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Article

Enhanced photodynamic therapy of TiO_2/*N*-succinyl-chitosan composite for killing cancer cells

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The aim of this study was to investigate the effect of TiO_2/*N*-succinyl-chitosan composite (TiO_2/NSCS) photodynamic therapy (PDT), while considering the effects of various light sources on the activation of photosensitizer. The methyl thiazolyl tetrazolium assay was used to examine the cell survival rate of the cells. The results showed that glioma cell strain (U251) was the most sensitive cancer cell strain to TiO_2/NSCS. When the concentration of TiO_2/NSCS was between 0 and 800 $\mu g \cdot mL^{-1}$, there was no obvious cytotoxicity to normal liver cells (HL-7702) and U251 cells. During the PDT process, the photokilling effect of TiO_2/NSCS on U251 cells under ultraviolet-A (UVA) light irradiation was stronger than that of pure TiO_2, and its killing effects were positively correlated with concentration and irradiation time. In addition, both UVA and visible light could excite TiO_2/NSCS, which had significant killing effect on U251 cells. The results of acridine orange/ethidium bromide fluorescent double staining and Annexin V/propidium iodide double staining indicated that TiO_2/NSCS under UVA and visible light irradiation could kill U251 cells by inducing apoptosis, and the apoptosis rate of TiO_2/NSCS treatment groups was higher than that of TiO_2 treatment groups. Therefore, TiO_2/NSCS might be used as a potential photosensitizer in PDT.

Keywords: Photodynamic therapy. TiO_2/*N*-succinyl-chitosan composite. Methyl thiazolyl tetrazolium assay. glioma cells (U251). Killing effect. Apoptosis.

INTRODUCTION

Cancer is the most distressing and life-threatening disease of modern times that continues to cause severe death worldwide (Ferlay *et al.*, 2013). Its incidence and mortality rate gradually increase every year. For cancer patients, the current option can only be non-targeted chemotherapy, surgical therapy, and radiotherapy in order to prolong the survival of patients, but they cannot reliably prevent secondary disease and fail to completely eradicate the tumor (Mitra *et al.*, 2015). One of the most promising cancer treatments is photodynamic therapy (PDT), which is a relatively new therapy to enhance anticancer therapy, reduce undesirable systemic toxicity and side effects (Schuitmaker *et al.*, 1996). In the process of PDT, a photosensitizer (PS) is exposed selectively to a particular wavelength of light, whereupon it transfers the photon energy to surrounding oxygen molecules to produce highly reactive oxygen species (ROS), resulting in toxicity to the targeted tissue (Gui *et al.*, 2013).

The unique characteristics of PS TiO_2 nanoparticles such as good biocompatibility, chemical stability, photoreactivity and economic viability made it the first choice for biological applications (Shakeel *et al.*, 2016). However, TiO_2 can only be excited by ultraviolet-A (UVA) light with the wavelength less than 387 nm

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(Yurt *et al*., 2018), and it is insoluble in water, which is detrimental and largely hinders its practical applications and developments in PDT (Wang *et al.*, 2015).

Fortunately, it had been reported that the absorption range of TiO_2 could be extended to the visible region by modifying methods (Ding *et al*., 2016; Li *et al*., 2015). *N*-succinyl-chitosan (NSCS) has been widely used in the biomedical field for drug delivery due to its superior biocompatibility and biodegradability, low-toxicity, nonimmunogenicity, long half-life and effective accumulation in tumor tissue (Kamoun, 2016; Wang, Li, Song, 2004; Yan *et al*., 2006). Therefore, in our previous study (Wang, Li, Zhang, 2016), the successfully prepared TiO_2/NSCS composite (Figure 1A) had better dispersion and stability, a wider photoresponsive range, and stronger absorption of light. For these reasons, TiO_2/NSCS composites were chosen as the PS to investigate PDT effect.

In the present work, we set out to explore the effect of PDT using TiO_2/NSCS as PS (TiO_2/NSCS-PDT). The most sensitive cancer cell strain to TiO_2/NSCS was screened, and the photokilling effect on the most sensitive cancer strain was tested. Methyl thiazolyl tetrazolium (MTT) assay was adopted to detect the inhibition effects of TiO_2/NSCS on the most sensitive cancer cells proliferation. And the killing effects of pure TiO_2 and TiO_2/NSCS were compared. Moreover, the mechanism of the photokilling effect was preliminarily explored.

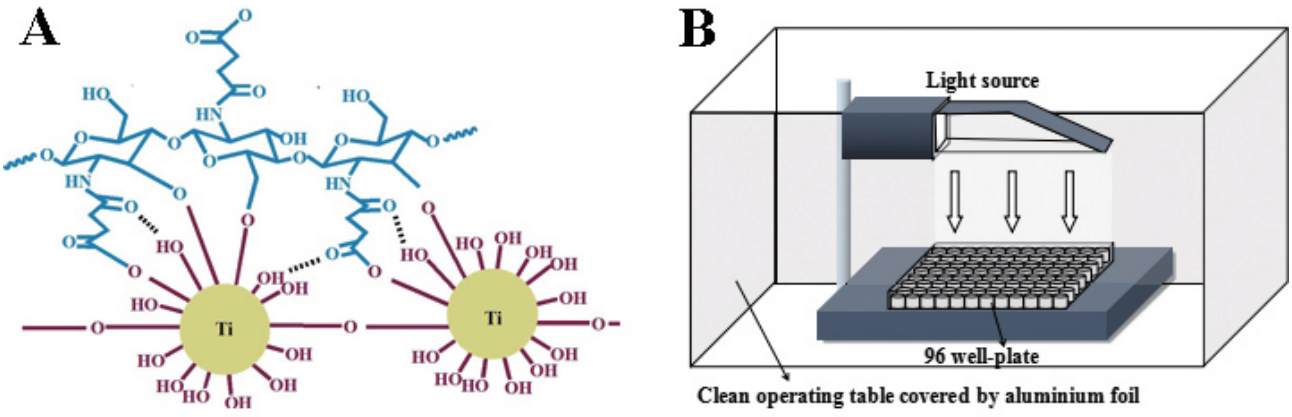

FIGURE 1 - (A) Schematic diagram of TiO_2/NSCS; (B) Schematic diagram of photocatalytic killing cancer cells experimental set-up.

MATERIAL AND METHODS

Cell, drug and equipments

Hepatocellular carcinoma cell strain (HepG2), cervical cancer cell strain (SiHa), glioma cell strain (U251), gastric cancer cell strain (SGC), embryonic cancer cell strain (EC), chromosome tumor cell strain (PC12) and normal liver cell strain (HL-7702) were supplied by Boster Biological Technology Co. Ltd., Wuhan, China.

TiO_2/NSCS composite was prepared and characterized in our previous research (Wang, Li, Zhang, 2016), which displayed good dispersion and high photocatalytic activity. The average particle size is 50 nm and the photoresponsive range of TiO_2 is extended to 450 nm.

3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied by Sigma (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), dimethyl sulfoxide (DMSO), penicillin-streptomycin (10%) and trypsin-EDTA peptic juice (0.25%) were obtained from Boster Biological Technology Co. Ltd. (Wuhan, China). Fetal bovine serum (FBS) was purchased from Beizhuo Biological Technology Co. Ltd. (Shanghai, China). Annexin V/propidium iodide (Annexin V/PI) double staining kit was purchased from Bender Medsystem (USA). Binding buffer was purchased from Biomiga (USA). All other chemicals

and solvents were of analytical grade or better and used without further purification. All solutions were prepared with Milli-Q (Millipore, Bedford, MA, USA) water with a resistivity of 18 MΩ cm.

HERA cell 150i Carbon dioxide incubator (Thermo Scientific, USA); pHS-25CW Microprocessor pH/mV/Temperature meter (Shanghai Bante Instrument Co. Ltd., China); Model 680 Micro-plate reader (Bio-RAD, USA); XD50R Inverted fluorescence microscope (Hongkong Xianda Analysis Instrument Co. Ltd., China). LED light (LX-1, 420 nm, 50 W, Shenzhen City Lüxing Lighting Technology Co. Ltd., China) and high pressure mercury lamp (GGZ-300W, 365 nm, 125 W, Shanghai Yaming Lighting Co. Ltd., China) were used as light sources. In order to avoid light scattering and interference of external light source, the clean operating table was surrounded by aluminium foil and its front was covered with an opaque black cloth. The light intensity in the experiment was 1.5 $mW \cdot cm^{-2}$ as measured by the VLX-3W radiometer-photometer (SP Company, USA). Schematic diagram of photocatalytic killing cancer cells was shown in Figure 1B.

Cell culture

The cells were grown in DMEM supplemented with 10% (*v*/*v*) FBS and 1% (*v*/*v*) penicillin-streptomycin, then incubated in a humidified incubator containing 5% CO_2 at 37ºC. After reaching 80% confluence, the above cells were washed twice with PBS. Then, the cells were trypsinized with trypsin-EDTA peptic juice (0.25%) and resuspended in DMEM. The cell concentration was adjusted to 10^5 $cells \cdot mL^{-1}$ by a cell counting plate. 100 μL of the cell suspension was added to each well of a 96-well plate.

Screen the most sensitive cancer cell strain

When reached 85% confluence, HepG2, SiHa, U251, SGC, EC and PC12 cells cultured with different concentrations of TiO_2/NSCS in serum-free medium for 24 h at 37ºC were irradiated by UVA light for 20 min. The most sensitive cancer cell strain was screened by evaluating cell viability.

Determination of cytotoxicity of TiO_2/NSCS

Briefly, the cells were exposed to different concentrations of TiO_2/NSCS without any irradiation for 24 h and then the cytotoxicity of TiO_2/NSCS was evaluated by determining cell viability.

Photodynamic efficacy *in vitro*

For PDT, the experiment was divided into seven groups, and each group was repeated three times. The PDT efficacy was evaluated by determining cell viability.

Group I: negative control group, routinely cultured cells without any treatment.
Group II: positive control group, cells cultured with TiO_2 were similarly treated with non-irradiation control group and PDT experimental group.
Group III: non-irradiation control group, cells were cultured with different concentrations of TiO_2/NSCS in the absence of light.
Group IV: irradiation control group, cells were irradiated by UVA light for 5, 10, 20, 30 and 40 min without any PS.
Group V: PDT experimental group of different concentrations, cells cultured with different concentrations of TiO_2/NSCS were irradiated by UVA light for 20 min.
Group VI: PDT experimental group of different irradiation times, cells cultured with a certain concentration of TiO_2/NSCS were irradiated by UVA light for 5, 10, 20, 30 and 40 min, respectively.
Group VII: PDT experimental group of different light sources, cells cultured with TiO_2/NSCS were irradiated by UVA or visible light for 20 min.

Cell viability analysis

To assess cell viability, the modified MTT assay protocol was used (van Meerloo, Kaspers, Cloos, 2011). MTT was dissolved in PBS (pH 7.40) at 1 $mg \cdot mL^{-1}$ and filtered to be sterilized. After treatment, cells seeded on the 96-well plates were washed by the PBS two times. Then culture medium (100 μL) and stock MTT solution (10 μL) were added to each well and incubated

at 37ºC for 4 h. Culture medium was removed, 100 µL of DMSO was added to each well and mixed thoroughly to dissolve the dark blue formazan crystals. Optical density (OD) values were measured on a micro-plate reader at a wavelength of 490 nm and the well contained 100 µL pure DMSO was regarded as the control group. The cell relative survival rate was calculated according to the following formula:

$$\text{Cell relative survival rate (\%)} = \frac{[OD]_t}{[OD]_i} \times 100\%.$$

Where $[OD]_t$ is the OD value of the experimental group, and $[OD]_i$ is the OD value of the control group.

Evaluation of cell death by acridine orange/ethidium bromide (AO/EB) staining

To investigate the cell damage mechanism by UVA and visible light irradiation, the treated cells were stained with AO/EB, in which AO stained live and dead cells, while EB only stained cells that lost membrane integrity. Live cells appeared uniformly green, early apoptotic cells stained green and contained bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells incorporated EB and therefore stained orange, but they showed condensed and often fragmented nuclei. Necrotic cells stained orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin (Kasibhatla *et al.*, 2006).

Briefly, the treated cells were washed three times with PBS, and stained with 100 µg·mL^{-1} AO/EB for 5 min in the dark. Cells were then observed under an inverted fluorescence microscope.

Apoptosis detection by flow cytometry

Apoptosis was detected by the Annexin V/PI double staining method (Chen *et al*., 2008). The treated cells were trypsinized, collected with PBS (pH = 7.40), then resuspended at a density of 10^6 cells·mL^{-1} in 200 µL of binding buffer and incubated with 5 µL of Annexin V-fluorescein isothiocyanate (Annexin V-FITC) for 10 min in the dark at room temperature. Subsequently, the cells were resuspended in 200 µL of binding buffer and incubated with 10 µL of PI for 10 min in the dark. Finally, the cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, USA).

Statistical analysis

The data were calculated and analyzed with Excel and the SPSS 22.0 software. Values were presented as mean ± standard deviation (SD). Statistical analysis was performed using the *t*-test, One-Way ANOVA. The *P* value less than 0.05 was considered as to be statistically significant.

RESULTS

Screen the most sensitive cancer cell strain

The effects of PDT after TiO_2/NSCS treatment on the following six types of cancer cell strains were investigated: U251, a glioma cell strain; SiHa, a cervical cancer cell strain; SGC, a gastric cancer cell strain; PC12, a chromosome tumor cell strain; HepG2, a hepatocellular carcinoma cell strain; EC, an embryonic cancer cell strain. Figure 2 showed the concentration curves for cell death after TiO_2/NSCS-PDT. The treatment had a higher impact on these cancer cells and displayed a maximal effect in the UVA light irradiation in the presence of 800 µg·mL^{-1} TiO_2/NSCS. Among the six kinds of cancer cells, the relative survival rate decreased as follows: PC12 cells (91.0% ± 2.2%) > EC cells (84.0% ± 6.2%) > HepG2 cells (63.0% ± 1.8%) > SiHa cells (59.0% ± 3.1%) > SGC cells (54.0% ± 2.7%) > U251 cells (49.0% ± 3.6%). In the presence of the highest TiO_2/NSCS concentration, U251 cells reached the maximal photokilling effect at the same time point. In addition, the PDT effect of TiO_2/NSCS-treated U251 cells was enhanced significantly with the increase of concentration in the UVA light irradiation.

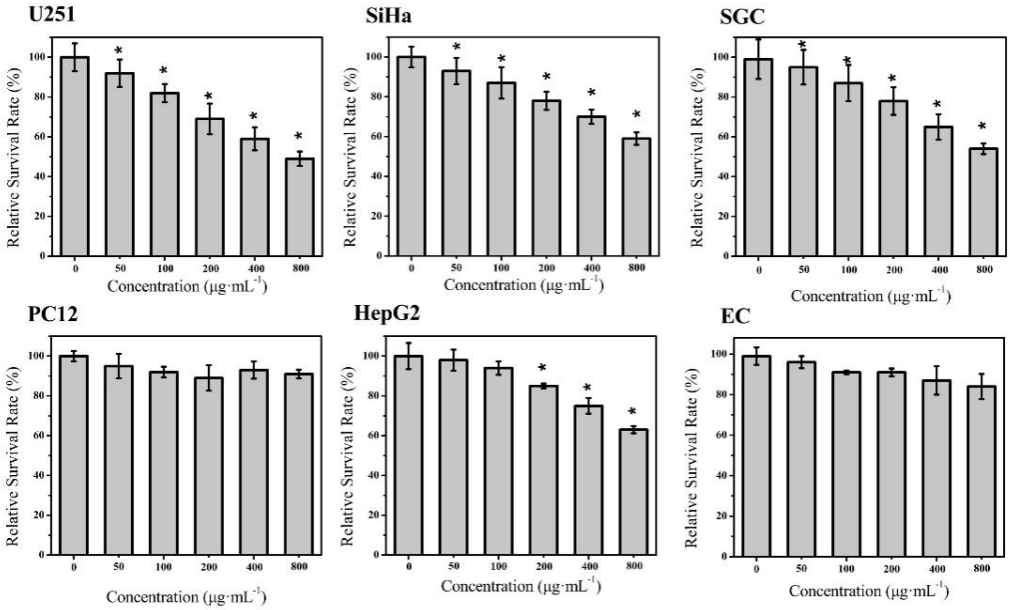

FIGURE 2 - The relative survival rate of six kinds of cancer cells treated with various concentrations of TiO_2/NSCS and the low radiance of UVA light (365 nm) at 1.5 mW·cm^{-2} for 20 min, TiO_2/NSCS concentration at 0, 50, 100, 200, 400 and 800 μg·mL^{-1}. The results are expressed as the mean ± SD (n = 3, *P < 0.05).

Cytotoxicity of TiO_2/NSCS

Here, the cytotoxicity of TiO_2/NSCS was measured by MTT assay. As shown in Figure 3, NSCS-modified TiO_2 showed no significant dark toxicity to U251 cells or HL-7702 cells in a concentration range of 0 - 800 μg·mL^{-1}. However, with the concentration of TiO_2/NSCS increased to 1600 μg·mL^{-1}, it demonstrated the significant dark cytotoxicity to U251 cells.

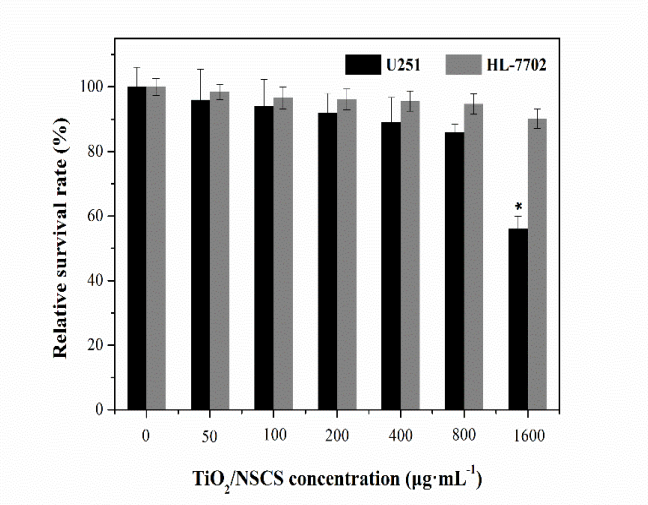

FIGURE 3 - The relative survival rate of U251 cells and HL-7702 cells treated with various concentrations of TiO_2/NSCS at dark, TiO_2/NSCS concentration at 0, 50, 100, 200, 400 800 and 1600 μg·mL^{-1}. The results are expressed as the mean ± SD (n = 3, *P < 0.05).

Photodynamic efficacy *in vitro*

Effect of concentration

The photodynamic effect of TiO_2/NSCS on U251 cells was evaluated, with the effect of different concentrations of PS on the relative survival rate of U251 cells presented in Figure 4A. TiO_2/NSCS and TiO_2 had no obvious toxic effect on U251 cells in the range of 0 - 800 μg·mL^{-1} without light irradiation. However, the phototoxicity obviously increased with the increasing concentration. There was a concentration-dependent photocytotoxic manner. The relative survival rate of U251 cells in TiO_2 group was higher than that in the TiO_2/NSCS group at different concentrations. Compared with TiO_2, TiO_2/NSCS exhibited a significant difference (*$P < 0.05$) at the concentration of 200 μg·mL^{-1} in the UVA light irradiation. Surprisingly, NSCS modified TiO_2 showed a better PDT effect than free TiO_2.

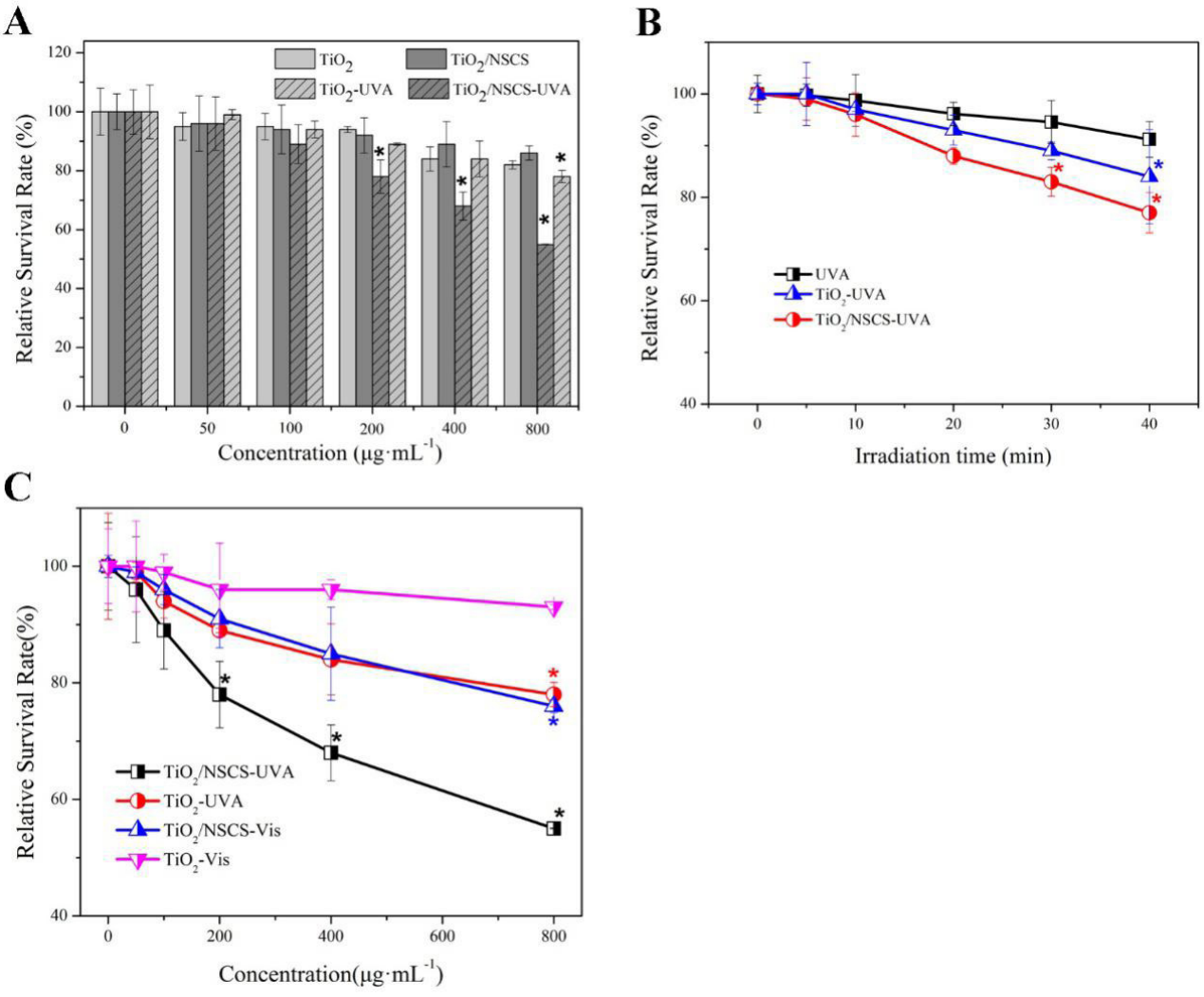

FIGURE 4 - (A) The effect of different concentrations of TiO_2/NSCS or TiO_2 on relative survival rate of U251 cells in the presence and absence of UVA light irradiation with 1.5 mW·cm^{-2} for 20 min, PS concentration at 0, 50, 100, 200, 400 and 800 μg·mL^{-1}; (B) The effect of different irradiation times on relative survival rate of U251 cells irradiated with UVA light for 5, 10, 20, 30 and 40 min with 1.5 mW·cm^{-2}. (C) The effect of different light sources on relative survival rate of U251 cells, under UVA or visible light irradiation with 1.5 mW·cm^{-2} for 20 min. The results are expressed as the mean ± SD ($n = 3$, *$P < 0.05$).

Effect of irradiation time

To investigate the influences of irradiation time on PDT, the effect of 200 $\mu g \cdot mL^{-1}$ PS on cell survival was tested in the UVA light irradiation. As shown in Figure 4B, the cell relative survival rate generally decreased with increasing irradiation time. After 40 min exposure, the relative survival rate of U251 cells was obviously reduced in the presence of TiO_2 and TiO_2/NSCS. In comparison with the untreated and TiO_2-treated cells, the relative survival rate of TiO_2/NSCS-treated cells was lower at any time point. Furthermore, more than 90% of U251 cells survived in the UVA light irradiation alone and did not show any significant differences, which was in agreement with previous report (Feng, Zhang, Lou, 2013). Therefore, we supposed that the inhibition effect was due mainly to the PDT effect, not in the heating process or to any other inhibitory effects.

Effect of light source

The effects of UVA and visible light irradiation on the relative survival rate of U251 cells were shown in Figure 4C. Compared with visible light, for TiO_2 group, UVA light had a more significant effect on photokilling efficiency, which was consistent with the fact that UVA light is necessary for TiO_2 photokilling cancer cells. However, the photokilling effect of TiO_2/NSCS obviously increased with the increasing concentration, with the relative survival rate of U251 cells remarkably reduced ($^*P < 0.05$) by about 40%, 30% after 20 min of UVA and visible light irradiation at its concentration of 800 $\mu g \cdot mL^{-1}$, respectively. It was suggested that NSCS modified TiO_2 made its light response range expanding from UVA to visible region (Wang, Li, Zhang, 2016).

Evaluation on cell death by AO/EB staining

To observe the PDT efficacy of PS at different concentrations, the treated U251 cells were stained by AO/EB. As could be clearly seen in Figure 5A, without PDT treatment, almost all cells showed green fluorescence, which was due to AO bound to DNA of normal cells nucleus and cells maintaining normal nucleus appearance (Cheng *et al.*, 2017). After TiO_2/NSCS-PDT treatment, U251 cells presented orange fluorescence due to loss of membrane integrity and condensed and fragmented chromatin (Figure 5E), suggesting that most cells were at a late stage of apoptosis. With the concentration increasing to 800 $\mu g \cdot mL^{-1}$, initial apoptosis was observed in U251 cells (Figure 5G), which presented intact membranes, and contained bright green dots in the nuclei and green fluorescence with condensed chromatin and nuclear fragmentation, with orange fluorescence observed in most of the cell nucleus. Under the same conditions, apoptosis of U251 cells in TiO_2/NSCS groups was more significant than that in TiO_2 groups (Figure 5D, F).

Fluorescent images of U251 cells irradiated with different light sources were shown in Figure 5. Compared with control cells (Figure 5A), the nuclei of cells treated with TiO_2 and TiO_2/NSCS in the dark did not show any apparent morphological changes (Figure 5B, C). However, orange fluorescence was observed after U251 cells treated with TiO_2/NSCS under UVA light, an indication that cells were in the late stages of apoptosis (Figure 5I), as cells lost the membrane integrity at this stage, thus allowing EB to enter and stain the DNA (Maity *et al.*, 2012). By contrast, minority of U251 cells underwent apoptosis after treatment with TiO_2 under UVA light (Figure 5H).

Treatment of U251 cells in the visible light irradiation caused chromatin condensation in the nucleus, followed by the formation of apoptotic bodies manifested as bright green plaques, indicating the early stages of apoptosis. At the same time, late apoptosis was observed in U251 cells, showing the significant photocytotoxic effect of TiO_2/NSCS (Figure 5K). In the case of TiO_2, there was no change in the nuclear morphology in visible light irradiation (Figure 5J).

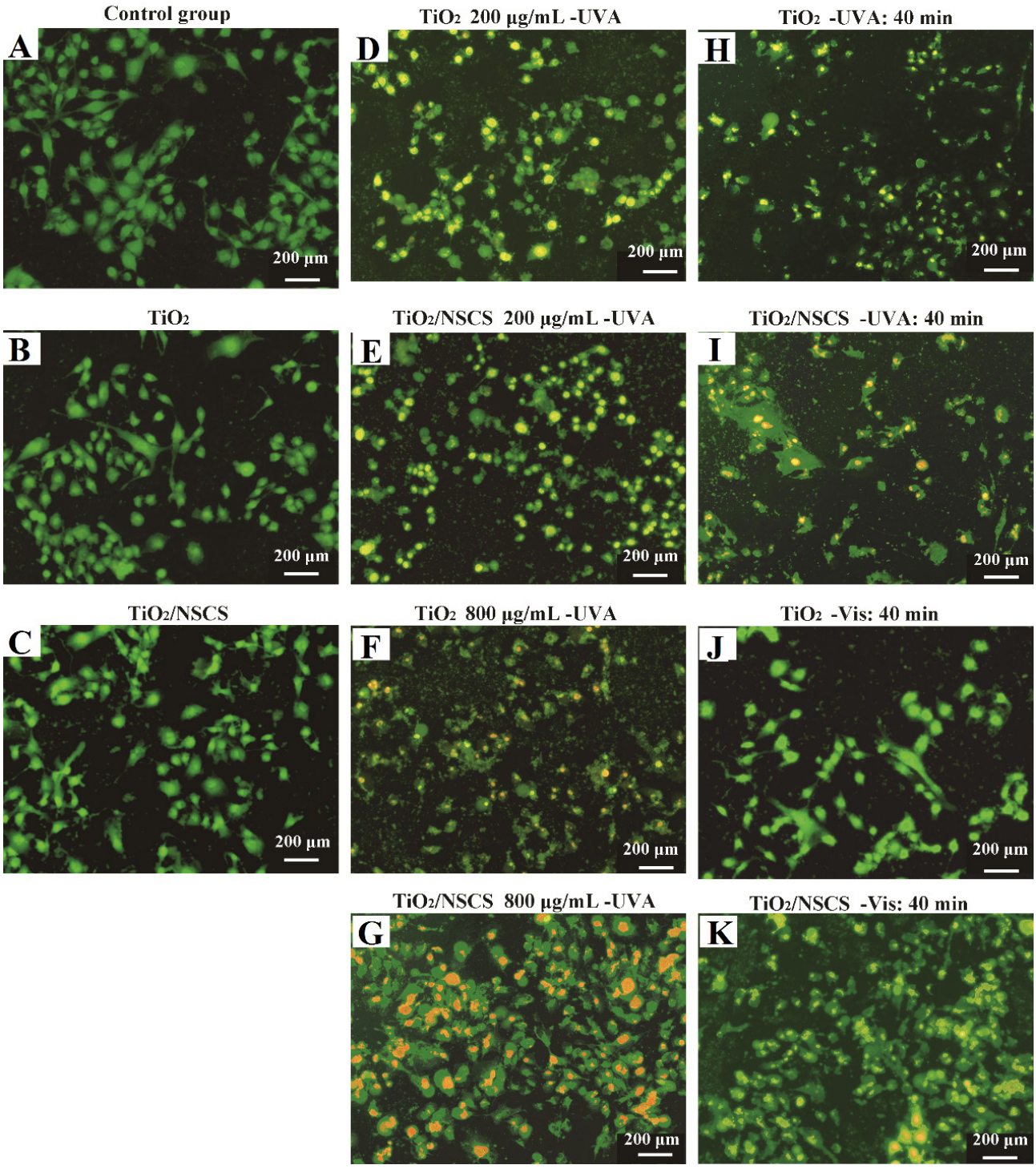

FIGURE 5 - The effect of different concentrations of PS and light sources on fluorescent image of U251 cells. AO/EB staining of U251 cells after treatment: (A) negative control, and treated with (B) 400 μg·mL^{-1} TiO_2, (C) 400 μg·mL^{-1} TiO_2/NSCS, (D-G) UVA light (1.5 mW·cm^{-2}) of 365 nm for 20 min, PS concentration at 200 and 800 μg·mL^{-1}, (H-I) UVA light at 365 nm for 40 min, PS of 400 μg·mL^{-1}, (G-K) visible light at 420 nm, irradiated (1.5 mW·cm^{-2}) for 40 min, PS of 400 μg·mL^{-1}, cells were separated with the medium (× 2.52).

Evaluation on cell death by Annexin V/PI staining

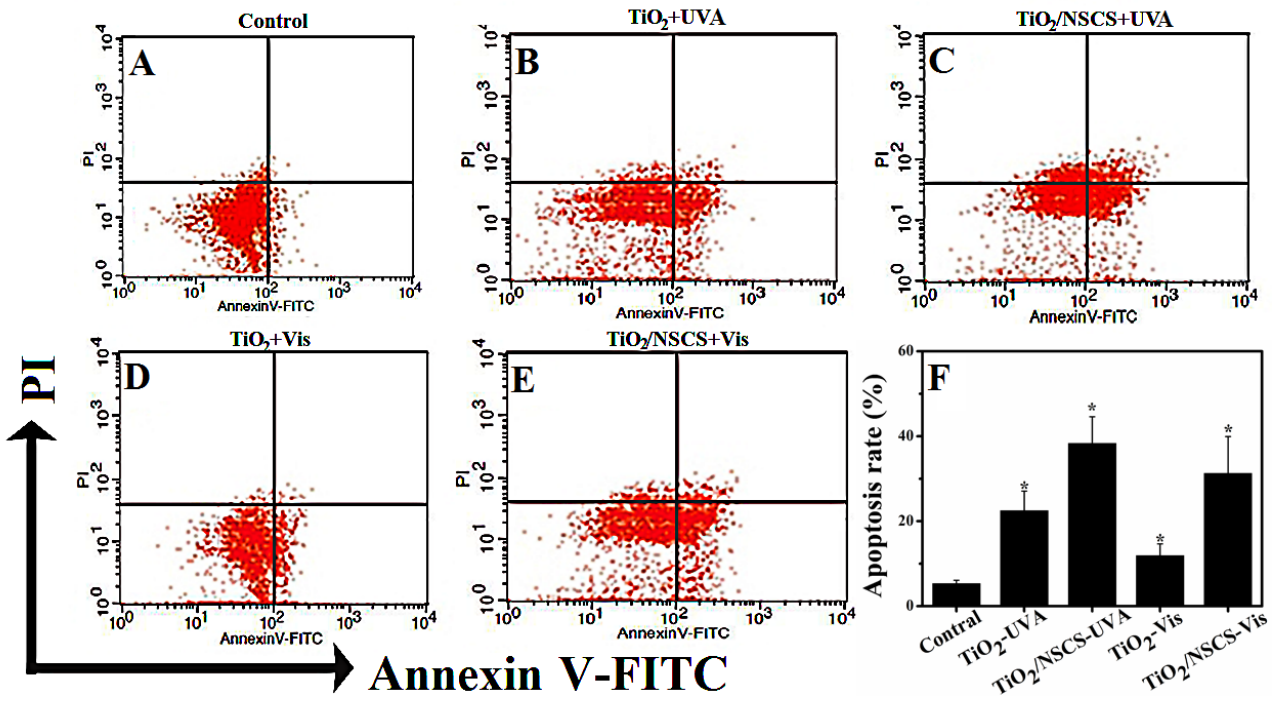

FIGURE 6 - Apoptotic analysis by Annexin V/PI double staining in U251 cells after treatment: (A) control, and treated with PS of 400 μg·mL^{-1} after (B-C) UVA light at 365 nm, (D-E) visible light at 420 nm, irradiated (1.5 mW·cm^{-2}) for 40 min. (F) The effects of PS under light irradiation on the apoptosis rate of U251 cells. The results are expressed as the mean ± SD (n = 3, *P < 0.05).

The apoptosis of U251 cells induced by different treatments was assayed by Annexin V/PI double staining *via* flow cytometry. As shown in Figure 6, less apoptosis cells were observed in the control group and TiO_2 treatment group in visible light irradiation. Moreover, the TiO_2 treatment group with UVA light irradiation exhibited 22.48% apoptosis, while TiO_2/NSCS treatment group in UVA and visible light irradiation significantly increased the apoptotic rate of U251 cells to 38.29% and 31.23%, respectively (*P < 0.05).

DISCUSSION

It has been reported that light-induced TiO_2 or modified TiO_2 causes many cancer cells death by the cytotoxic effect of PDT (Rozhkova *et al.*, 2009; Zhang, Sun, 2004; Zhang, Shan, Dong, 2014; Pan *et al.*, 2017; Shang *et al.*, 2017). In this case, we considered it necessary to confirm whether TiO_2/NSCS cytocidal changes occurred in various types of cancer cells. Therefore, the effects of PDT after TiO_2/NSCS treatment on the six types of cancer cell strains were investigated. In this study, we have demonstrated that TiO_2/NSCS-PDT induced massive cells death in six cancer cells displayed different invasive properties, especially for U251 cells. Therefore, the most sensitivity to TiO_2/NSCS was observed in U251 cells and used as a model for TiO_2/NSCS-PDT.

Biosafety evaluation is critical in assessing the potential application of TiO_2/NSCS in clinics. Recent studies have shown that the cell compatibility of TiO_2 nanoparticles and NSCS are very good (Li *et al.*, 2013; Wang *et al.*, 2018). According to our obtained results, it is clear that NSCS modified TiO_2 does not show any significant dark toxicity to normal cells (HL-7702), which proves that TiO_2/NSCS is cytocompatible. Furthermore,

the cytotoxicity of TiO_2/NSCS to cancer cells (U251) is negligible in the absence of light experiments in the concentration range of 0 - 800 $\mu g \cdot mL^{-1}$, while it showed significant dark cytotoxicity at the concentration of 1600 $\mu g \cdot mL^{-1}$. These effects might be recommended by the nano size structure of nanocomposites. The tiny particle size of TiO_2/NSCS facilitated cellular penetration at the highest concentration, which might damage the cell membrane or accumulate in the U251 cells to cause necrosis (Sarin *et al*., 2008). The TiO_2/NSCS without any light irradiation had a small effect on cell viability over a wide range of concentrations, which indicated low dark toxicity, thus ensuring its large potential range of applications in the field of cancer PDT. Therefore, the concentration of TiO_2/NSCS between 0 and 800 $\mu g \cdot mL^{-1}$ was used in further PDT experiments *in vitro*.

In this experiment, phototoxicity increased significantly with the concentration increasing, and there was a concentration-dependent photocytotoxic manner, with the results that TiO_2/NSCS nanoparticles had a better photodynamic performance in UVA light irradiation than that of pure TiO_2. The differences between pure TiO_2 and TiO_2/NSCS might be mainly attributed to nanoparticles aggregation, which affected their cytosolic distribution, with pure TiO_2 presenting high aggregation, conversely, TiO_2/NSCS presented less aggregation. Aggregation of pure TiO_2 could be internalized by the endocytic pathway, resulting in their concentration in small vacuolar intracellular regions, while modified TiO_2 by NSCS avoided TiO_2 aggregation and allowed them to be evenly distributed throughout the cytoplasm. In addition, TiO_2/NSCS could protect TiO_2 from media interference (Tada *et al*., 2014; Tada *et al*., 2010).

As shown by transmission electron microscope and zeta potential results, the average particle diameter of TiO_2/NSCS was 50 nm and the surface charge + 39 mV. It had been reported that nanoparticles with surface charge of + 30 mV had been shown to be stable due to the fact the surface charge was sufficient to prevent aggregation of the particles (Ing *et al*., 2012; Wang, Li, Zhang, 2016). The results of this study were in accordance with the work by Venkatasubbu and collaborators (Venkatasubbu *et al*., 2013).

In the other experiment, we consider the influences of irradiation time on PDT efficacy. The results showed that TiO_2/NSCS killed U251 cells in a time-dependent manner.

Our results demonstrated that compared with TiO_2, the PDT activity of NSCS modified TiO_2 was obviously improved, especially the high photokilling efficiency under visible light. These results strongly suggested that TiO_2/NSCS might be a novel PS for PDT, because visible light-induced U251 cell damage was significantly enhanced *in vitro* compared to TiO_2. According to the study, excitation of TiO_2/NSCS could also be achieved using other wavelengths with visible light which is no detrimental, lest tissues burned and mutations exposed to UVA light. This clearly demonstrated that NSCS modification played a certain role in improving the photocatalytic and PDT activities of the TiO_2 nanostructures. Therefore, visible light could be selected as its excitation light source.

After light exposure, PDT can induce cell death *via* apoptosis and/or necrosis (Feuser *et al*., 2016). The morphological changes of U251 cells clearly indicated that visible light-irradiation TiO_2/NSCS could induce U251 cells apoptosis and lead to their damage, consistent with the enhanced PDT activity of TiO_2/NSCS. Simultaneously, the results of Annexin V/PI double staining suggested that TiO_2/NSCS in the UVA and visible light irradiation could kill U251 cells by inducing apoptosis. This study's results were similar to the work by Maity and collaborators (Maity *et al*., 2012) whose PDT was used to induce cell death by apoptosis in U251 cells. Moreover, the cell death by apoptosis was observed in a variety of tumor cell strains after PDT application with different PS (Siqueira-Moura *et al*., 2013). Apoptosis is an intrinsic physiological activity, which can also be triggered by external stimuli such as oxidative stress attributable to PDT. In the presence of oxygen, light absorption of PS triggers photophysical and in biological processes form ROS, which damages cellular components and causes cell death by apoptosis (Xu *et al*., 2015). Our results suggest that TiO_2/NSCS and low radiance visible light could effectively induce cell death by apoptosis (Feuser *et al*., 2016).

CONCLUSIONS

In this paper, the U251 cell strain was screened as the most sensitive cancer cell strain to TiO_2/NSCS. Furthermore, it reported TiO_2/NSCS-PDT against U251

cells *in vitro*. The cell viability assay confirmed that TiO_2/NSCS showed less dark toxicity without irradiation, but their photokilling effect was significantly higher than that of TiO_2 in the UVA and visible light irradiation. The photokilling effect of TiO_2/NSCS was positively correlated with concentration of TiO_2/NSCS and irradiation time. The results of microscopic fluorescent images and flow cytometry showed that TiO_2/NSCS could kill U251 cells by inducing apoptosis in the UVA and visible light irradiation. Therefore, TiO_2/NSCS composite can be used as a promising drug for anticancer PDT, with our work providing a new idea for anticancer therapy.

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