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Doxorubicin-loaded *N*-naphthyl-*N,O*-succinyl chitosan micelles for colon cancer treatment

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ABSTRACT

Doxorubicin (Dox) is one of the effective treatments in many types of cancer, such as breast cancer and ovarian cancer. However, the clinical settings of this drug are limited by its low therapeutic efficiency and high toxicity to normal cells. To achieve better anticancer efficacy, Dox-loaded micelles were developed using the amphiphilic chitosan derivative *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) through the dropping technique. The physicochemical properties including particle size, zeta potential, morphology, encapsulation efficiency (%EE), and loading capacity (LC) were evaluated. Results revealed that the Dox-loaded micelles were spherical in shape with a nanosized diameter (<200 nm) and negative charge (-30 mV). Incorporation of Dox into the micelles at the initial drug concentration of 40% w/w to the polymer provided the highest values of %EE (76.42%) and LC (305.68 µg/mg), reflecting that a high amount of drug could be encapsulated into the inner core of the micelles. Moreover, the Dox-loaded micelles exhibited higher cellular uptake compared with the free drug. *In vitro* cytotoxicity against HT29 colorectal cancer cells revealed that the Dox-loaded micelles had a greater inhibitory effect than the free drug with lower IC_{50} values. Therefore, these NSCS micelles may be potential nanocarriers to deliver Dox for colon cancer treatment.

Keywords: doxorubicin; chitosan; polymeric micelles; colon cancer

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common type of malignant tumor and is associated with high morbidity and mortality worldwide. Patients with CRC, who usually are diagnosed in the advanced states, are mainly treated with conventional chemotherapy using FDA-approved anticancer drugs such as oxaliplatin, 5-fluorouracil, or irinotecan to eradicate the cancer cells and improve a patient's quality of life (American Cancer Society, 2017; American Cancer Society, 2018). However, traditional anticancer drugs still need to address several problems, including serious side effects and lack of specific drug distribution and accumulation. Moreover, most anticancer drugs have poor aqueous solubility, leading to the restriction of antitumor efficacy and treatment failure (Cisterna et al., 2016). Doxorubicin (Dox; Figure 1) has been approved for use in single or combination regimens for the treatment of several cancer types such as leukemia, breast cancer, ovarian cancer, and lung cancer. In spite of

the treatment of many cancer types using Dox administration, it is not currently approved for CRC therapy due to a high level of cardiotoxicity and development of chemoresistance. Current studies in the clinical stage have attempted to understand the mechanism of CRC resistance to Dox and overcome this issue with a reduced dose to decrease the risk of cardiotoxicity (Hu et al., 2016; Sonowal et al., 2017). The main obstacle of the formulation development of this drug is its low anticancer effect, which hinders its clinical applications (Fan et al., 2018). In recent years, nanocarriers, such as polymeric micelles, liposomes, and dendrimers, have been extensively developed to obtain an effective drug vehicle for the delivery of hydrophobic anticancer agents to targeted tumor sites due to their good solubilization; they can also protect the drug from degradation, control drug release, enhance tumor targeting, improve biodistribution, and reduce toxicity to normal cells (Hossen et al., 2019; Attia et al., 2019). Amphiphilic copolymers can create nanoparticles with a core-shell structure in an aqueous environment through the self-assembly process. The hydrophobic part of polymers can form an inner core that entraps an anticancer drug inside, while their hydrophilic part can form an outer shell that helps increase the hydrophilicity of a micelle's surface; these copolymers are compatible with the aqueous environment and improve the carrier's stability in plasma by evading elimination by the reticuloendothelial system (RES) (Deshmukh et al., 2017). After drug administration, the nanoscale polymeric micelles can reach into tumor sites via an enhanced permeation and retention (EPR) effect through a process known as passive targeting (Biswas et al., 2016). Various polymers with biodegradable and biocompatible features, such as polyethylene glycol, poly L-lactic acid, poly lactic-co-glycolic acid, and chitosan, have gained extensive research attention for the development of nanocarriers of hydrophobic anticancer drugs (Gothwal et al., 2016). In this study, a chitosan derivative *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) was employed to prepare polymeric micelles; NSCS has been used in previous studies to deliver various hydrophobic drugs successfully (Kansom et al., 2018; Woraphatphadung et al., 2015; Soodvilai et al., 2019). Despite the use of various anticancer agents in CRC, only a few studies reported the use of Dox-loaded micelles for CRC treatment. Therefore, in this study, Dox-loaded NSCS micelles were constructed by the dropping technique. The physicochemical properties of the micelles were methodically investigated, and *in vitro* drug release was also studied. Finally, *in vitro* cellular uptake and cytotoxic activity of these micelles were assessed in HT29 cells.

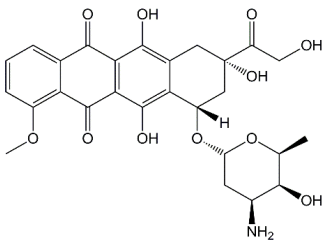

Figure 1. Chemical structure of doxorubicin

2. MATERIALS AND METHODS

2.1 Materials and cell culture

Dox HCl ($C_{27}H_{29}NO_{11}$·HCl) with at least 98% purity, naphthaldehyde, succinic anhydride, and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Chitosan (degree of deacetylation = 96%) was purchased from OilZac Technologies Co., Ltd. (Bangkok, Thailand). Dialysis tube (Cellusep® T1, molecular weight cut off (MWCO): 6-8 kDa) was purchased from Membrane Filtration Products (Seguin, TX, USA). The HT29 (human colorectal adenocarcinoma) cell line was obtained from the American Type Culture Collection (ATCC® HTB-38™, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-activated fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator. All reagents for cell culture were obtained from Gibco (BRL, USA). The other chemical substances were commercially available and used without any purification.

2.2 Synthesis of NSCS

The amphiphilic chitosan derivative NSCS was synthesized through reductive *N*-amination and *N,O*-succinylation in accordance with our previous reports (Woraphatphadung et al., 2015). In brief, 2.0 g of CS was solubilized in 150 mL of aqueous acetic acid, and 100 mL of ethanol was added to the solution. 2-Naphthaldehyde was added and stirred for 24 h. The precipitate was obtained by filtration, washed with ethanol, and dried under a vacuum at room temperature. This protocol created *N*-naphthyl CS (NCS). The *N,O*-succinylation of NCS was conducted using succinic anhydride. In brief, 1.0 g of NCS was dispersed in 40 mL of *N,N*-dimethylformamide (DMF)/dimethyl sulfoxide (DMSO), and 3.0 g of succinic anhydride was added. The reaction was heated at 100°C in a nitrogen atmosphere for 24 h. The clear solution was dialyzed and lyophilized. This NSCS copolymer had the CMC value of 0.0678 mg/mL (Woraphatphadung et al., 2015).

2.3 Formulation of Dox-loaded polymeric micelles

The NSCS micelles containing Dox were formulated via the dropping method. Prior to the use of Dox·HCl, the solution of Dox·HCl in DMSO was mixed with triethylamine to remove the HCl salt from Dox·HCl. The Dox molecule (free base) became more hydrophobic. In brief, Dox (0, 5, 10, 20, and 40% w/w to polymer) and NSCS polymer (5 mg) were liquefied in 0.5 mL of DMSO. This liquid was added dropwise into distilled water at a dropping rate of 2 mL/min under a stirring rate of 100 rpm and completely blended overnight (at least 8 h). The final volume ratio of DMSO:water was set to 1:5. Subsequently, the mixture was placed into a dialysis tube (MWCO: 6-8 kDa) and dialyzed against water for 24 h with altering water every 4 h to remove DMSO residue. The micellar solution was centrifuged at 1,500 rpm for 3 min to remove the unentrapped drug, and the supernatant was collected for additional experiments (Kansom et al., 2018). Blank micelles of NSCS which did not contain Dox were also prepared via the same steps as described above.

2.4 Characterization of Dox-loaded polymeric micelles

2.4.1 Morphology

The surface morphology of the empty and Dox-loaded NSCS micelles was investigated by transmission electron microscopy (TEM; Philips® Model TECHNAI 20, Netherlands). Subsequently, 1% uranyl acetate as a negative staining solution was applied with samples that were placed onto a formvar-coated copper grid. After staining and drying in the air, the sample grids were examined by TEM at an accelerating voltage of 80 kV.

2.4.2 Micelle size and size distribution

The micelle size, size distribution (PDI), and zeta potential of these micelles were investigated by dynamic light scattering on a Zetasizer Nano ZS instrument (Malvern, UK) at 25°C. The micelles were properly diluted in distilled water prior to measurement. Each value was expressed as the mean ± SD of three measurements.

2.4.3 Drug loading into micelles

To determine the quantity of Dox in the Dox-loaded polymeric micelles, 100 µL of the Dox-loaded micelles was added into 900 µL of DMSO:H_2O (9:1) to destroy the micellar structure. The solution was properly diluted within the linearity of the calibration curve (0.25–5 µg/mL). The Dox content was quantified using a fluorescence spectrophotometer (Shimazu, Japan) at excitation and emission wavelengths of 470 and 550 nm, respectively. The encapsulation efficiency (%EE) and loading capacity (LC) were computed using the following formulas:

$$\%EE = \frac{\text{amount of loaded drug in micelles}}{\text{amount of initially added drug}} \times 100 \quad (1)$$

$$LC\ (\mu g/mg) = \frac{\text{amount of loaded drug in micelles}}{\text{amount of polymer in micelles}} \quad (2)$$

2.5 *In vitro* drug release

In brief, Dox solution and Dox-loaded NSCS micelles (equivalent Dox concentration of 400 µg/mL) was sealed in a dialysis tube (MWCO: 6-8 kDa) and then soaked into 10 mL of PBS pH 7.4 as a release medium at 37± 0.5°C with shaking speed of 150 rpm. The released Dox from micelles transferred to PBS, and 1.0 mL of release medium was collected at sampling times (0.5, 1, 2, 4, 8, 12, and 24 h) and replaced by adding fresh medium. The released drug amount in each sample was quantified by fluorescence spectroscopy as described in section 2.4.3.

2.6 *In vitro* cellular uptake

The cellular uptake of Dox into HT29 cells was checked by flow cytometric analysis. In brief, HT29 cells at a density 1 × 10^6 cells/well were grown in a six-well plate and incubated overnight at 37°C. The cells were treated with free Dox or the Dox-loaded NSCS micelles (equivalent Dox concentration: 2.0 µM) in the fresh culture medium for 12 and 24 h. The cells were rinsed twice with PBS (pH 7.4) to discard any free Dox or micelles and then visualized by a fluorescence imaging system (Nikon DS-Ri2 camera connected with NIS element D4.60 software; Nikon, Japan) at 4× objective lens. Moreover, the cell suspensions in PBS of each treatment were analyzed by using a flow cytometer (BD Bioscience, USA) to confirm the extent of Dox in the cells. Excitation and emission wavelengths at 488 nm and 575 nm were applied to detect the fluorescent intensity and fluorescent histogram of Dox. Typically, 30,000 events were counted for each sample. Finally, the mean fluorescent intensity (MFI) of Dox from individual three detections was computed and reported as mean ± SD.

2.7 *In vitro* cytotoxicity

To evaluate the anticancer activity of Dox and the Dox-loaded NSCS micelles against HT29 cells by MTT colorimetric assay, the cells at a density 1 × 10^4 cells/well were seeded onto a 96-well plate and allowed to attach for 24 h. The free Dox that was dissolved in DMSO and the Dox-loaded NSCS micelles with different concentrations (equivalent to Dox concentration of 0.125-40 µM) were added to the cells and incubated at 37°C for 24 and 48 h. After drug exposure, the fresh medium containing 1.0 mg/mL MTT was filled to each well and further incubated for 3 h. The solution was removed and replaced with DMSO to dissolve the formazan crystal formed. Optical density (OD) was measured at 550 nm by using an automated microplate reader (VICTOR Nivo™, PerkinElmer, Hamburg, Germany), and the relative cell viability compared with the control was computed by the following formula:

$$\text{Cell viability } (\%) = \frac{\text{OD of treament}}{\text{OD of control}} \times 100 \quad (3)$$

IC_{50}, defined as the concentration of drug that inhibits 50% of cell growth, was estimated from the logarithm dose-growth curve created by GraphPad Prism. In a similar procedure, the cytotoxic effect of the blank NSCS micelle (100, 250, 500, and 1000 µg/mL) was observed as well. All experiments were conducted in triplicate.

2.8 Statistical analysis

All experiments were carried out in triplicate. Each result was reported as the mean ± SD. The independent t-test was used, and one-way ANOVA and Tukey multiple comparisons were conducted to analyze differences between groups using GraphPad Prism version 5.01 (GraphPad Inc., USA). Statistical significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Preparation and characterization of Dox-loaded polymeric micelles

In this work, the amphiphilic chitosan derivative NSCS, composed of naphthyl groups as hydrophobic part and succinyl groups as hydrophilic part, was used to prepare the empty and Dox-loaded micelles. When Dox-loaded micelles were formulated by dropping technique, the hydrophobic anticancer molecule Dox could be encapsulated and self-assembled into core–shell micellar structure. The physical appearance of freshly prepared micelle formulations was observed by visual inspection, as presented in Figure 2(a). The Dox-loaded micelles were found to be homogenously dispersible red liquid in the water, while the coarse suspension of Dox formed precipitated powder at the bottom of the container because Dox could not be dissolved in water. The surface morphology of the empty and 40% w/w Dox-loaded micelles is illustrated in Figure 2(b). The TEM images revealed that both micelles with and without drug were spherical in shape and nanometer in size (<200 nm).

The physicochemical characteristics of the micelles are summarized in Table 1. When the initial drug amount

increased, the micelle size gradually increased. However, the zeta potential did not change and still presented a negatively charged surface (about −30 mV) due to the anionic nature of succinyl groups. The particle size of the Dox-loaded micelles was bigger than that of the empty micelles due to the drug encasement into the inner compartment of the micelles. Overall, the Dox-loaded micelles at different percentages of drug to polymer had an average size under 200 nm in diameter with a narrow size distribution (PDI<0.3). These appropriate properties could be beneficial to effectively deliver the anticancer drug into a targeted tumor through the EPR effect. Moreover, the amount of starting drug was correlated with the drug-loading content, presented in terms of %EE and LC. The Dox-loaded micelles exhibited the maximum value of %EE (76.42±1.45%) and LC (305.68±5.81 μg/mg). This high drug content might be attributed to the strong hydrophobic interaction between the anthracycline part of the Dox molecule and the inner core of the NSCS polymer.

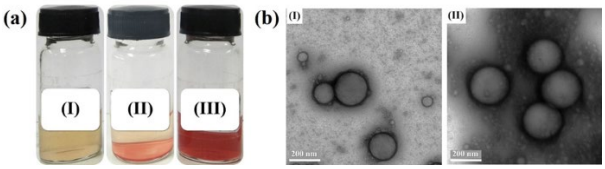

Figure 2. (a) Appearance of (I) empty NSCS micelles, (II) Dox coarse suspension in water, and (III) 40% w/w Dox-loaded NSCS micelles. (b) TEM photographs of (I) empty NSCS micelles and (II) 40% w/w Dox-loaded NSCS micelles. The scale bar is equal to 200 nm

Table 1. Physicochemical properties of the empty and Dox-loaded NSCS micelles

DOX (% to polymer)	Particle size (nm)[a]	PDI[b]	Zeta potential (mV)	%EE[c]	LC (μg/mg)[d]
0	102.80 ± 0.86	0.237 ± 0.056	(-) 32.17 ± 2.23	-	-
5	123.63 ± 0.59	0.274 ± 0.049	(-) 30.40 ± 0.50	43.84 ± 1.52	21.92 ± 0.76
10	161.10 ± 0.70	0.111 ± 0.035	(-) 29.73 ± 0.19	55.27 ± 1.04	55.27 ± 1.04
20	174.27 ± 0.66	0.118 ± 0.048	(-) 31.80 ± 0.40	69.07 ± 1.28	138.14 ± 2.55
40	193.83 ± 1.65	0.207 ± 0.054	(-) 31.40 ± 0.43	76.42 ± 1.45	305.68 ± 5.81

Note: [a]Measured by dynamic light scattering, [b]polydispersity index, [c]encapsulation efficiency, [d]loading capacity

3.2 *In vitro* drug release

The release patterns of Dox from the Dox solution and Dox-loaded micelles in PBS buffer are displayed in Figure 3. Free Dox showed a quick release profile into buffer medium with a release rate of around 60% within 1 h, and free drug released from Dox solution led to complete release rate of almost 100% after 24 h of observation. Concurrently, the Dox-loaded micelles exhibited significantly slower release rate (p<0.05) in the initial stage for 4 h followed by continuous release until its release rate reached 81.40% over a test period. This sustained release behavior might be explained by the fact that Dox entrapped into micelles diffused out from the inner core to aqueous medium, and the release rate of Dox was manipulated by the hydrophobic interaction between drug and copolymer. The biphasic Dox release from micelles was consistent with previous reports (Fan et al., 2018; Gao et al., 2013).

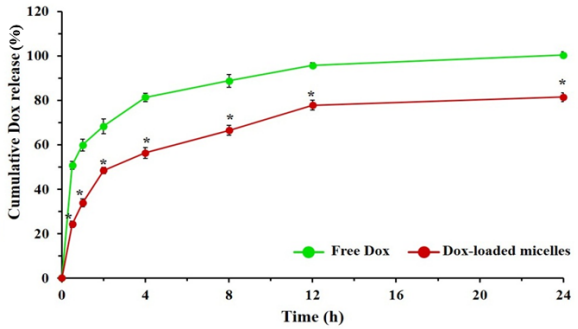

Figure 3. *In vitro* release profile of Dox from Dox solution and Dox-loaded micelles in PBS solution (pH 7.4) at 37°C for 24 h. *, p < 0.05 indicated a statistically significant difference compared with free Dox

3.3 *In vitro* cellular uptake

The cellular uptake of free Dox and the Dox-loaded micelles was examined qualitatively using fluorescence microscopy, as displayed in Figure 4. HT29 cells were exposed to Dox solution and micelles for two different time periods (12 and 24 h). The results showed that cells incubated with the Dox-loaded micelles emitted higher fluorescent intensity than those incubated with the free drug at both incubation times. To verify this result, the cells were analyzed by flow cytometry for quantitative cellular uptake. From the fluorescent histograms displayed in Figure 5a, the cells incubated with the Dox-loaded micelles emitted stronger fluorescent intensity than those of the free drug; this result was consistent with the fluorescent image study. As shown in Figure 5b, the MFI in Dox-loaded micelles was significantly higher (p<0.05) than that in the free drug after 24 h of treatment, representing that the micelle-encapsulated Dox was effectively taken up by cancer cells. The MFI in empty micelles was not different from that in the control group. Thus, the Dox-loaded micelles could enhance drug internalization and accumulation into the cancer cells.

3.4 *In vitro* cytotoxicity

The cytotoxic activity against HT29 cells of the 40% w/w Dox-loaded micelles compared with free Dox following incubation for 24 and 48 h was determined via a tetrazolium MTT assay, as shown in Figures 6a and 6b. In this experiment, both the free drug and Dox-loaded micelles showed a dose-dependent and time-dependent killing manner. The results revealed that the IC_{50} values of Dox-loaded micelles at 24 and 48 h were 2.04±0.13 and 0.67±0.05 μM, respectively, which were significantly lower (p<0.05) than those of free Dox at 24 (3.04±0.19 μM) and 48 h (1.48±0.06 μM). Thus, the micelles could enhance the anticancer activity of Dox in HT29 cells. This result could be explained by the augmented cellular

uptake of the Dox-loaded micelles relative to the free drug. The empty NSCS micelles had less cytotoxicity on HT29 cells at concentrations lower than 1000 µg/mL. The cell viability was still higher than 80% at 24 and 48 h of treatment (Figure 6c). Therefore, the NSCS polymer could be used to deliver Dox for improving its anticancer effect and was also a safe carrier material for anticancer drug delivery.

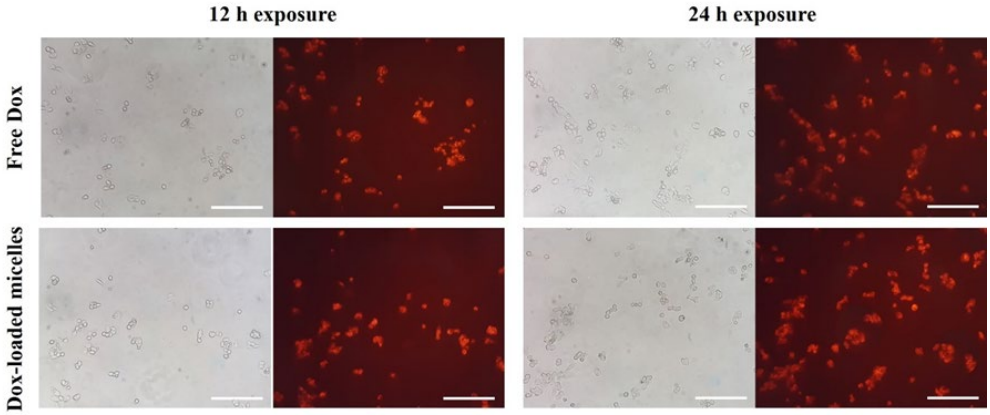

Figure 4. Fluorescent images of HT29 cells that were exposed to 40% w/w Dox-loaded NSCS micelles compared with free Dox for 12 and 24 h. The scale bars in all images are 200 µm

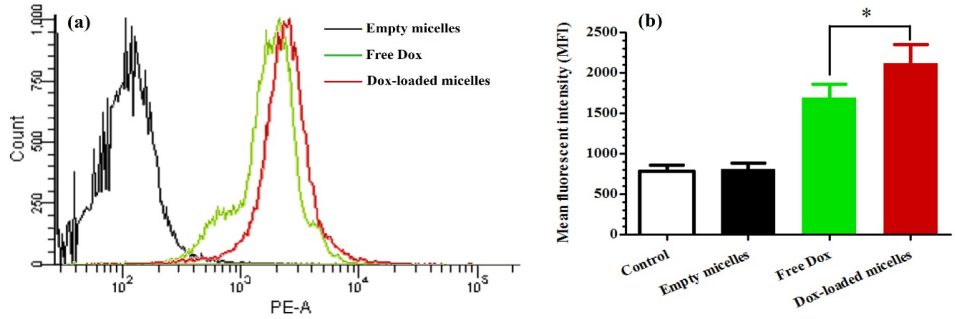

Figure 5. Drug uptake from 40% w/w Dox-loaded NSCS micelles and free drug in HT29 cells determined by flow cytometry. (a) Histogram of fluorescent events and (b) mean fluorescent intensity of Dox. *, $p<0.05$ was statistically significant compared with free Dox

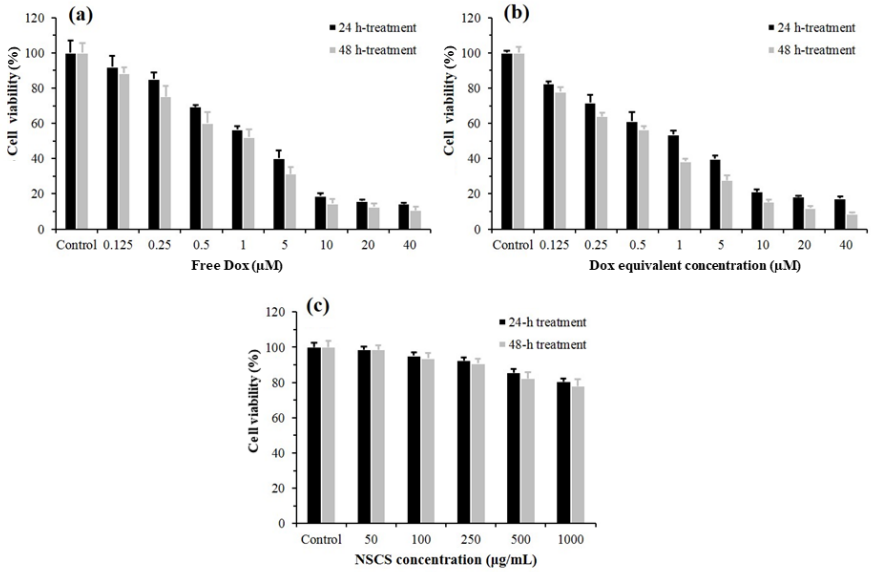

Figure 6. Cytotoxic effect of (a) free Dox, (b) 40% w/w Dox-loaded NSCS micelles, and (c) empty NSCS micelles against HT29 cell following incubation for 24 and 48 h

4. CONCLUSION

The Dox-loaded NSCS micelles were successfully formulated through the dropping method. The micelles were spherical nanosized particles with size of <200 nm with sufficient drug-loading content. The drug-loaded micelles enhanced the anticancer activity against HT29 cells and improved drug internalization into the cancer cells compared with its free drug. Thus, the Dox-loaded NSCS micelles may be a promising nanocarrier for Dox delivery for colon cancer treatment.

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