Sonodynamic effect in A375 melanoma cells with chlorin e6 induced by 20 kHz ultrasound

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Abstract
In this research, combined effect of chlorin e6 and noninvasive 20 kHz, low-powered ultrasound on melanoma cells is proposed for the first time. The proposed combination incorporates the application of safe low-frequency ultrasound that is used for transdermal drug delivery. We demonstrate that impact of the 20 kHz ultrasound on chlorin e6 leads to strong sonodynamic effect in melanoma cells. We also show that chlorin e6 keeps its monomeric form in the cells and does not aggregate after exposure to ultrasound. We propose a two-step model where sonoluminescence serves as an internal light source for the chlorin e6 photoactivation.

Keywords: sonodynamic therapy, cancer, chlorin e6, ultrasound

(Some figures may appear in colour only in the online journal)

1. Introduction
Currently, the spread of cancer is rampant, and the search for new methods for the effective treatment of cancer is a socially significant challenge. Several traditional ways are known to treat cancer, such as chemotherapy, surgical removal, hormone therapy, radiation therapy; each of them has reported side effects [1–3]. At the same time, advanced methods of treating oncological diseases are developing. For example, chemodynamic therapy, photoacoustic therapy, and Plasmonic Photothermal Therapy using biocompatible nanoparticles [4]. Photodynamic therapy (PDT) is one of the most sparing and at the same time effective methods for the diagnosis and treatment of cancer [5–7]. PDT is based on the generation of reactive oxygen species (ROS) by photoexcitation of photosensitizer molecules. The distinctive ability of photosensitizer is its accumulation mostly in the tumor tissues. Once exposed to visible light, a photosensitizer molecule transfers into an excited state. The collision of an excited photosensitizer with surrounding medium leads to the generation of ROS [8, 9]. ROS is capable of triggering the mechanisms of cancer cells apoptosis or necrosis [10, 11]. Tetrpyrrole molecules can be considered as an ideal material to develop an approach for cancer visualization and treatment. This possibility exists as tetrpyrrole molecules as a photosensitizer selectively accumulate in cancer tissues [12, 13] and efficiently generate singlet oxygen (SO), which can be produced by transferring tetrpyrrole to its excited state. Despite the advantages of PDT over traditional cancer therapy methods, it possesses a significant drawback that limits its wide application in clinical practice. Biological tissues strongly absorb visible radiation, which is necessary for transferring the sensitizer to an excited state. This fact reduces the application of this method exclusively to the treatment of epithelial forms of cancer and small tumors [14].

Thus, several approaches to overcome this hurdle are now being considered. The first is to reveal a way to create an internal source for photoexcitation of the tetrpyrrole molecule.
The second one is to find an alternative way to transfer the tetrapyrrole molecule to a triplet state, so that it can generate ROS. Some research groups have recently demonstrated that ultrasound (US) at frequencies of 0.88–3 MHz and intensities of 0.1–2.4 W cm\(^{-2}\) can activate traditional photosensitizers, like tetrapyrrole molecules or titania nanoparticles, in cancer cells [17–19]. It has opened an avenue to a new non-invasive and extremely efficient approach called sonodynamic therapy (SDT) for cancer treatment, especially for inhomogeneous, large and deeply localized tumors. SDT effect is more complex as it results from the combination of different mechanisms. Currently, there are two most likely mechanisms to explain the sonodynamic effect on cancer cells, i.e. cavitation and ROS generation (see figure 1). Cavitation is the process of formation and collapse of bubbles producing colossal energy; the released energy is converted into chemical or mechanical forms, which leads to cell destruction [20]. It has been shown that the use of nanoparticles, or their aggregates at appropriate amount and size, causes an increase in the yield of the sonochemical reaction. In this case, the addition of nanoparticles can increase the number of cavitation bubbles, thus enhancing the yield of the sonochemical reaction for US frequencies at 20 kHz and higher [21, 22]. The mechanism of ROS generation implies the interaction of US with sonosensitizers. US can produce sonoluminescence (SL) that can serve as an internal light source to excite sensitizer species [23–25]. Recently, it has been demonstrated that traditional photosensitizers exposed to US with a frequency of 1.866 MHz and intensity of 1.5 W cm\(^{-2}\) can lead to strong apoptotic and necrotic effects in cancer cells [26]. Despite obvious benefits, like deep penetration in the human body, SDT has some side effects because the US frequencies used in SDT can destroy normal cells and tissues [27]. At the same time, the treatments by low-intensity US at frequency of 20–100 kHz, which have no damage to human tissues, are used for transdermal drug delivery in a wide range of diseases [28–32]. At very high power, 20 kHz ultrasound is used for food processing and wastewater treatment [33, 34]. In addition, the penetration depth of US at a frequency of 20 kHz is much greater than that of US with a frequency of 1 MHz. Mitragotri studied the influence of 20 kHz on the integrity of the epidermis and other living tissues and showed that no cell damage has been detected [35]. In fact, 20 kHz US allows to increase the concentration of drugs in cells and tissues without triggering the mechanisms of cells apoptosis or necrosis [36]. To the best of our knowledge, low-intensity 20 kHz US has never been studied in SDT applications up to now. It is surprising because it is a well-known fact that low-intensity 20 kHz US can generate SL [37, 38]. Therefore, we hypothesized that low-frequency US can be potentially used as an internal energy
source to excite molecular and nanostructured sensitizers in cancer cells and tumors. We, therefore, study the impact of low-intensity 20 kHz US on a photosensitizer in melanoma cells. The second-generation photosensitizer chlorin e6 (Ce6) was applied as a sensitiser. Ce6 is actively used as an effective drug for PDT of cancer due to its ability to generate SO \(\text{SO}_x\) \[39–41\]. Unlike other tetrapyrrrole molecules, Ce6 is not toxic to the body \[42\]. Moreover, Ce6 is quickly removed from healthy tissues in comparison to other photosensitizers \[43–47\]. We demonstrate here that monomers of Ce6 loaded into melanoma cells can be efficiently activated by 20 kHz US that triggers the destruction of melanoma cells. We also show that short-time treatment of melanoma cells with Ce6 by 20 kHz US obtains the SDT effect that is comparable with the PDT effect for the same Ce6 concentration and the same incident energy dose. The difference between the efficiency of SDT and PDT may be related to the limited penetration depth of light as a result of PDT. This is the first demonstration of the efficacy of 20 kHz US in SDT.

2. Material and methods

2.1. Chemical reagents

Dimethyl sulfoxide (DMSO) and phosphate buffer solution (PBS) were purchased from Sigma-Aldrich Inc. RPMI-1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, L-glutamine, HEPES, Nystatin and sodium pyruvate were purchased from Biological Industries. Presto Blue assay was purchased from Fisher Scientific. Chlorin e6 (Ce6) was purchased from Frontier Scientific (USA). Bi-distilled water was used throughout the experiments. All reagents were used without further purification.

2.2. Preparation of Chlorin e6 solution

Ce6 powder was dissolved in PBS solution at a concentration of \(2 \times 10^{-5}\) M.

2.3. Cell culture

Melanoma A375 was used as a cell line. RPMI-1640 was used as a nutrient medium, containing 10% FBS, 1% penicillin–streptomycin, 1% L-glutamine, 0.175% HEPES, 0.075% Nystatin, and 1% sodium pyruvate. The cells were kept in an incubator (5% CO\(_2\)) and every 2 days were washed twice with PBS solution, and a new nutrient medium was added. Ce6 (dissolved in PBS) (5% of medium volume) were added to cells in RPMI-1640 medium, so each plate’s units contained (950 \(\mu\)L) medium and 50 \(\mu\)L Ce6 solutions with concentration \(2 \times 10^{-5}\) M. Ce6 concentration in the medium was kept \(10^{-5}\) M for all experiments.

2.4. Cell viability assessment

Presto Blue test assay (Fisher Scientific) was used as a test for determining the viability of cells. Cell’s viability is assessed before and after cells exposure to light or US. Presto Blue is a resazurin-based chemical sensor. The addition of resazurin to the cells leads to modification of the reagent by the reducing medium of viable cells. The reagent turns red and develops strong sensor photoluminescence (PL). This color change can be detected by measuring fluorescence or absorption spectra. Presto Blue was added to the cells seeded on the plates in the amount of 10% of the total nutrient medium, then plates were kept in the incubator for 10 min. The number of living cells was estimated by measurement of PL intensity of Presto Blue using Infinite M200 (Tecan) spectrophotometer.

2.5. Spectroscopic measurements

Absorption, PL and PL excitation spectra of Ce6 molecules in cells were measured and analyzed before and after exposure to 20 kHz US. The measurements were performed on multifunctional microplate reader Tecan Infinite M200.

2.6. Formation of Ce6 aggregates

To obtain solutions with the monomeric form and aggregates of Ce6, pure PBS solution and PBS diluted in bidistilled water with the ratio of 1:500 were prepared. Concentration of Ce6 was \(2 \times 10^{-5}\) M in each solution. Ce6 dissolved in PBS at a concentration of \(2 \times 10^{-5}\) M was used as a reference sample.

2.7. PDT test

For the photodynamic test, A-375 melanoma cells were seeded in a 12-well plate \((4 \times 10^5\) cells/well). After 24 h, the cells were washed twice with PBS (pH = 7.4) and a Ce6 solution at a concentration of \(2 \times 10^{-5}\) M (in PBS) was added to the cells in the amount of 5% of the total volume of the nutrient medium. After 24 h of incubation at a temperature of 37°C (with 5% CO\(_2\)), the cells were washed three times with a solution of PBS and nutrient medium was added. This protocol of washing melanoma cells guarantees that all Ce6 molecules that did not penetrate into the cells were washed off with PBS from the plate walls or the cell surface. We used 650 nm LED with an intensity of 7 mW cm\(^{-2}\) to study PDT on A375 cell line. The cells for PDT experiments were incubated with Ce6 for 4, 24 and 72 h, and then subsequently irradiated by the LED for 117 s. An estimation of PDT effect, produced by Ce6 cells, was performed as a ratio of cell viability before and after irradiation of samples. Cells incubated with Ce6 at the same conditions but without LED irradiation were used as a control for the PDT effect evaluation. Cells without Ce6 irradiated by the LED at the same conditions were used as control of light influence on cell viability. We also checked the viability of the cells grown at the same conditions with light exposure and Ce6 as a control of viability of the cell line. Cells were moved to an incubator 1 h after light exposure, then cells were washed three times with PBS, filled with fresh medium and their viability was checked using Presto Blue test.
2.8. SDT test

Samples were prepared in the same way as PDT test. Ultrasonic Q-SONICA system (Q-700, Newtown, CT, USA) with microplate horn (MPH) was used in SDT test of A375 cell line. The US exposure time was 10 s (10% amplitude). The intensity of US was determined with the calorimetric method (0.083 W cm⁻² per well). MPH is an indirect sonication device capable of processing an entire 12-well plate. MPH was fill up the water, and the 12-well plate sank to the surface of the water. After that, sonication was performed on the A375 cells. Estimation of SDT effect of Ce6 on cells was performed in the same way as PDT test, i.e. as ratio of cell viability before and after the action of US on samples.

2.9. Statistical analysis

All data were expressed as mean ± standard deviation. The statistical analyses were performed using GraphPad Prism 9 software, by using one-way analysis of variance. Asterisk (*), sharp (#) and ampersand (#) denote statistical significance between bars (p < 0.05, p < 0.01, p < 0.001, p < 0.0001).

3. Results and discussion

3.1. SDT test on A375 cells

Figure 2 shows the viability of A375 cells incubated with Ce6 (10⁻⁶ M) for 4, 24 and 72 h after exposure to 20 kHz US with power 0.8 W for 10 s (for details see the section 2.8 at section 2). The US energy incident on the sample was calculated as 8 J. According to data presented in figure 2, exposure of 20 kHz US on A375 cells incubated with Ce6 leads to more than 25% cells death, demonstrating for the first time that combining Ce6 with the low-frequency US can produce SDT effect. It is well known that 20 kHz ultrasound is used to increase the intracellular concentration of drugs, but in our case, the accumulation of Ce6 in cells does not depend on sonication. Cells, after incubation with Ce6 molecules, were thoroughly washed several times (for details see section 2.7). Accordingly, there is no mechanism for an increase in the intracellular concentration of Ce6 due to ultrasound. The works presented in literature have been discussing the causes of the [48, 49] sonodynamic effect only as a result of exposure to therapeutic US (1–3 MHz, 0.5–3 W cm⁻²).

Figure 2 clearly demonstrates that Ce6 does not show any cytotoxicity effect on A375 melanoma cells. At the same time, the situation changes when cells incubated with the same amount of Ce6 were exposed to US. The US treatment of A375 melanoma cells with Ce6 molecules results in the death of the cell, while the US irradiation only on A375 melanoma cells shows no effect. It is of interest to find out the mechanism that is responsible for the appearance of the sonodynamic effect. Previously, we discussed two mechanisms that can lead to the destruction of cancer cells [50, 51]. The first mechanism is cavitation and the use of aggregates of Ce6 molecules to enhance the cavitation effect that leads to the destruction of cancerous cells [52, 53]. This mechanism has not been demonstrated for molecules in monomeric form. The presence of aggregates of Ce6 molecules can enhance this cavitation effect, leading to some thermal or chemical local effects that destroy the cells due to cavitation. In this case, the aggregates of molecules are used as centers of cavitation [54]. The second mechanism is based on SO generation by the Ce6 molecules under US exposure. It requires the presence of molecules in monomeric form because their aggregates are usually less efficient SO generator [55].

Therefore, the main SDT mechanism is not clear and can be elucidated relying on the Ce6 intracellular form (monomers or aggregates) in cancer cells. The presence of monomers or aggregates of molecules can be assessed by the traditional PL and PL excitation spectra [56]. If Ce6 molecules take the aggregate form, the main sonodynamic mechanism is cavitation. If Ce6 molecules take the monomeric form, the main sonodynamic mechanism is Ce6 molecules SO generation by SL.

3.2. Chlorin e6 optical properties in melanoma cells

PDT effect of tetrapyrrole molecules strongly depends on their SO generation efficiency. It is well known that aggregation of tetrapyrrole molecules leads to the loss of their ability to generate SO because of the increase of non-radiative rate in tetrapyrrole aggregates [57, 58]. Ce6 is a tetrapyrrrole molecule and its optical properties are well studied in water solution at different pH levels, DMSO and PBS [59, 60]. At the same time, there are no data on absorption and PL spectra of Ce6 in any cancer cells. Therefore, we have examined PL and PL excitation spectra of Ce6 in A375 melanoma cells. According to the spectra, we can evaluate the state of our molecules directly in the cancer cells. All measurements were performed in 12-well plates (for details see the section 2.5 in section 2).
Figure 3. (a) PL excitation spectra of Ce6 in bare RPMI medium (black curve) and Ce6 in cells (red curve). PL excitation spectra are normalized by maximum of first absorption band of Ce6. (b) Normalized PL spectra of Ce6 in bare RPMI medium (black curve) and Ce6 in cells with RPMI (red curve). PL excitation wavelength is 405 nm, PL registration wavelength is 760 nm.

Figure 3 shows the PL and PL excitation spectra of Ce6 in a RPMI nutrient medium and inside the cancer cells.

Figure 3 shows the Ce6 PL excitation and PL spectra, which confirms the monomeric form of Ce6, because of the position of the Ce6 PL band at 676 nm, the Q(IV) absorption band at 500 nm, and the position of the Q(I) band at 670 nm. It should also be noted that the Ce6 monomer in A375 cells demonstrates the ‘reddest’ position of its PL band in comparison with RPMI medium and PBS solutions, representing a sample in a strong alkaline environment that is not characteristic of cancer cells [61]. We believe that Ce6 accumulates in mitochondria, and this allows the Ce6 to remain in its monomeric form [62, 63]. Also, in the section A of supplementary materials (available online at stacks.iop.org/JPD/55/045402/mmedia), the stability of Ce6 monomers was shown under the action of 20 kHz US. Consequently, US of this frequency and power, does not lead to Ce6 aggregation. According to the results, Ce6 molecules do not aggregate in cancer cells with low pH or when exposed to US. It was previously shown (in figure 2) that US at this intensity and frequency itself does not lead to the destruction of cancer cells, for which also Ce6 molecules are needed. It means that the SDT mechanism due to the formation of a cavitation nucleus and Ce6 aggregation is unlikely in our case.

3.3. SDT vs PDT efficiency in melanoma cells incubated with Ce6 molecules

Considering the second SDT mechanism, which leads to the generation of SO, by the combined action of US and photosensitizer molecules. In this model the SDT effect is due to the SO generation by photosensitizers, which is a two-step process. At the first step, US produces cavitation effect in cells’ medium or in cells, that can produce SL characterized with a wide band in visible spectral range (400–1100 nm) [64]. At the next step, this light can be absorbed by photosensitizer, i.e. Ce6 in our case, transferring it to $T_1$ state [65]. Thus, US is used to obtain SL, which serves as a source, similar to visible light. It is well documented that US at a frequency of 20 kHz is capable to generate a SL [66]. We assume that SL is formed under the action of US, then the efficiency of the photodynamic effect and the sonodynamic effect at the same energies should be similar. Comparison between the impact of US and light on A375 cells loaded with Ce6 is shown below (figure 4). The photodynamic test is used as a reference experiment to confirm that Ce6 can efficiently generate SO in cancer cells. The viability of cells incubated with Ce6 under exposed by light is shown in figure 4.

Viability data presented in figure 4 confirms PDT effect on A375 cells that has led to 19%, 22% and 23% decrease in cells viability with incubation time of 4, 24 and 72 h, respectively.
As clearly seen in figure 4, Ce6 does not demonstrate any toxic effect on A375 cells after 24 h of incubation. At the same time, the situation is dramatically changed when cells incubated with the same amount of Ce6 are irradiated by 650 nm LED. It is not surprising effect because Ce6 is a second-generation PDT drug that is widely used in the clinic [67]. Thus, it becomes clear that under the influence of light, Ce6 behaves as a traditional photosensitizer. We suggest that the increased time of incubation enhanced the Ce6 intracellular concentration, which cause the increase in PDT effect. Figure 5 shows the dependence of PDT and SDT efficiency on the incubation time of cells with Ce6 and with the same incident energy per sample.

Figure 5 compares PDT and SDT tests with the same incident energy on the sample. According to the results, the efficiency of photodynamic and sonodynamic tests increases with the increase in incubation time of cells with Ce6. In addition, it is clearly seen that an increase in the incubation time leads to an increase in the difference between PDT and SDT effects. At the same time, exposure to US in combination with Ce6 leads to greater cell death rate than exposure to light with Ce6. It is the first demonstration of the SDT effect with these configurations and further research is needed to clarify the observed effect. If we assume the second mechanism of SDT is active, then the sonodynamic effect can be associated with the formation of SL microbubbles inside the nutrient medium [68]. Probably, Ce6 excitation arose from chaotic microbubbles formation is more efficient than exposure to external light. SL can appear at the temperatures from 20°C and higher at the frequency 20 kHz [69]. In those conditions, SL will be observed at US power density of about 0.09–0.2 W cm⁻² [70]. The presented literary parameters are consistent with our experimental parameters, so we assume that SL can occur in our case. K Yasui showed the appearance of SL from microbubbles at an ultrasonic frequency of 20–30 kHz [71]. In that work, the author calculated the energy of the light emitted during collapse of a single bubble in water to be is 7.2 pJ. At the same time, the energy of LED light absorbed by Ce6 molecules in cells equals 16 nJ (details are discussed in the section C of supplementary materials). Kazachek showed that at an US frequency of 20 kHz, about 10⁵ sonoluminescent microbubbles can appear during one US period (50 µs) [72]. An increase in the time of US exposure can lead to a greater number of sonoluminescent bubbles. Our results demonstrate that the combination of 20 kHz US with Ce6 leads to SDT effect probably due to the generation of SO by Ce6. We suggest that cavitation in the nutrient medium leads to the SL, which in its turn serves as a local internal light source for photoexcitation of Ce6 molecules. SDT is comparable to PDT in our conditions and indicates the perspective for the development of this area and the importance of obtaining the results.

4. Conclusion

Optical properties of Ce6 and effect of US and visible light have been studied. The impact of low-frequency US on the monomeric form of Ce6 molecules did not lead to structural changes in the Ce6 molecule. The combined effect of 20 kHz US and Ce6 on melanoma cells leads to a pronounced SDT effect. The mechanism of SDT in A375 melanoma cells is most likely based on photoexcitation of Ce6 in resulting the absorption of light by SL. The SL can originate from cavitation in the nutrient medium or cells.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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