In-vitro cell tests using doxorubicin-loaded polymeric TiO_2 nanotubes used for cancer photothermotherapy

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To determine the appropriate surfactant to be added to TiO_2 nanotubes (TNTs) for use in cancer photothermotherapy, this study measured the increase in temperature and examined the size distribution of TNT particles loaded with different surfactants during near-infrared irradiation. In addition, in-vitro cell (fluorescein isothiocyanate and MTT assay) tests were carried out to examine the cytotoxic effect of doxorubicin-loaded and polyvinyl alcohol-added TNTs (pTNTs). The mean particle size of the pTNTs was 151.8 nm with a particle size variation of less than 3 nm, which is low enough to flow through blood vessels without causing a blockage. The temperature of the pTNTs was ~47°C, which is high enough to destroy cancer cells. Doxorubicin-loaded TNTs and pTNTs in combination with a near-infrared laser showed a cell viability of 4.5% – a sufficiently high cytotoxic effect. *Anti-Cancer Drugs* 23:553–560 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **23**:553–560

Keywords: antibody, near-infrared light, photothermotherapy, surfactant, TiO_2 nanotubes

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Received 21 October 2011 **Revised form accepted** 11 December 2011

Introduction

In general, an increase in tissue temperature to higher than 40–41°C is known as hyperthermal therapy or hyperthermia. Treatments with tissue temperatures higher than 46°C are referred to as thermoablation and have direct cytotoxic effects. Early thermotherapy, that is, hyperthermia, comprised simple heating techniques using thermal energy sources, such as lasers, microwaves, ultrasound, magnetic fields, and tubes with hot water. These therapies have a common problem in that it is difficult to heat the tumor tissues selectively as it is difficult to discriminating tumor cells from the surrounding healthy cells. In these techniques, treatment failure results from an insufficient increase in temperature in parts of the tumor, enabling tumor regrowth. However, excessive intratumoral temperatures might damage the adjacent structures. Therefore, hyperthermal therapies have shortcomings in that the treatment times are long and the lesion boundaries are not well defined. Thus, despite its long history, tumor ablation using heat is not used widely in clinics because of these technical difficulties.

In contrast, second-generation thermotherapies, that is, photothermotherapies (PTTs), are typically used in combination with chemotherapy and radiography but with fewer limitations compared with either of these therapies. Localized heating of tumor tissues using these techniques can be accomplished either by functional modification of the nanomaterial surface using an antibody or a ligand with some affinity for the tumor tissue, such as folic acid (active targeting), or by exploiting the extravasation phenomenon (passive targeting). In contrast, PTTs utilize the heat generated by nanoparticles under optical illumination. PTT is a combination of chemotherapy and radiotherapy. This technique does not have the above-mentioned shortcomings of hypertherapy because only the tissues near the nanoparticles are heated selectively. Therefore, this technique can remove tumors by repeated ablation without side effects [1].

In recent times, photothermal therapy based on photothermal agents in combination with a near-infrared (NIR) laser has attracted considerable attention because of its efficiency in destroying cancer cells more selectively. Early PTT used natural chromophores or dye molecules in the tissue as photothermal agents; however, both have problems of very low optical absorption and photobleaching under laser irradiation [2]. In recent years, inorganic nanomaterials, such as gold nanospheres [3], gold nanorods [4], gold nanoshells [5], gold nanocages [6], carbon nanotubes [7], porous silicon nanoparticles [8], and TiO_2 nanotubes (TNTs) [9,10], have been demonstrated in PTT owing to their high optical absorption properties in the NIR region. Of these nanomaterials, this study focused on TNTs because of its excellent photothermal properties [9] and biocompatibility [11–15]. In addition, TNTs can be prepared easily by the simple electrochemical anodization of a Ti foil [16]. Regarding the biomedical applications of TNTs, their use in drug delivery has been reported [17,18]; however, there are no reports on the applications of TNTs in cancer thermotherapy.

The eventual goal in the development of cancer therapy is the systematic administration of cancer markers by

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328350446b

inducing the selective destruction of cancer cells. In state-of-the-art systematic administration, cancer markers, such as antibodies or folic acid, are used to help therapeutic agents access cancer cells spontaneously despite the very low probability of the markers accessing the antibody receptors in the cancer cells successfully. However, it is important to prevent the agglomeration of as-prepared TNT nanoparticles using a surfactant, similar to other material nanoparticles. This study examined whether the high photothermal properties of TNTs can be maintained after adding a surfactant and whether the TNTs can destroy cancer cells effectively even after being mixed with surfactants and loaded with antibodies, such as doxorubicin (Dox). The excellent heat-generating ability of TNTs, the ability to destroy cancer cells irreversibly under NIR laser irradiation, and the ability to inhibit tumor growth were examined previously by measuring the increase in temperature [8,10]. However, the results were not sufficient to confirm the efficacy of TNTs as a photothermal agent for cancer treatment as the tests were carried out under very limited conditions. Furthermore, there are no reports on the efficacy of TNTs treated with both surfactants and antibodies in destroying cancer cells to date.

Materials and methods

Preparation of TiO_2 nanotube colloid

TNT layers were formed by the electrochemical anodization of a thin Ti foil in an electrolyte containing 0.3 wt.% NH_4F and 2 vol.% H_2O in ethylene glycol at 60 V for 17 h. The inner diameter and thickness of the TNTs was $\sim$100 nm and $\sim$160 μm, respectively. The TNT layers were fragmented into numerous small pieces with sizes smaller than 220 nm using an ultrasonicator (model: 2510E-DTH, Branson Ultrasonics Corp., Danbury, Connecticut, USA) for 24 h and filtered sequentially through 220 and 450 nm microfilters (model: SLGV 033 RS, Millipore, Billerica, Massachusetts, USA) to obtain a TNT colloid.

Particle size analysis

The sizes of the TNT nanoparticles in the prepared TNT colloids were measured using a zeta potential particle size analyzer (model: ELS-Z, Photal, Otsuka Electronics Co., Hirakata-shi, Osaka, Japan). The mean particle sizes of the TNT colloids treated with surfactants, such as polyvinyl alcohol (PVA), sodium dodecylbenzenesulfonate (SDBS), dimethyl sulfoxide (DMSO), and Triton X-100, were also measured.

Preparation of purified doxorubicin-loaded and polyvinyl alcohol-added TiO_2 nanotubes

Figure 1a shows a transmission electron microscopy (model: JEM2100F; JEOL, Akishima-shi, Tokyo, Japan) image of the prepared PVA-added TNTs (polymeric TNTs or pTNTs). PVA stuck fast to the TNTs, which were loaded with 2 μmol/l doxorubicin. The Dox-loaded pTNT colloid was placed in a 10 kDa dialysis membrane, and both ends of the dialysis membrane were sealed tightly. This membrane was placed in a beaker filled with deionized water and stirred to remove the unloaded Dox. Purified Dox-pTNTs can be obtained by this process because pTNTs with a mean particle size of $\sim$117 nm cannot pass through the dialysis membrane, whereas the small unreacted Dox can. Figure 1b shows a schematic diagram of the purified Dox-pTNTs used as a therapeutic agent for cancer PTT.

Measurement of heating of TiO_2 nanotube colloid samples by near-infrared irradiation

The photothermal properties of the TNT colloids treated with different surfactants were measured using an Infrared Thermography Camera (model: Thermovision A20M Fire Wire; FLIR, Billerica, Massachusetts, USA). The TNT colloids are hereafter called TNTs. Their photothermal properties were examined using NIR light from a high-power NIR laser (808 nm) source with a water cooling system. The laser diode bar was coupled to a fiber with a length, core, and numerical aperture of 120 cm, 375 μm, and 0.22, respectively. The fiber was connected to a laser diode driver (model: KS3-11321-503; BWT Beijing Ltd, Fengtai, Beijing, China) with a maximum power of 10 W. The samples were irradiated continuously with an NIR laser at 2.0 W/cm^2 for 20 min. The laser beam size and distance between the laser diode and samples were 2 and 5 cm, respectively.

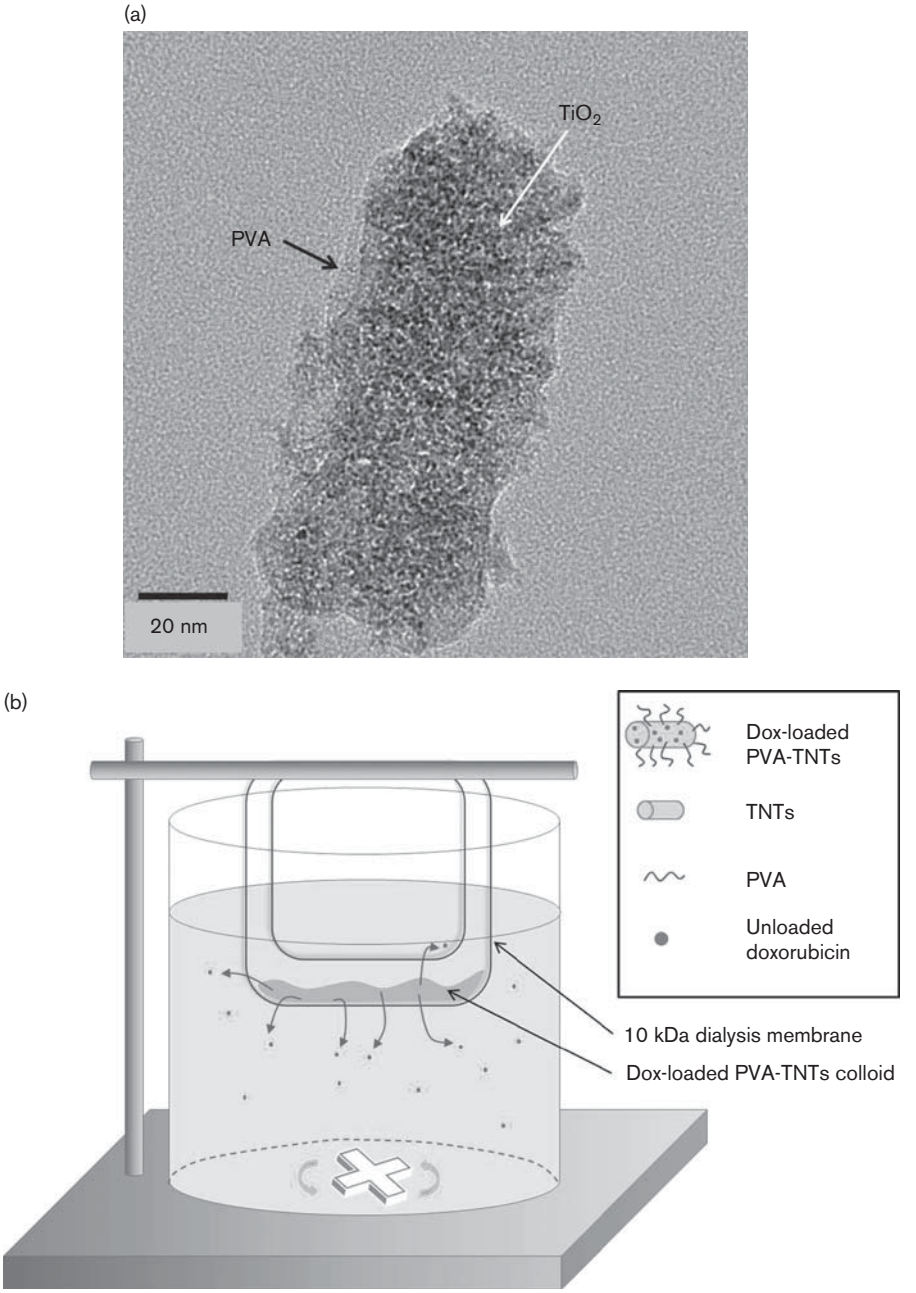
Cells line and materials

Murine colon cancer cell lines (CT-26) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The CT-26 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Fetal bovine serum, cell culture media, penicillin/streptomycin, and all the other agents used in the cell culture studies were purchased from Invitrogen (Gibco, Grand Island, New York, USA). After seeding the cells on 24-well plates, the cells (1×10^5) were incubated for $\sim$24 h at 37°C in an atmosphere containing 5% CO_2. After incubation, the cell media were removed from the wells, and the cells were washed with PBS. Incomplete DMEM was then added to each well. The pTNT colloid was then added to each well. The attached CT-26 cells with or without an incubation treatment in the pTNT colloid were transferred to a circular quartz cuvette and exposed to an NIR laser.

MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were carried out to monitor cell viability after NIR laser irradiation. The MTT assay solution was prepared by dissolving 50 mg of the MTT powder in 10 ml of PBS solution and filtering the mixed solution. After the cell medium was removed, 180 μl of

Fig. 1



(a) Transmission electron microscopy image of the TiO_2 nanotubes (TNTs) treated with polyvinyl alcohol (PVA). (b) Schematic diagram of the doxorubicin (Dox)-loaded TNTs treated with polyvinyl alcohol.

incomplete medium was added. Subsequently, 20 µl of the MTT solution was added to each cell sample, and the samples were incubated for 5 h at 37°C in DMEM containing 5% CO_2. Finally, each sample was injected with Dox-pTNT colloid and exposed to an NIR laser at 2 W/cm^2 for 20 min. After removing the solution, 200 µl of DMSO was added to each cell sample. After pipetting and shaking, the solution absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay reader.

Annexin V–fluorescein isothiocyanate apoptosis assays tests

CT-26 cells were cultured in DMEM. CT-26 cells (1×10^6) were seeded in a 100 nm dish for 18 h before incubating in 24-well plates at 37°C in an atmosphere containing 5% CO_2 for 24 h. After incubation, the cell media were removed from the wells, the cells were washed with PBS, and incomplete DMEM was added to each well. The pTNT colloid was then added to each

well. Annexin V–fluorescein isothiocyanate (FITC) apoptosis assays were carried out on the following five CT-26 cell sample groups to observe their modes of cell death (apoptosis or necrosis):

(1) the CT-26 cell control group – given neither pTNT nor laser treatment (control),
(2) the CT-26 cell group treated with the NIR laser only,
(3) the group treated with Dox only,
(4) the group treated with pTNTs only,
(5) the group treated with both Dox-pTNTs and the NIR laser.

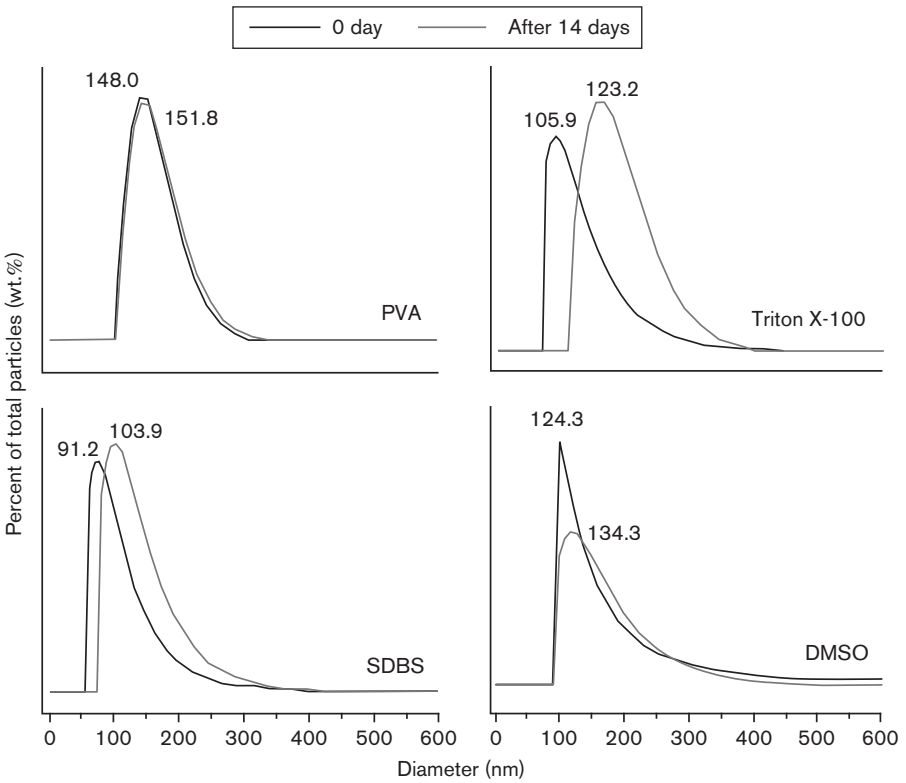
For the annexin V–FITC apoptosis assay, the CT-26 cells were treated with Dox-pTNTs and the NIR laser, and 3×10^5 cells were removed from the culture, washed twice with cold PBS, and double stained with annexin V–FITC and propidium iodide (Becton Dickinson Biosciences, San Jose, California, USA) in an annexin-binding buffer. The cells were then analyzed using a FACS-calibur flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. To avoid any nonspecific fluorescence from dead cells, live cells were gated using a forward and side scatter. Another set of cell samples was prepared in the same manner described earlier using Dox-pTNTs instead of pTNTs.

Results

Particle size analysis

As mentioned earlier, surfactants need to be added to the as-prepared TNTs to prevent agglomeration. The kinetic stabilization behavior of the TNTs treated with different surfactants was examined by measuring the mean particle size as a function of time. Figure 2 shows the mean sizes of the TNT nanoparticles in the TNTs at 0 and 14 days after mixing them with the surfactants. All the TNT samples were stored in a colloidal state after being sealed tightly at room temperature for 14 days. The nanoparticles in the colloids were crushed for 1 h by ultrasonication to redistribute them before measuring their sizes on the 14th day, because nanoparticles generally agglomerate and precipitate, even in colloids mixed with surfactants. Figure 2 suggests that the TNTs treated with the PVA surfactant is the most stable steric colloid among the TNTs treated with four different surfactants (PVA, SDBS, DMSO, and Triton X-100), because the particle size variation in the TNTs is less than 3 nm.

Fig. 2



TiO_2 nanotube particle size distributions in the TiO_2 nanotubes treated with different surfactants: polyvinyl alcohol (PVA), sodium dodecylbenzenesulfonate (SDBS), dimethyl sulfoxide (DMSO), and Triton X-100.

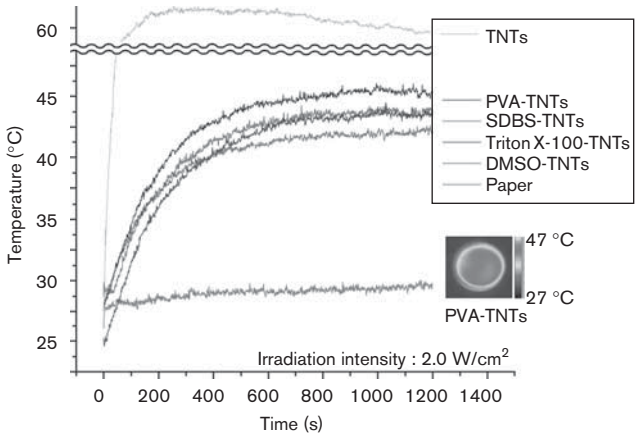
Photothermal effects of the surfactants-added TiO_2 nanotubes samples

Figure 3 compares the temperatures of the TNTs treated with different surfactants during NIR laser irradiation. The temperature of a sheet of paper increased to ~29°C immediately after exposure to the NIR laser at 2.0 W/cm^2 but did not increase any further. The temperature of the sheet of paper after NIR exposure was 28.5°C, which was ~2.5°C higher than before NIR laser irradiation (26°C). This suggests that the temperature 28.5°C of the sheet of paper at 0 exposure time is entirely due to the heat from the NIR laser source. The temperatures of the TNTs treated with the surfactants after NIR exposure for sufficient times ranged from 43 to 47°C, which is 18–19°C higher than those before NIR exposure. In particular, the PVA-treated TNTs showed the highest photothermal effect among those treated with different surfactant. In contrast, ~200 s after NIR exposure, the temperature of the TNTs not treated with the surfactants increased to ~62°C. Therefore, the addition of surfactants to the TNTs reduces the photothermal effect by 15–17°C. In contrast, the temperature of the PVA-added TNTs was increased to 47°C, which is high enough for thermoablation. The inset in Fig. 2 shows a thermal image of the TNTs treated with PVA exposed to NIR laser for 20 min.

MTT assay

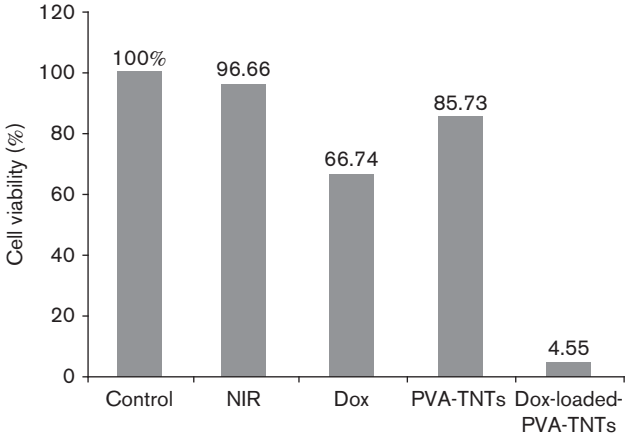
An MTT assay test was carried out on the CT-26 cells exposed to Dox-pTNTs in combination with NIR light irradiation to determine whether localized, irreversible photothermal ablation of tumor tissue could be achieved. The results in Fig. 4 show that among the four different cell samples, only the cells treated with both Dox-pTNTs and NIR light had been killed. The cells exposed to NIR light at 2.0 W/cm^2 for 20 min without the Dox-pTNTs treatment showed a cell viability of 96.7%. Similarly, the cells treated with Dox-pTNTs but not with NIR irradiation also showed a cell viability of 85.7%. A combination of these two techniques, however, resulted in a cell viability of 4.5%. This indicates that more than 95% of the cells treated with Dox-pTNTs were destroyed during NIR light irradiation at 2.0 W/cm^2 for 20 min. In contrast, the cells treated with Dox showed only a cell viability of 66.7%. This low cell viability might be due to the cytotoxicity of Dox itself. Dox is also used in cancer chemotherapy because of its cytotoxic effect. In contrast, the low cell viability of the cells treated with both Dox-pTNTs and NIR light was attributed to a combination of the photothermal effect of the TNTs under NIR laser irradiation and the cytotoxic effect of Dox.

Fig. 3



Temperature of the TiO_2 nanotube (TNT) colloids treated with different surfactants as a function of the near-infrared laser irradiation time (laser intensity, 2.0 W/cm^2). DMSO, dimethyl sulfoxide; PVA, polyvinyl alcohol; SDBS, sodium dodecylbenzenesulfonate.

Fig. 4

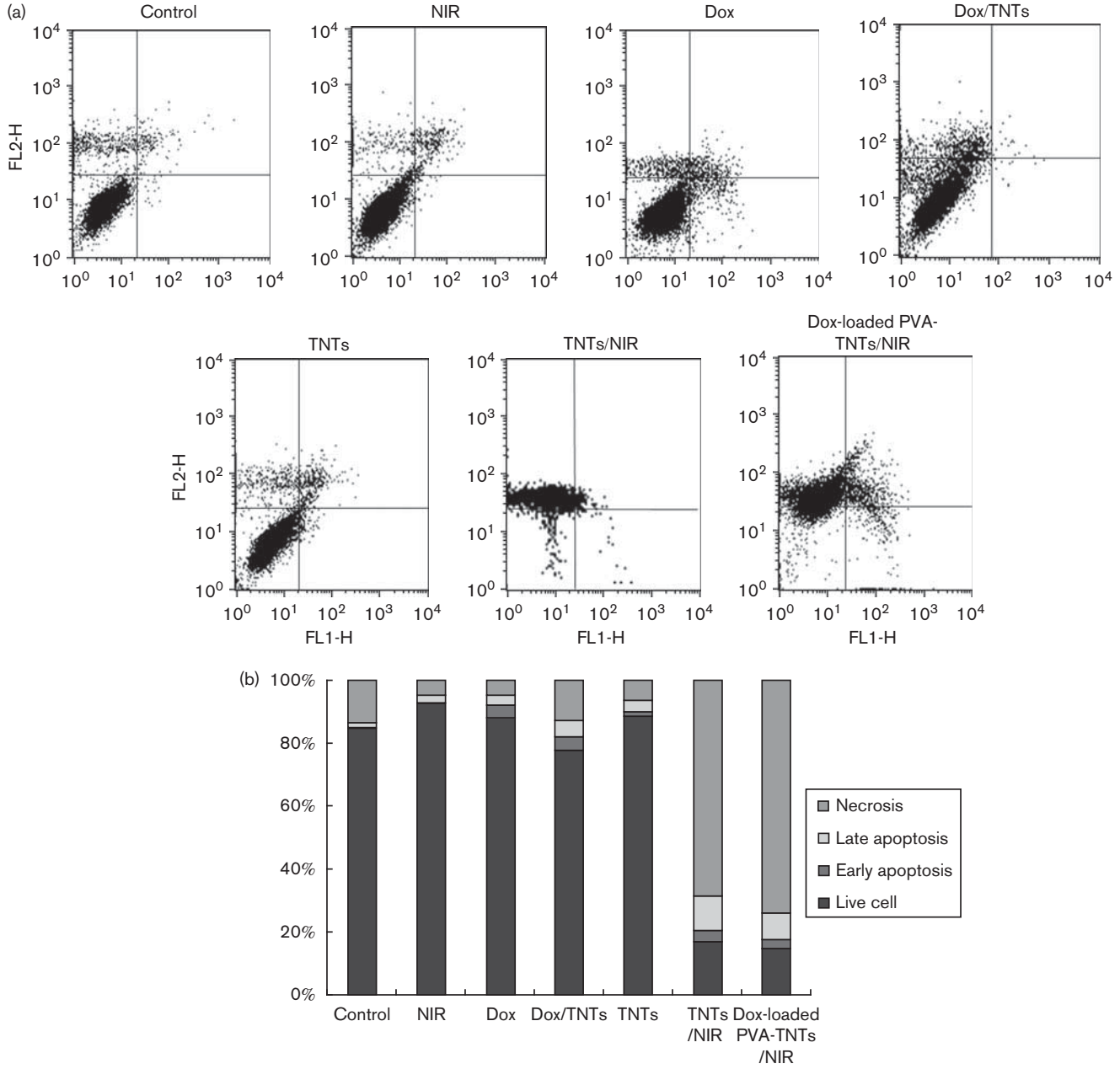


Cell viability. Near-infrared (NIR) laser irradiation was carried out with an illumination intensity of 2.0 W/cm^2 for 20 min. The CT-26 cells treated with the Dox-pTNT colloid and NIR laser showed a considerable level of cell death. In contrast, neither those treated with only a pTNTs colloid nor those treated with only a NIR laser were changed compared with the control group ($P<0.05$). Dox, doxorubicin; PVA, polyvinyl alcohol; TNTs, TiO_2 nanotubes.

Annexin V–fluorescein isothiocyanate apoptosis assay tests

An annexin V–FITC apoptosis assay test was carried out on CT-26 cancer cells to determine the ability of Dox-pTNTs and pTNTs to kill cancer cells and identify the death modes. The fluorescent activated cell sorter flow cytometry profiles obtained from the annexin V–FITC apoptosis assay show annexin V–FITC staining in the *X*-axis and propidium iodide in the *Y*-axis (Fig. 5a). The four sections of the quadrant in each profile from the upper left in a clockwise direction represent necrosis, late apoptosis, early apoptosis, and living cells, respectively. Of the four cell modes, necrosis and late apoptosis are generally considered to be cell death. The group treated with both Dox-pTNTs and NIR laser showed

Fig. 5



Annexin V–fluorescein isothiocyanate (FITC) apoptosis assay results: (a) Flow cytometry profiles represent annexin V–FITC staining in the *X*-axis and propidium iodide in the *Y*-axis. (b) Summary of the annexin V–FITC apoptosis assay results showing the percentage of cell-death modes: necrosis, late apoptosis, early apoptosis, and live cell. DOX, doxorubicin; NIR, near-infrared; PVA, polyvinyl alcohol; TNT, TiO_2 nanotube.

substantially higher cell death (necrosis + late apoptosis) rates than those not given both treatments (Fig. 5b). The group treated with neither Dox-pTNTs nor NIR laser (the control group), that treated with Dox-pTNTs only, and that treated with the laser only showed a cell viability of more than 85%. However, a combination of these two techniques resulted in a cell viability of less than 25%. The histogram shows that the cell deaths were mainly due to necrosis (~75%) but also partly due to late apoptosis (~10%). Therefore, this in-vitro cell test result suggests that only a combination of Dox-pTNTs and NIR laser treatments can destroy cells. This confirms that a combination of Dox-pTNTs and NIR light results in a high cell death rate due to a combination of the photothermal effect of pTNTs under NIR laser irradiation and the cytotoxic effect of Dox.

Very recently, Qin *et al.* [19] reported that Dox and TiO_2 nanoparticles without NIR treatment were very effective in killing cancer cells. In their study, TNT was used as a vehicle for carrying anticancer agent Dox. Additional tests were carried out in this study to check the cytotoxic effect of Dox and TNT without NIR treatment. The cell death rate of the group treated with Dox + TNT without NIR treatment was somewhat higher than that of the group treated with only Dox without NIR treatment. However, the cell death rate of the group with Dox + TNT without NIR treatment was not as high as those reported by Qin and colleagues. In contrast, the group treated with both TNT and NIR treatments showed a very high cell death rate that is almost the same as that of the group treated with both Dox + TNT and NIR treatments. These results indicate that the cell-destroying ability of TNT combined with NIR is very high but that the cytotoxic effect of TNT without NIR treatment is not so high. The results also suggest that the cell-destroying ability of TNT by drug delivery cannot be effectively evaluated by in-vitro cell tests. Instead, it should be evaluated by in-vivo animal tests. In contrast, the cell-destroying ability of TNT in combination with laser irradiation through photothermal therapy can be well evaluated by in-vitro cell tests.

Discussion

The TNTs mixed with a surfactant PVA showed a larger increase in temperature than the TNTs mixed with PVA, SDBS, DMSO, and Triton X-100. The temperature of the PVA-added TNTs was $\sim 47^{\circ}C$, which is high enough to destroy cancer cells. The mean TNT particle size in the TNTs mixed with PVA (pTNTs) was 151.8 nm with a size variation of less than 3 nm, which is sufficiently low to enable blood flow in vessels without causing a blockage. Cells exposed to NIR light at $2.0\,W/cm^2$ for 20 min without the Dox-pTNTs treatment showed a cell viability of 96.7%. Similarly, the cells treated with Dox-pTNTs but not with NIR irradiation showed a cell viability of 85.7%. A combination of these two techniques, however, resulted in a cell viability of 4.5%. This suggests that Dox-pTNTs have a sufficiently high cytotoxic effect due to the combined photothermal effect of pTNTs under NIR laser irradiation and the cytotoxic effect of Dox. Figure 5 shows that there is quite a high percentage of dead cells in the control. There are several possible causes for the death of cells in the control:

(1) Contamination from impurities from DMEM and DMSO solutions as they were supplied from large bottles containing those solutions that many people use in common;
(2) Damage of the cells in contact with walls or exposed to air by environmental causes such as sunlight and moisture in the air;
(3) Insufficient DMEM solution used for sample preparation.

In this preliminary study, only in-vitro cell tests were conducted with the Dox-pTNTs. The in-vitro cell test results suggest that the thermotherapy based on Dox-pTNTs combined with NIR laser is an efficient technique in destroying cancer cells. However, they do not necessarily show that the Dox-pTNTs can inhibit tumor growth. Therefore, in-vivo animal tests are currently under way to determine whether the Dox-pTNTs can indeed inhibit tumor growth. In addition, the interaction of Dox-pTNTs with other blood components, for example, albumin, and the renal clearance of Dox-pTNTs within a reasonable period of time should also be examined before a Dox-pTNTs drug solution is used for cancer treatment in clinics. The in-vitro cell test results show the photothermal effect of thermotherapy based on Dox-pTNTs combined with laser on cell death, but it does not guarantee that the thermotherapy can inhibit tumor growth. To confirm that the photothermal effect of Dox-pTNTs combined with a NIR laser could efficiently destroy tumor cells without damaging surrounding healthy cells, in-vivo therapeutic examinations against Balb/c mice bearing CT-26 on their backs are under way.

Acknowledgements

This study was supported by the Korea Engineering and Science Foundation (KOSEF) through 'the 2010 Core Research Program'.

Conflicts of interest

There are no conflicts of interest.

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