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Enhancement of single-walled carbon nanotube accumulation in glioma cells exposed to low-strength electric field: Promising approach in cancer nanotherapy

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ABSTRACT

The objective of the study is to determine the patterns of regulation of single-walled carbon nanotube accumulation, distribution, and agglomeration in glioma cells exposed to an external electric field. C6 glioma cells were treated with 5 μg/ml DNA wrapped single-walled carbon nanotubes and exposed to bi-phasic electric pulses (6.6 V/m, 200 Hz, pulse duration 1 ms). Nanotube accumulation was determined by Raman microspectroscopy and their intracellular local concentration was evaluated using the G-band intensity in Raman spectra of single-walled carbon nanotubes. It was revealed that the low-frequency and low-strength electric field stimulation of glioma cells exposed to single-walled carbon nanotubes led to facilitation and, thus, to amplification of nanotube accumulation inside the cells. The number of nanotubes in intracellular agglomerates increased from (28.8 ± 13.1) un./agglom. and (84.0 ± 28.7) un./agglom. in control samples to (60.6 ± 21.4) un./agglom. and (184.2 ± 53.4) un./agglom. for 1 h and 2 h stimulation, respectively. Thus, the tumor exposure to an external electric field makes it possible to more effectively regulate the accumulation and distribution of carbon nanotubes inside glioma cells allowing to reduce the applied therapeutic doses of carbon nanomaterial delivered anticancer drugs.

1. Introduction

Carbon nanomaterials have great therapeutic potential in the field of antitumor therapy. Among a wide class of nanomaterials, a special place belongs to carbon nanotubes (CNTs).

CNTs are promising materials for nanopharmacology as nano-carriers of anticancer drugs including doxorubicin (DOX) and cisplatin [1,2]. They were shown to reduce toxicity, extend drug half-life, and retain their activity and stability in biological environments [1–3].

Owing to the high intensity of the Raman scattering of single- and double-walled CNTs, they can be easily detected with confocal Raman spectroscopy. It has been shown that Raman spectroscopy allows (i) visualizing the distribution of CNTs in living cells [4] (ii) tracking the dynamic of their accumulation [5], and (iii) determination of the local concentrations of CNTs in cells [6].

The elongated shape of CNTs promotes them to penetrate cell membranes. Functionalization of CNTs with different groups as well as their conjugation with various biomacromolecules allows them to be rendered hydrophobic/hydrophilic, thus providing the redistribution of CNTs between various organelles within cells or their selective intracellular accumulation [6]. On the other hand, CNTs of various types with various surface modifications can have a cytotoxic [7] or, on the contrary, activating effect on cells [8].

In cancer therapy, the usage of an external electric field (EF) is usually aimed at the destruction of cells due to the irreversible permeabilization of the plasma membrane [9]. Application of the high-frequency and high-strength EF for this purpose results in

Abbreviations: SWCNT, single-walled carbon nanotubes; CNT, carbon nanotube; EF, electric field; C6, rat C6 glioma cell line.

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electroporation of cells [10], impaired membrane integrity, etc. [11]. This approach has also found application in antitumor therapy. However, it is non-selective, since the high-strength EF leads to the destruction of not only tumor cells, but also normal cells adjacent to the tumor, although to a lesser degree [12].

The cell stimulation with EF of lower frequencies and strengths is accompanied by (i) a decrease in the elasticity of the cell membrane [13], (ii) the change in cell proliferation [14] and plasma membrane potential [15]. The latter is caused by the change in the activity of the plasma membrane ion channels [16].

Thus, the use of the external EF of low frequency and strength is a very attractive approach for therapeutic applications and for antitumor therapy in particular, since it does not have a destructive effect on cells, but allows one to regulate cellular activity and, potentially, intracellular drug accumulation. Consequently, the combined effect of CNTs and an external EF on cells will allow, on the one hand, to reduce the necessary concentration of CNTs and, on the other hand, to select protocols of electrical stimulation that does not affect the functioning of normal cells, but sensitizes only cancer cells containing CNTs.

This is especially true for antitumor therapy: if the targeted delivery of CNTs is carried out only into the tumor cells, it is possible to apply an EF at the organism level without damaging normal tissues.

In this study, for the first time, we demonstrate that the low-strength EF enhances the delivery of single-walled carbon nanotubes (SWCNTs) into glioma cells without plasma membrane destruction and pore formation, but via delicate regulation of physical properties of the cell membrane. The effect is demonstrated using Raman spectroscopy imaging of the cells loaded with SWCNTs with and without exposure to the external electric field.

2. Materials and methods

2.1. Cell culture

Rat C6 glioma cell line (ATCC ® CCL-107 ™) was purchased from ATCC, LGC Standards (Ogrodowa 27/29, Kielpin, Poland). The C6 glioma cells were cultured in DME/F-12 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum HyClone (USA) and 50 μg/ml gentamicin (Belmedpreparaty, Minsk, Belarus) at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. $2.5 \cdot 10^5$ cells in 3 ml were seeded on silicon wafers per each well in a 12-well plate. After 20 h, the cells were incubated with 5 μg/ml suspension of SWCNTs conjugated with salmon DNA (Sigma-Aldrich, USA) [17] and in 30 min some of the wells were exposed to the external EF stimulation for 1 h and 2 h. We used SWCNTs obtained by the gas-phase catalysis (HiPCO process, Nanointegris Technology Inc.). Previously, tube lengths were shortened to 100–400 nm using soft cutting approach [18].

2.2. External EF cells stimulation

The EF for stimulation was formed using steel electrodes connected to a programmable power source. The electrodes were placed in pairs directly in a Petri dish with a cell culture (Fig. 1a). Biphasic rectangular pulses (Fig. 1b) were applied to the electrodes with a duration of 1 ms for each phase (total pulse duration was 2 ms), the EF strength was 6.6 V/m, and the pulse repetition rate was 200 Hz. The start of electrical stimulation was carried out in 30 min after SWCNT addition. Stimulation was carried out for 1 h and 2 h.

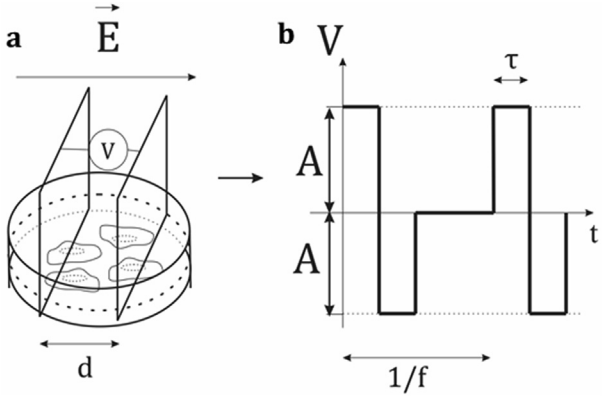

Fig. 1. EF setup for cell stimulation (a) and form of the biphasic rectangular pulse (b). E is the EF strength (V/m), d is a distance between the steel electrodes, V is applied voltage (V), f is a frequency (Hz), τ is pulse duration (s), A is voltage amplitude (V).

2.3. Raman microscopy

After EF stimulation, the cell monolayers on silicon wafers were washed twice with Hepes buffer (NaCl – 126 mM, KCl – 3 mM, $MgSO_4$ – 2 mM, $CaCl_2$ – 2 mM, Hepes – 10 mM, and glucose – 6 mM). Raman measurements at an excitation wavelength of 785 nm were performed using micro-Raman spectrometer NanoFinder HE (Lotis TII-Tokyo Instruments). The power of the excitation laser was restricted to 25 mW. 2D mapping was done with a scanning step of 1.5 μm and an accumulation time of 1 s. Images of SWCNT agglomerates were compiled using the integral intensity of the G-band in the Raman spectrum of carbon nanotubes. The integral intensity was calculated as an integral over the highlighted area of the G-band (see Fig. 2). Image analysis was implemented with NanoFinderViewer software and open source program ImageJ.

2.4. Local SWCNTs concentration evaluation

The local concentration of SWCNTs in agglomerates was evaluated using the G-band intensity in the Raman spectra of SWCNT, as described in Ref. [17]. Briefly, the local SWCNT concentration can be evaluated by the formula

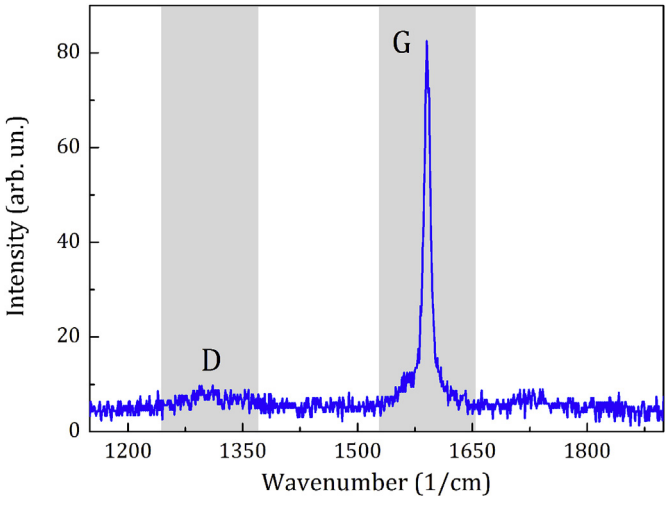

Fig. 2. Typical Raman spectrum of SWCNT agglomerate. D- and G-bands are highlighted by grey area.

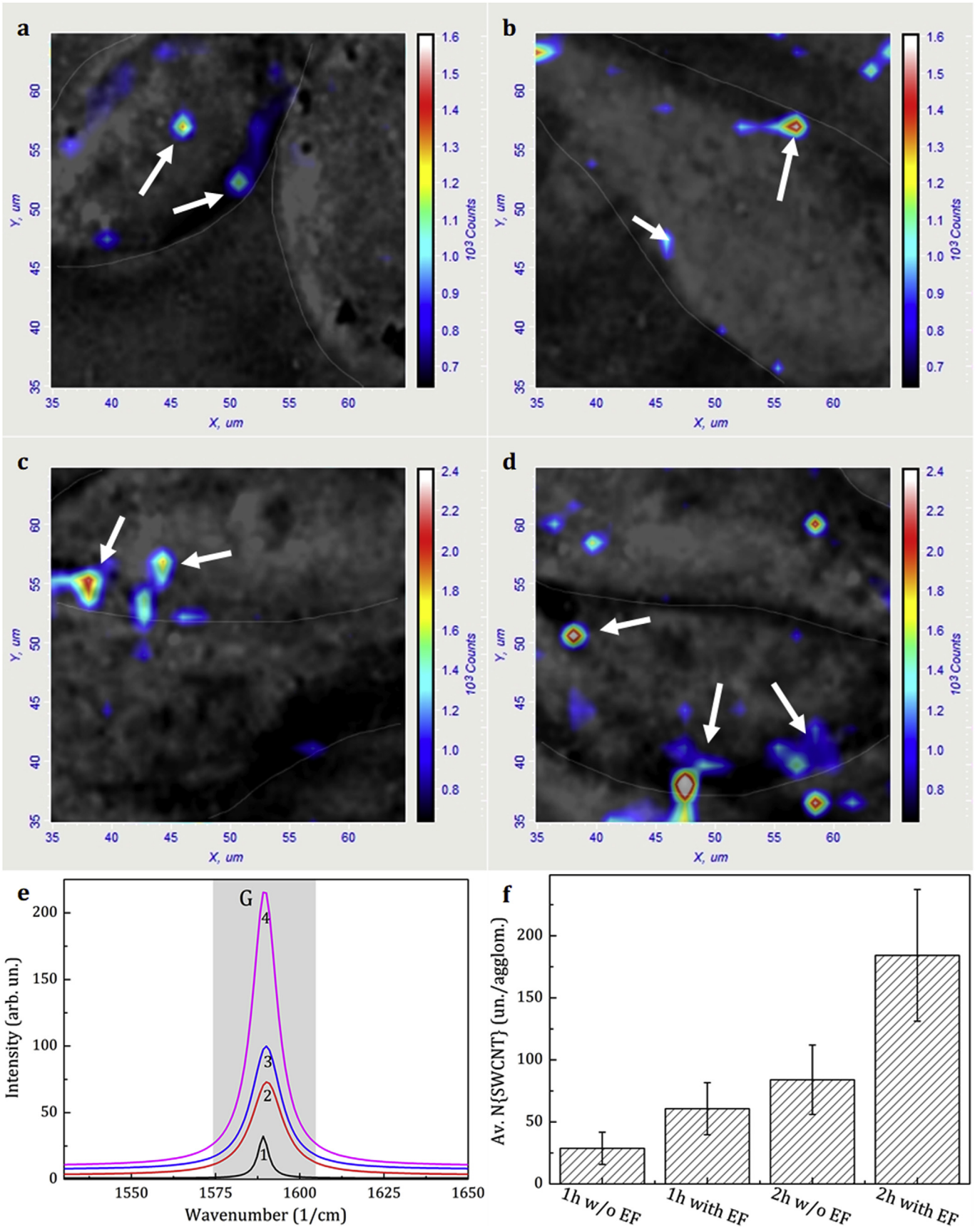

Fig. 3. Distribution of SWCNTs in glioma cells after 1 h (a, b) and 2 h (c,d) exposure to SWCNTs suspension. (a, c) – SWCNTs accumulation in cells without EF stimulation; (b, d) – SWCNTs accumulation in cells after stimulation with EF. SWCNT agglomerates are marked with arrows. Images are merged from reflected light image of cells and Raman mapping, obtained using G-band at 1591 cm^{-1}. (e) – Average Raman spectra of SWCNTs accumulated in glioma cells after exposure to SWCNTs: 1 – for 1 h, 2 – for 1 h under EF stimulation, 3 – for 2 h, 4 – for 2 h under the EF stimulation. (f) – Calculated average number of SWCNTs per agglomerate for spectra 1–4 in (e). Data are presented as mean value ± standard deviation.

$$N = \frac{I_G}{\alpha \cdot P \cdot t},$$

where N is a number of SWCNTs in a waist of the focused laser beam; α is the proportionality coefficient which was found from experiments [17]; I_G is an intensity of the G-band; P is a laser power; t is signal accumulation time.

Scanning areas with $I_G > 15$ units were considered as the SWCNT agglomerates. The averaging of SWCNT Raman spectra was performed over all agglomerates detected in the cross-section of each cell (see Fig. 3e).

3. Results and discussion

It was shown earlier, that SWCNTs conjugated with DNA are efficiently accumulated in glioma cells C6 [19]. This process is time-dependent; the intracellular concentration of SWCNTs reaches saturation after 18 h of cell exposure to SWCNTs [19]. Despite such a fairly rapid accumulation of nanoobjects inside the cell, nevertheless, it is important to reduce the time of biological material exposure to CNTs in order to reduce undesirable cytotoxic effects. Typical Raman spectrum of the SWCNT agglomerate has a small D-band (1310 cm^{-1}) and rather prominent G-band (1591 cm^{-1}); see Fig. 2. The ratio of the intensity of the D-band to that of the G-band is quite small, 0.05, meaning that SWCNTs are of a good crystalline quality. Fig. 3 shows typical images of SWCNT distributions in glioma cells after 1 h and 2 h of their accumulation with (Fig. 3 b,d) and without (Fig. 3 a,c) additional stimulation by an external EF.

As shown in Fig. 3 a-d, the SWCNTs are localized mainly near the cell membrane as individual agglomerates indicating the initial stage of endocytosis [19]. However, it is worth noting that the additional exposure to an external EF for 2 h promotes the translocation of SWCNTs into cells increasing the number of agglomerates in the cytoplasm as compared with the case without EF action (Fig. 3d, agglomerates are marked by arrows). The intensity of the G-band in the Raman spectrum of SWCNTs can be used not only for a qualitative description of the distribution of SWCNTs in the cell (2D mapping, Fig. 3a-d) but also for quantitative estimation of the local concentrations of SWCNTs inside the cell [17]. Fig. 3e demonstrates the average Raman spectra of SWCNT agglomerates accumulated in glioma cells after exposure to SWCNTs for 1 h and 2 h with and without additional stimulation with EF. Averaging has been done overall agglomerates detected within each cell. The calculated average number of SWCNTs per agglomerate is shown in Fig. 3f.

From the data presented in Fig. 3e-f, we can conclude that the influence of the external EF leads to a significant increase in a number of SWCNTs per agglomerate in C6 glioma cells, and the local concentration of SWCNTs during cell stimulation for 1 h is quite comparable to the local concentrations of SWCNTs during their accumulation in cells for 2 h without exposure to the external EF. Moreover, the application of the external EF leads to a more than two-fold increase in local SWCNT concentrations as compared to CNT accumulation without EF (60.6 ± 21.4 vs. 28.8 ± 13.1) un./agglom. and (184.2 ± 53.4 vs. 84.0 ± 28.7) un./agglom. for 1 h and 2 h, respectively. Thus, we observe an increase in the number of SWCNTs per each agglomerate, as well as in the number of agglomerates per cell after cell exposure to the external EF.

The use of external EF for targeted delivery of biomolecules into the cells is mainly based on the formation of pores in plasma membranes due to the high field strength and pulse repetition rate [20]. This approach has been successfully used for cell transfection [21], as well as for targeted delivery of antitumor drugs such as bleomycin, cisplatin, and DOX [22]. Electroporation can be effectively combined with nanoparticles for targeted drug delivery as described in Ref. [23] where the authors used external EF (50V–1600V) and CNTs to load the cells with DOX. However, the effect of high-strength electric fields can lead to irreversible pore formation and tissue ablation and necrosis [9].

In this study, an external EF with a voltage of 6.6 V/m and a pulse repetition rate of 200 Hz does not lead to electroporation and cell viability disturbance [15]. The increased accumulation of SWCNTs in cells is primarily associated with a decrease in the transmembrane potential caused by EF stimulation [15]. A decrease in the transmembrane potential leads to a change in the rigidity of the membrane [19] and facilitates the processes of SWCNT endocytosis by tumor cells. Thus, low-frequency EF enhances SWCNT accumulation in C6 glioma cells causing a modification of plasma membrane physical properties and enhancement of SWCNT endocytosis by cells.

In order to minimize possible side effects and toxic load on the body during antitumor therapy, one needs to enhance the therapeutic effect of drugs. For the first time, the non-destructing low-strength and low-frequency electrical stimulation of tumor cells were applied to accelerate CNT penetration through cell membranes. The enhanced accumulation of SWCNTs was revealed in cells exposed to external EF. Low-strength EF does not damage cellular membrane, but tiny regulates its properties and promotes endocytosis of SWCNTs inside glioma cells. Our results will contribute to the reduction of therapeutic CNT concentration and procedure duration increasing the antitumor effectiveness of drug-carrying CNTs, which will provide faster post-therapeutic recovery of the body. In the future, the combination of therapeutic action of drug-loaded CNTs and an external EF could potentially provide the basis for the development of synergistic antitumor nanotherapy.

Declaration of competing interest

The authors declare no conflicts of interest.

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