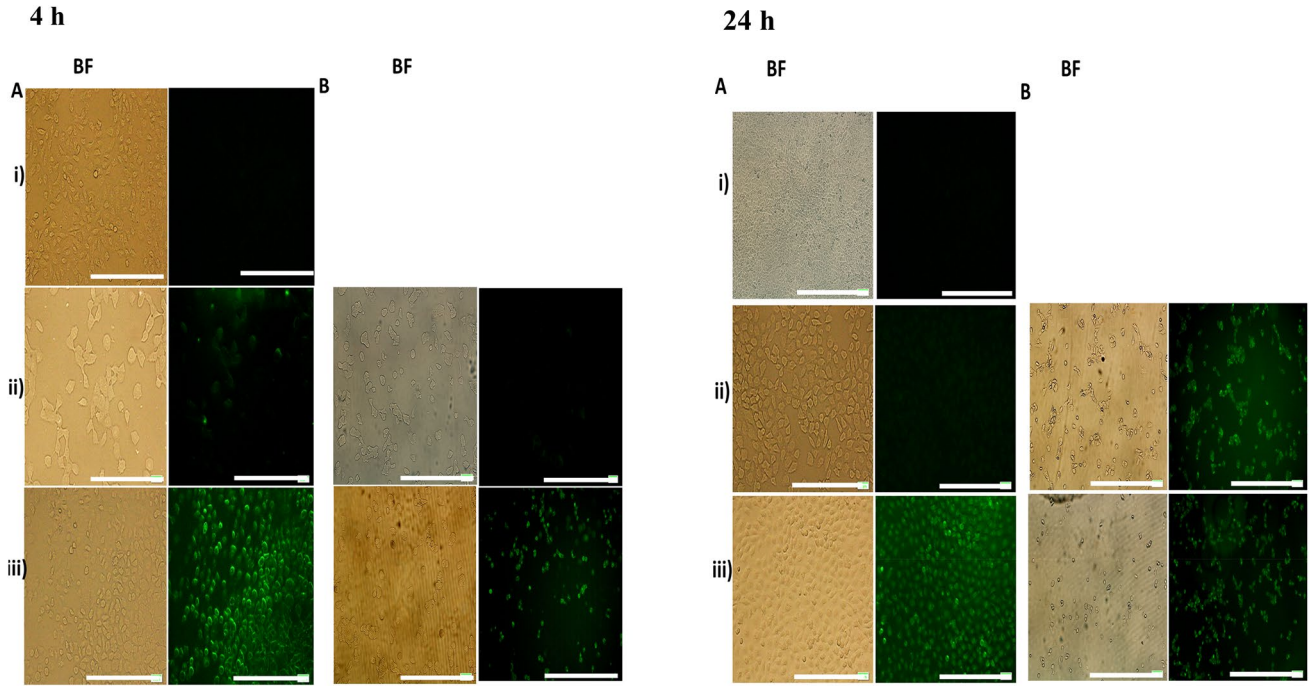

Fig. 3 Fluorescence images of MCF-7 cells treated with (i) media only (control) (ii) free DOX (iii) DOX-NaCNs at **A** 5 µM and **B** 10 µM; observed after 4 h and 24 h of treatment with 10× magnification at a scale bar of 50 µM

NaCNs, thereby enhancing the therapeutic efficacy of DOX. From this, it can also be ascertained that casein is an excellent carrier for cancer treatment.

In vivo anti-tumor activity of intravenously administered DOX-NaCNs

The mice injected with PBS, blank NaCNs, and free DOX formed large tumors with continuous growth throughout the experimental period (Fig. 4). Moreover, the tumor excised from the groups treated with the PBS, blank NaCNs, and free DOX appeared to have massive growth (Figs. S7, S8). The tumor excised from the mice group treated intravenously with DOX-NaCNs tumor demonstrated a comparatively slower growth (Fig. S8). The tumor shrank significantly after only two doses of DOX-NaCNs, demonstrating that DOX-NaCNs has a potent anti-tumor effect (Fig. 4).

In vivo anti-tumor activity of orally administered DOX-NaCNs

To validate the benefits of using an oral route, one set of four groups ($n=5$) of mice were treated with orally administered saline, drug-free NaCNs, free DOX solution, and the DOX-NaCNs at 5 mg/kg/day via gavaging.

Tumor volume recorded significant reduction (Figs. 5, S9, S10) in the case of orally administered DOX-NaCNs on day 17 (5.66 ± 4.36 mm^3) after the two doses, compared to the intravenously administered DOX-NaCNs (10.29 ± 4.86 mm^3). At the end of the study, a comparably slower growth of the tumor was observed in orally administered DOX-NaCNs (42.80 mm^3) and intravenously administered DOX-NaCNs (51.86 mm^3) compared to free DOX solution administered orally and intravenously (372.92 and 438.56 mm^3, respectively) (Fig. 5A, B). Hence, the anti-cancer activity of DOX is significantly enhanced when (1) loaded onto micelles ($p<0.001$) and (2) administered via the oral route.

The significant enhancement of the anti-cancer activity of the DOX-NaCNs seen via oral delivery may be attributed to the longer residence time in the circulation system (Swarnakar et al. 2014) when compared with intravenously administered DOX-NaCNs. This hypothesis was further validated through our biodistribution studies. Moreover, the tumor growth was suppressed until day 20, followed by a slow growth rate up to day 28 in both orally and intravenously administered DOX-NaCNs. At the same time, the free DOX showed an exponential curve in tumor growth, causing sufficient tumor burden in animals which is in line with in vivo study conducted by Kim et al. for polymeric micelles (2009). Compared to the free DOX, the tumor growth rate of group treated with blank micelles was initially delayed, but the later growth was enhanced with no sign of toxicity towards the tumor.

The successful tumor regression by DOX-NaCNs, administered both intravenously and orally, may also be

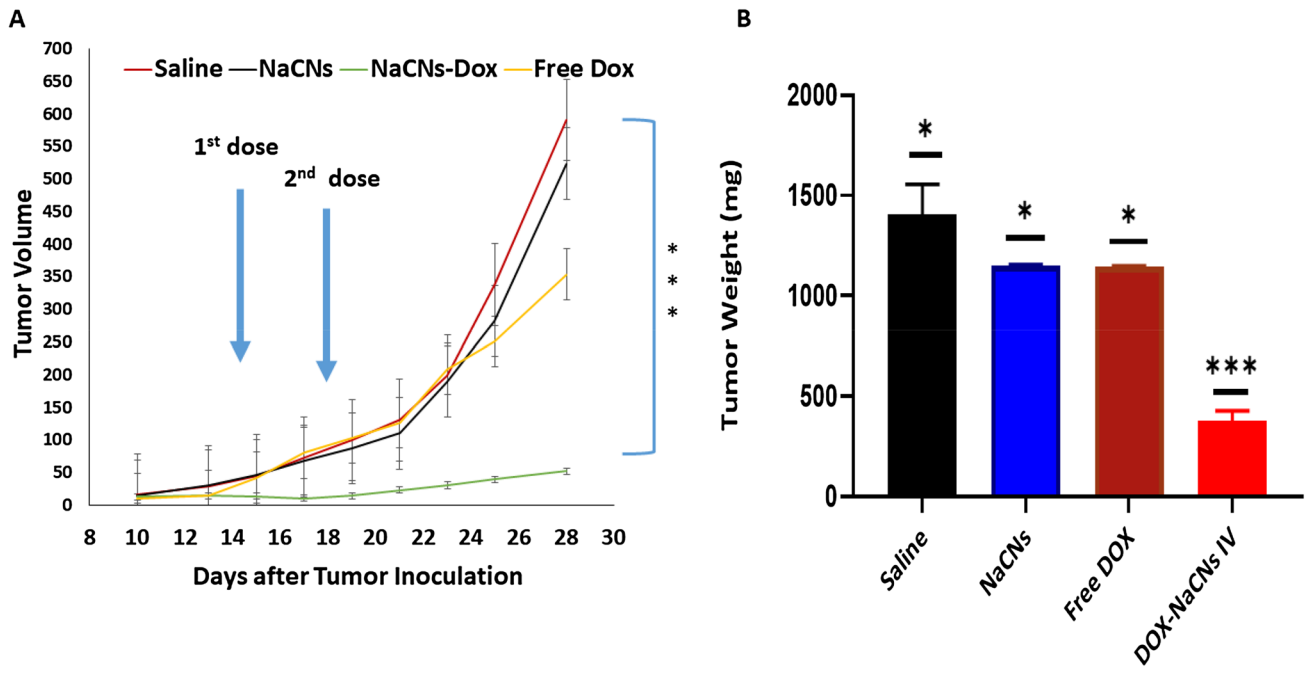

Fig. 4 In vivo tumor growth inhibition of free DOX and DOX-NaCNs following IV treatment: In vivo anti-tumor effects showing **A** mean tumor volume (mm^3) of negative control, NaCNs, free DOX and DOX-NaCNs-treated groups throughout the experimental period following intravenous administration and **B** the mean weight of the tumour of negative control, NaCNs, free DOX and DOX-NaCNs used to treat the mice on day 28 after the mice were sacrificed [values were considered statistically significant (*) at $p < 0.05$, very significant (**) at $p < 0.01$ and highly significant (***) at $p < 0.001$]

attributed to the controlled and biphasic release of DOX through NaCNs as revealed in our drug release assay or due to the rapid and higher internalization of DOX in the DOX-NaCNs compared to the free DOX. The significant enhancement of the anti-cancer activity of the DOX-NaCNs through oral delivery may be because casein can enhance the cellular uptake by penetrating the plasma membrane in an energy-dependent manner (Huang et al. 2015) compared to free DOX (intravenously and/or orally) and intravenously administered DOX-NaCNs groups. Kanwal et al. (2018) reported that the encapsulation of DOX in the biodegradable and non-toxic delivery system may prevent its degradation, increasing the residence time of the drug in the circulation. Secondly, it is plausible that the natural assembling property of sodium caseinate into micelles facilitates the passive drug delivery to tumor cells through EPR effect (Elbialy and Mohamed, 2020). Furthermore, casein molecules allow DOX to reach the gut epithelium surface and also control the release rate for DOX, thus promoting its absorption and oral bioavailability as observed by Penalva et al. (2018) and conferring enhanced anti-tumor effects.

Figure 5B showed the average weight of the excised tumor in each group. The group treated with DOX-NaCNs had a noticeable reduction ($p < 0.05$) in average tumor weight when compared with other groups, thus demonstrating the good anti-tumor effects of the natural protein micelles. Furthermore, sodium caseinate micelles also prevented the premature release of anti-cancer drug and maximized the drug concentration in the tumor, which resulted in the enhanced therapeutic effect of the loaded nano-system.

No apparent change in body weight was observed in the group treated with DOX-NaCNs formulations and NaCNs in both sets (Figs. S11, S12A), signifying the safeness of the treatment method and the non-toxicity of the delivery system. However, there was a slight decrease in the weight of mice in the free DOX-treated group which may be due to the high cytotoxicity of DOX in the free form. Furthermore, no significant abnormal behavior and activity of mice was seen and all mice survived the treatment.

To further confirm the toxicity of the micelles, the vital organs (heart, brain, liver, lung, kidney, and spleen) were removed after the treatment period of all treated groups and weighed. Both orally and intravenously treated DOX-NaCNs groups showed a significant decrease ($p < 0.001$) in the spleen and liver weights compared to the free DOX-treated group, (Figs. S11, 12B) indicating that NaCNs can prevent mice from suffering the adverse effects of the anti-cancer drug (El-Far et al. 2018). Furthermore, the liver was smaller (Fig. S11B) in orally administered DOX-NaCNs group, where DOX exhibited a remarkable therapeutic efficacy of orally administered DOX-NaCNs, when compared

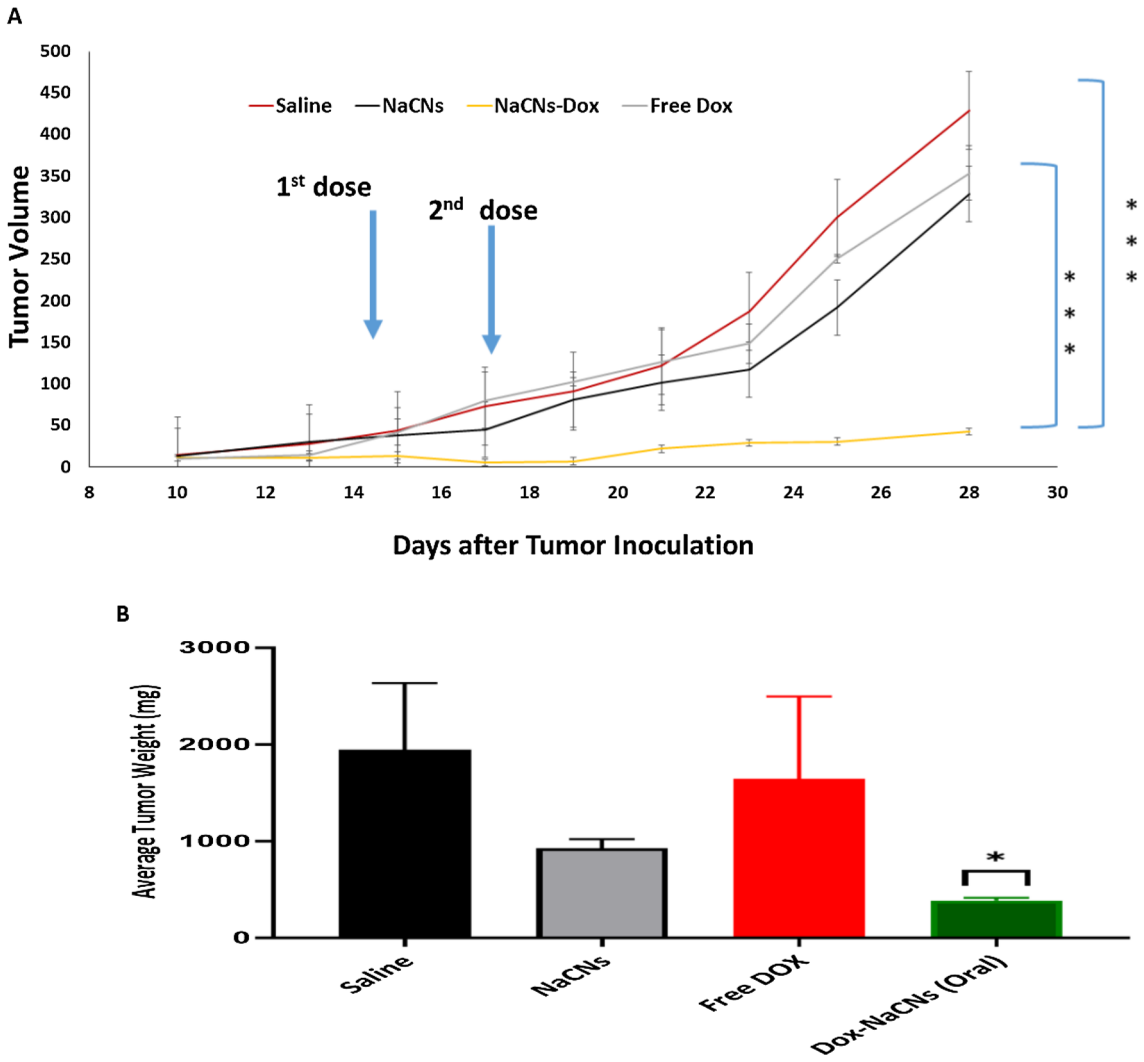

Fig. 5 In vivo tumor growth inhibition of free DOX and DOX-NaCNs following oral treatment: In vivo anti-tumor effects **A** average tumor volume (mm^3) for negative control compared with NaCNs, free DOX and DOX-NaCNs given orally **B** the mean weight of the tumor of negative control vs NaCNs, free DOX and DOX-NaCNs. Data were presented as mean ± SD and were considered as statistically significant (*) at $p < 0.05$, very significant (**) at $p < 0.01$ and highly significant (***) at $p < 0.001$

to both intravenously administered DOX-NaCNs and free DOX-treated groups.

Biodistribution study in a xenograft mouse model

In vivo biodistribution of the nano-formulations and their mechanisms of biodegradation and excretion define the fate, viability, and applicability of such a nano-delivery platform in the practical clinical translation (Souris et al. 2010). Furthermore, the biocompatibility of the synthesized nano-formulations at the levels of cell, blood, and tissue is always considered crucial for efficient drug delivery (Zhang et al. 2018).

Considering the aforementioned facts, biodistribution studies of the DOX-NaCNs and free DOX were performed in Balb/c mice (5–6 weeks old) after intravenous administration of an equivalent DOX dose of 5 mg/kg at the tail vein and through oral gavage. In order to monitor the formulation biodistribution and to determine the therapeutic efficacy of the orally administered DOX-NaCNs, a comparative study was carried out by orally administering one set of mice (three groups with 4 mice per group) with NaCNs, free DOX, and DOX-NaCNs, while intravenously administering the other set of mice (three groups with 4 mice per group) with the same set of drug formulations. All other conditions were otherwise identical between the two sets. The drug formulations were administered at an equivalent DOX dose of 5 mg/kg. Blank NaCNs was used as control in both set of mice. 24 h post-administration of the formulations in both sets of mice, the animals were sacrificed to collect their

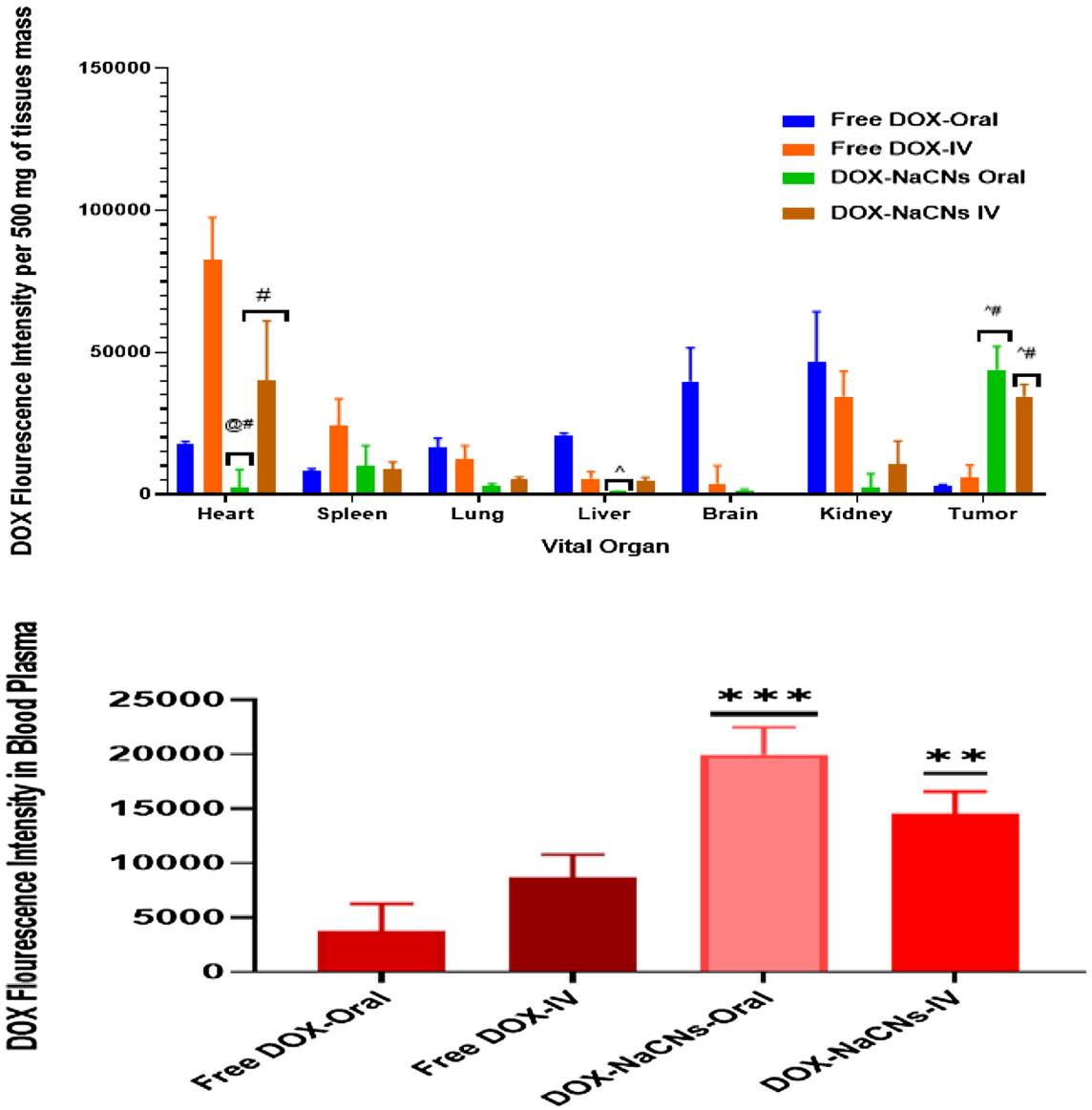

Fig. 6 Tumor drug distribution comparison of the orally administered DOX-NaCNs with intravenously administered DOX-NaCNs and the DOX fluorescence intensity in the blood plasma: In vivo biodistribution of both intravenously and orally treated with free DOX and DOX-NaCNs at an equivalent dose of 5 mg/kg inside mice ($n=4$) bearing a xenograft 4T1 tumor. Values represented biodistribution of both intravenously and orally treated with free DOX and DOX-NaCNs at 24 h after treatment. Data was shown as mean ± SD where $n=4$ and #$p<0.001$vs Free DOX (IV), @$p<0.001$ vs DOX-NaCNs (IV), ^$p<0.001$ vs Free DOX (oral). The DOX fluorescence intensity in the blood plasma of Balb/c 4T1 tumor-bearing mice after 24 h of both intravenous and oral treatments with DOX-NaCNs when compared to free DOX following an intravenous injection. Values were considered very significant (**) at $p<0.01$ and highly significant (****) at $p<0.001$ and vs. the same treatment with free DOX at a CI of 95% of representative samples. Values were blank-corrected with the control group

blood, organ tissues, and tumor. The orally administered bound DOX formulation evidently lead to a higher tumor distribution (Fig. 6A) when compared with other organs 24 h post-administration of a single dose.

Moreover, Fig. 6A indicates that the tumor drug distribution of the orally administered bound DOX was 1.27, 6.8 and 8.34-fold higher than intravenously administered bound DOX, intravenously administered free DOX, and the orally administered free DOX respectively, whereas intravenously administered bound DOX exhibited the 4.9-fold and 6.65-fold increase over the intravenously administered free DOX and the orally administered free DOX respectively. The

orally administered bound DOX reduced the drug disposition into the heart significantly ($p < 0.001$) when compared to free DOX and intravenously administered bound DOX, thus suggesting the potential of DOX-NaCNs to reduce cardiotoxicity caused by DOX.

Figure 5A also showed that a negligible amount of bound DOX administered orally and intravenously was found in the liver, lung, kidney, and spleen (RES). The hydrophilic coat of the NaCNs reduced plasma protein adsorption, decreased surface charge, enhanced hydrophilicity, and inhibited the electrostatic and hydrophobic interactions that permitted opsonin to be attached to the micelles. All these factors explicitly reduced the drug's accumulation in the liver and the spleen, thus preventing RES elimination (Kim et al. 2009) and eventually causing higher tumor accumulation of bound drugs. This hypothesis was further tested by quantifying the DOX level in the mice blood plasma 24 h post formulation administration. Orally administered free DOX showed approximately a five-fold lower drug concentration ($p < 0.001$) in the blood compared to orally administered bound DOX at 24 h. The orally administered bound DOX represented a 1.4-fold increase in blood plasma concentration compared to the intravenously administered bound DOX (Fig. 6B). Interestingly, the lower brain accumulation of DOX-NaCNs observed here may be attributed to endocytosis via brain endothelial cells, which account for only 1% of the brain mass as reported by Ambruosi et al. for poly (butyl cyanoacrylate) nanoparticles (2005).

The enhanced plasma level of orally administered bound DOX might be due to the longer circulation time as a result of increased membrane permeability of intestinal epithelium and inhibition of P-gp efflux pump through entrapment of DOX inside NaCNs (Perlstein et al. 2014). Thus, the enhanced therapeutic efficacy of the orally administered DOX-NaCNs may also contribute to the increased accumulation of DOX by tumor-resistant cells, making DOX-NaCNs a better formulation for oral delivery for cancer treatment. Oral delivery is more desirable over IV infusions, which may cause hospitalization and contribute to increased cost while exposing immune-compromised patients to infections (Bar-Zeev et al. 2016). Additionally, the formulation is desirable since there was no significant change in the organ's weight of mice treated intravenously and/or orally with DOX-NaCNs compared with free DOX and normal mice groups (Fig. S13). To further support the notion of enhanced plasma level of orally administered bound DOX, we need to perform the bioavailability studies by applying the pK (pharmacokinetic) models in both oral and I/V administration.

Acute oral toxicity study

In this study, NaCNs treatment at an oral dose of 2000 mg/kg did not confer any morbidity or toxicity in the animals. Moreover, there was no significant toxicity, behavioral changes (such as increased in breathing, postural changes) or other abnormalities including skin and fur changes, hair loss nor any change in organ weight observed after 14 days of experiment. Additionally, there was no significant weight gain, weight loss, or noteworthy necroscopy findings recorded after the 2 weeks indicating that there was no significant acute toxicity associated with NaCNs. The LD_{50} for female Balb/C mice is > 2000 mg/kg. Furthermore, all animals survived with no mortality seen until the end of the observation period of almost 2 weeks.

Bodyweight, water, and food consumption analysis

There was no apparent weight change observed among the control mice and the NaCNs-treated mice (Fig. S14). All mice showed a regular increase in weight (Fig. S14A) throughout the observation period with no apparent difference in food and water intake (Fig. S14B, C). However, particular deviations in food and water consumption occurred among both groups, which may be attributed to various factors including stress caused by oral treatment, distress from fighting among animals housed together, the impact of light or noise exposure, or simply measurement error (Kim et al. 2002; Lee et al. 2011). Furthermore, there was no significant difference between the food and water consumption among both control mice and NaCNs-treated mice irrespective of the treatment, further indicating that NaCNs are non-toxic (Anadón et al. 2014).

Conclusion

In the current study, DOX-NaCNs were fabricated and characterized to explore their antitumor effects and to conduct a biodistribution analysis in a murine breast cancer model. The developed DOX-NaCNs showed a maximum DLE of 78.99 ± 1.55% with a particle size of 270.86 ± 17.95 nm and had colloidal stability, when stored at 4˚C for three months. Since NaCNs were well-tolerated in mice when administered at a single dose of 2000 mg/kg, the safety of NaCNs was further confirmed in an in vivo test. An almost eight-fold reduction in tumor size in the group that was treated orally with DOX-NaCNs was observed compared to the group treated with free DOX, which may be attributed to the controlled release of DOX from the delivery system when compared to the free DOX or DOX-NaCNs intravenous formulation. Thus, the sodium caseinate micelles can improve the drug's bioavailability when administered orally. NaCNs is a potential emerging oral drug delivery system that should be further explored for tumor-directed delivery of drugs while validating the biodistribution patterns of the loaded drugs in various cancer models.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest All authors (F. Rehan, M.E. Karim, N. Ahemad, M.F. Shaikh, M. Gupta, S.H. Gan, and E.H. Chowdhury) declare that they have no conflict of interest.

Ethical approval Animal study was approved by Monash University Malaysia Animal Ethics Committee (Project ID: 2020-19843-39399) following standard protocols for animal handling and care.

Consent to participate Not applicable.

Consent for publication All authors gave their consent to publication of the data.

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