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BNNT-Mediated Irreversible Electroporation: Its Potential on Cancer Cells

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Irreversible lethal electroporation (IRE) is a new non-thermal ablation modality that uses short pulses of high amplitude static electric fields (up 1000 V/cm) to create irreversible pores in the cell membrane, thus, causing cell death. Recently, IRE has emerged as a promising clinical modality for cancer disease treatment. Here, we investigated the responses of tumour human HeLa cells when subjected to IRE in the presence of BNNTs. These consist of tiny tubes of B and N atoms (arranged in hexagons) with diameters ranging from a 1 to 3 nanometres and lengths $<2\mu$m. BNNTs have attracted wide attention because of their unique electrical properties. We speculate that BNNTs, when interacting with cells exposed to static electrical fields, amplify locally the electric field, leading to cell death. In this work, electroporation assays were performed with a commercial electroporator using the cell-specific protocol suggested by the supplier (exponential decay wave, time constant 20 ms) with the specific aim to compare IRE in absence and in presence of BNNTs. We observed that BNNTs have the capacity to decrease substantially the voltage required for IRE. When cells were pulsed at 800 V/cm, we observed a 2,2-fold reduction in cell survival in the presence of BNNTs compared to controls. We conclude that the death of the tumour cells exposed to IRE is strongly enhanced in the presence of BNNTs, indicating their potential therapeutic application.

Key words: Boron nitride nanotubes; Irreversible electroporation.

Introduction

In-situ ablation of diseased tissues, most commonly of neoplastic tissues, has become an important minimally invasive treatment alternative to surgical resection. Most methods for *in-situ* ablation are based on thermal techniques using rapid freezing below -40°C as with cryosurgery (1) or heat, *e.g.*, radiofrequency (2) and high-intensity focused ultrasound (3) or nanoparticle-mediated irradiation (4). Irreversible (lethal) electroporation (IRE) has been proposed as an alternative non-thermal technique for minimally invasive *in-situ* ablation based on the use of electrical pulses. When an electric field is applied to a cell, a change in trans-membrane potential is induced, which can cause biochemical and physiological changes of the cell. When the threshold value of the trans-membrane potential is exceeded, the cell membrane becomes permeable, thus allowing entrance of molecules that otherwise cannot cross the membrane (5). A further increase in the electric field intensity may cause irreversible membrane permeabilization when these pulses create extensive defects (pores) in the cell membrane lipid bilayer such that the integrity of the cell membrane cannot be restored (irreversible

Abbreviation: Boron Nitride (BN); Boron Nitride Nanotubes (BNNT); Irreversible Electroporation (IRE); Trasmission Electron Microscopy (TEM); Carbon Nanotubes (CNT).

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electroporation). This leads to cell death by apoptosis through loss of cell homeostasis (6). Irreversible electroporation is thus capable of efficient *in-situ* ablation targeted to the tumour site. A study by Davalos et al. showed that IRE can ablate substantial volumes of tissue without inducing a thermal effect, thereby providing a novel tissue ablation modality and has opened the way for the use of IRE in clinical practice (7). The promising results of this study were subsequently confirmed by other reports involving *in-vitro* studies on cells (8), *in-vivo* small animal models (9) and in large animal models of IRE targeted to the liver (10) and the heart (11). The most important finding reported by these studies is that irreversible electroporation produces precisely delineated ablation zones with cell scale resolution between ablated and non-ablated areas, without any intermediate zone of partial damage as is encountered with thermal ablation. Furthermore, these reports have demonstrated that irreversible electroporation affects only the membrane of living cells and spares the supporting stromal tissues. Rubinsky et al. (10) were the first to highlight the beneficial clinical features of IRE, including avoidance of collateral damage to large blood vessels and bile ducts, rapid tissue regeneration (attributed to the retained integrity of large blood vessels scaffolds), rapid activation of the immune system, minimal fibrosis and the potential ability to treat tumours adjacent to large blood vessels by avoiding the heat sink effect of circulating blood (12).

Despite the potential and advantages of the technique for clinical *in-situ* ablation, to date, IRE has been limited by the need of strong electrical fields of the order of 1000-1500 V/cm (8, 9). In particular, there are justifiable concerns regarding possible adverse effects of strong electric fields created by IRE ablation on other non-targeted tissues, *e.g.,* muscle contractions, cardiac arrhythmias which may occur especially during the "vulnerable" period of the cardiac cycle, *i.e.,* atrial or ventricular systole (13, 11). Currently, all clinical IRE ablation units utilize cardiac synchronization to ensure patient safety (14). Another clinical limitation concerns the pain induced by the electrical field itself or by the implanted electrodes for energy delivery, which requires that IRE is performed under general anaesthesia. Additionally, work has be done to address the limitation of muscle contractions induced by conventional electroporation and IRE. Daskalov *et al.* have used biphasic pulses to reduce patient discomfort during electrochemotherapy (15) and Arena *et al.* have used high-frequency bipolar bursts to eliminate muscle contractions during non-thermal IRE (16).

The present study is the first report of an attempt to reduce the magnitude of the electric field required for IRE in order to overcome its current limitations. Specifically, the study concerns the use of IRE mediated by nanomaterials with tailored properties, exemplified by boron nitride nanotubes (BNNT). BNNTs represent an important class of non-carbon inorganic nanotubes. Structurally, BNNT is similar to a carbon nanotube (CNT). However, electronically, BNNT is quite different from its carbon analogue. Normally, BNNTs are insulating, possess excellent mechanical properties, and are much more thermally and chemically stable compared to CNTs (17). Nevertheless, to date BNNTs have attracted much less attention than CNTs, due to the difficulties in their synthesis. Although currently the biological behaviour of BNNTs remains largely unexplored (18, 19), various biomedical applications have been recently suggested (20, 21) and initial attempts to explore their potential have been reported (22, 23). Our group was the first to report on the interactions of polymer coated singly dispersed BNNTs with living cells, demonstrating their cytocompatibility (20). Lahiri *et al.* studied the interactions of the osteoblasts and macrophages with bare BNNTs and reported their non-cytotoxicity (24). Subsequently, these findings were confirmed by other reports (18, 25). Recently, Horvath and colleagues tested the cytocompatibility of BNNTs on cells from the lung since they were interested in human exposure through inhalation (19). Both pulmonary epithelial cells and resident macrophages exhibited toxic effects when exposed to BNNTs in a dose and time-dependent manner. However, the toxicity of BNNTs for HEK cells observed in this study contradicts the findings of the study by Chen *et al.* who reported that HEK and CHO cells are not affected by the presence of BNNTs in the culture medium up to a dose of 100 μg/ml (25). This apparent controversy arises from the different length of the nanotubes employed in the different studies. BNNTs used by Horvath *et al.* were longer (>10 μm) than those used by Ciofani *et al.* (<2 μm) (18, 20) and Chen *et al.* (<6 μm) (25). Evidently, length is a crucial parameter for the cytotoxicity of the nanofibers. It has been clearly demonstrated for the structurally analogue CNTs, that nanotube lengths >10 μm induce the formation of granulomas similar to those caused by asbestos fibres following intraperitoneal implantation in mice. This pathology is attributed to 'frustrated phagocytosis' by macrophage cells unable to endocytose the long tubes with the subsequent formation of giant cells by fusion of single cells (26).

All together, the preliminary data available in literature seem to confirm the potential of BNNTs in the biomedical field, provided due attention is paid to the parameters influencing the toxicity of nanomaterials (*i.e.,* morphology, methods of production, impurities, *etc.*).

In a previous study, we described that BNNTs can be used as nanotools to enable reversible cell permeabilization with low electric fields (27). In the present study, we compare BNNT-mediated IRE with conventional IRE using a commercial electroporator. The results demonstrate that BNNTs allow a substantial reduction of the electric field required for irreversible electroporation of human HeLa cell line.

Materials and Methods

BNNT Production and Characterization

The BNNTs used in the study were synthesized via the pressurized vapour/condenser (PVC) method (28). BNNTs manufactured by this technique have been reported to have a unique morphology, namely high crystallinity, very high aspect ratio, high mechanical flexibility, and very small diameter, consisting of one to a few walls. The morphology of the BNNTs was determined by high-resolution transmission electron microscopy (TEM). Images were taken at 100 kV on a JEOL JEM-2100 F electron microscope.

In the present study, an aqueous dispersion of BNNTs was realized with glycol-chitosan (Sigma, St. Louis, MO, U.S.A.). Glycol-chitosan was dissolved in Phosphate Buffer Solution (PBS, Lonza, Milan, Italy) at a concentration of 0.1% (w/v), after which 1 mg/ml of BNNTs was added. The resulting mixture was sonicated with a sonicator (Bransonic2510, CT, USA) at 20 W for 16 h. Spectrophotometric analysis was performed with a LIBRA S12 Spectrophotometer UV/Vis/NIR (Biochrom).

Cell Cultures

Human epithelial carcinoma cell line (HeLa) was obtained from American Type Culture Collection (ATCC, Rockville, MD). HeLa cells were grown in a complete culture medium consisting of DMEM supplemented with 2 mM Lglutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated calf serum (ECS0040L, EuroClone, Italy). Cells were maintained at 37°C in a saturated humidity atmosphere of 95% air and 5% CO_2.

Electroporation Assays

Electroporation assays were performed with a commercial electroporator (Gene Pulser Xcell, Biorad). The protocol for cell permeabilization was as follows: cells were trypsinized, centrifuged for 5 min at 350 g and re-suspended in pulsing medium at a concentration of 10^6 cells/ml. Cells were electroporated using an exponential decay wave, a 2-mm cuvette and a volume of cell suspension of 100 µl, according to the protocol provided by the supplier. The voltage suggested by the supplier for reversible electroporation was 160 V (corresponding to an electric field of 800 V/cm). The range of voltage tested in the present work was 0-220 V (corresponding to an electric field of 0-1100 V/cm). In all experiments, the time constant was 20 ± 3 ms.

Three experimental groups were used in the study:

1. Sample. The pulsing medium was Opti-MEM added with polymer: BNNT (polymer and nanotube concentrations were 1.25 µg/ml).
2. Control 1. The pulsing medium used was Opti-MEM added with polymer (concentration of 1.25 µg/ml).
3. Control 2. The pulsing medium used was Opti-MEM.

After electroporation, cell suspension was diluted 1:5 in fresh cell culture medium. 100 µl of this suspension was seeded in each well of a 96-well plate to assess cell viability. Cell viability was assessed after 24 h of incubation.

Cell Viability

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT, Sigma, St. Louis, MO, U.S.A.) cell proliferation assay was used to assess cell viability. For MTT assay 20×10^3 cells were seeded into each well of a 96-well plate and then incubated with the culture medium. This was then replaced with 100 µl of medium containing 0.5 mg/ml of MTT and cells were incubated for 2.5 h at 37°C, 5% CO_2. In this assay, the mitochondrial dehydrogenase of viable cells reduces the water-soluble MTT to water-insoluble formazan crystals. The MTT-containing medium was then replaced with 100 µl of dimethylsulfoxide (DMSO, Sigma St. Louis, MO, U.S.A.) and left for 5 min on a platform shaker to solubilize the converted formazan. The absorbance was measured on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at a wavelength of 570 nm with background subtracted at 690 nm. All data are normalized with respect to the negative control (cells incubated with normal medium).

Cell counting assay was performed with ScepterTM cytometer (Merck Millipore, Milan, Italy).

Statistical Analysis

At least 12 replicates were used for each experiment. Values are expressed as means ± standard error of the mean (S.E.M.). Statistical significance was assessed by one way analysis of variance (ANOVA) followed by post-hoc comparison test (Tukey). Significance was set at 5%.

Results

We initially tested the fabricated BNNTs by TEM. A typical isolated, as-grown, PVC-synthesized BNNT is shown in Figure 1. Such BNNTs are highly crystalline, have one or just a few walls and, and the tube walls have few defects and are parallel to the axis. These BNNTs are highly flexible, as can be seen by the close conformation of the tube in Figure 1 to the carbon support grid, presumably by electrostatic forces. No catalysts are used to produce PVC-grown BNNTs, so the raw material contains only boron and BN. There are impurities, namely BN flakes and rods on the 100 nm to 1 micron size range (similar in scale to ball-mill-grown BNNTs) and some solidified, nanoscale boron droplets. The BNNTs themselves have typically

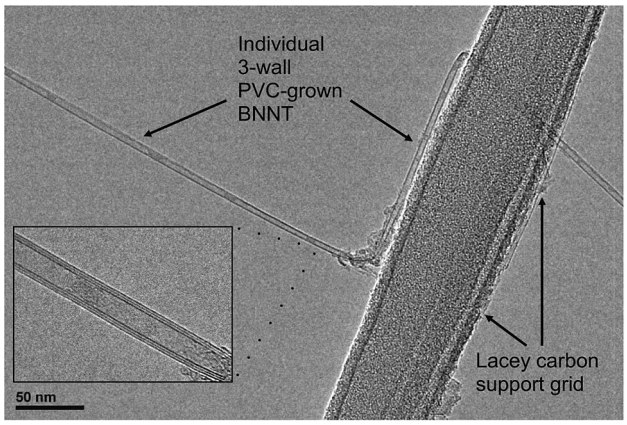

Figure 1: High resolution TEM image of an individual 3-wall PVC-grown BNNT.

1 to 5 walls, 2 and 3 wall tubes being the most common. These tubes have diameters ranging from about 2.5 to 5 nm.

As-produced nanotubes are up to millimeters in length (28). This extraordinary length gives the raw material the appearance of conventional textile fibres. These macroscopic bundles are broken up efficiently by the sonication procedure. Sonication causes rupture of hydrophobic and Van der Waals forces between BNNTs and breaks the tubes at the defect sites. The presence of the surfactant (glycol-chitosan) has the effect of stabilizing the tubes in aqueous solution and hence dispersion. The resulting mixture contains nanotubes singly dispersed (or as aggregates of few tubes) with an average length of $1 \pm 0.5\,\mu m$. UV-sis analysis of the dispersion confirmed the characteristic peak of BNNTs (Figure 2) (22).

Prior to validation of their biological use, BNNTs were tested for their biocompatibility by cell viability assays using aqueous dispersions of glycol-chitosan (GC: BNNT) at a nanotube/polymer mass ratio of 1:1. Specifically, dose response assays were performed in order to assess the ED25, *i.e.,* the nanotube dose reducing cell viability of 25%. GC: BNNTs and GC were tested in the range 10 ng/ml – 1 µg/ml. GC was found not to induce cytotoxicity at any tested concentration (data not showed). Experimental results showed that the cytotoxicity

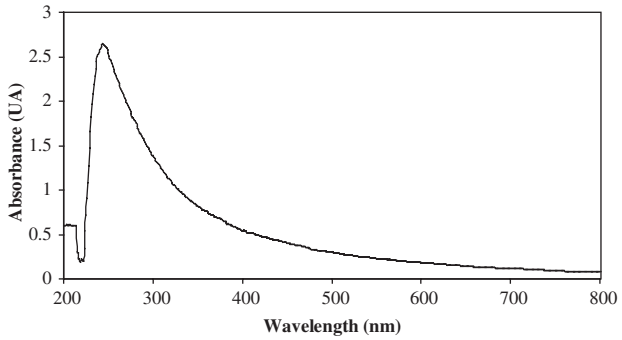

Figure 2: UV-sis spectrum of BNNT dispersion.

induced by the nanotubes is dose-dependent (Figure 3). ED25 was observed at about 253 ng/ml of BNNTs ($R^2 = 0.9971$). Based on these experimental data, further experiments involving cells involved incubation with a BNNTs concentration of 250 ng/ml in the culture medium.

The next stage of the study involved the performance of standard IRE against BNNT-mediated IRE. HeLa cells were subjected to electroporation in GC: BNNT modified Opti-MEM or GC modified Opti-MEM or pure Opti-MEM. Electroporated cells were diluted in normal cell growth medium (dilution ratio 1:5) and incubated for 24 h before to assess cell viability. Figure 4 shows viability of cells electroporated in presence of GC: BNNT or GC as a function of the applied electrical fields. Viabilities of cell electroporated in GC modified Opti-MEM or pure Opti-MEM were not statistically different (data not showed). Cells electroporated in GC: BNNT modified Opti-MEM was subjected to cytometric analysis. The results revealed that the increment of the applied electric field causes a decrease of cell density and a corresponding increase of cellular debris (Figure 5).

Discussion and Conclusion

BNNTs used in the study were produced by a pressurized vapour/condenser (PVC) method described by the authors in a previous report (28). This method produces, without catalysts, highly crystalline, very long, small-diameter BNNTs. The uniformity, small diameter (<5 nm), and high flexibility of the nanotube are clearly evident by the ready deformation and adhesion of the molecule to the microscope support grid (Figure 1). This flexibility demonstrates that these high aspect ratio BNNTs are able to conform to cellular-scale structures with minimal applied mechanical force, which may greatly reduce their cytotoxic response. An important advantage of the morphology of PVC-grown BNNTs may be thus improved baseline cytotoxicity. Work by Horváth et al. (19) on the inherent toxicity of BNNTs on epithelial cells,

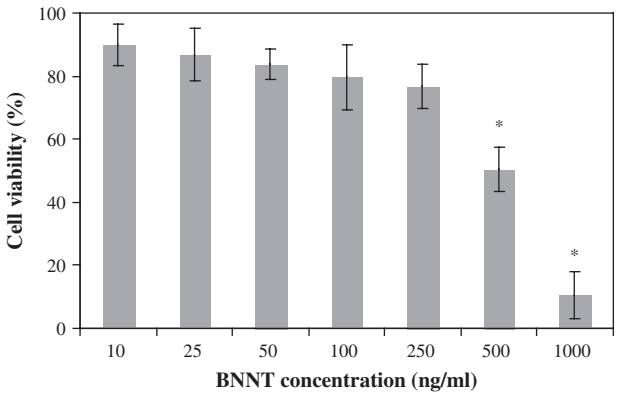

Figure 3: Dose response curve. Cells incubated 24 hours at various concentrations of polymer coated nanotubes. Cell viability expressed as percentage of the control (cells treated with polymer alone). N = 12. $p^* < 0.0001$.

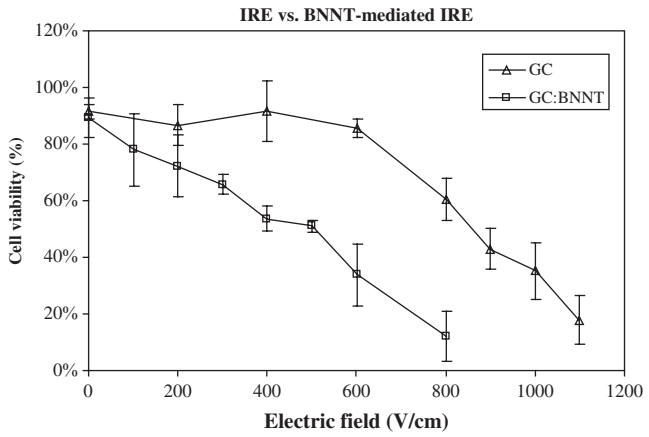

Figure 4: HeLa cells treated with polymer coated nanotubes (CG:BNNT) and polymer alone (CG): cell viability after 24 hours of incubation following electroporation assay. Cell viability expressed as percentage of the control (cells not electroporated). N = 14.

alveolar macrophages, fibroblast cells, and human embryonic kidney cells, reported that the toxicity is highly morphology dependent. In particular, mechanically stiff BNNTs, in their case up to 80 nm in diameter and up to 10 microns in length, were found to be more toxic than relatively flexible (20 nm diameter) carbon nanotubes. PVC-Grown BNNTs, at less than 5 nm diameter, appear to be much more flexible still, and may prove to be proportionally less toxic, completely avoiding the 'rod-like' disruption of cell membranes observed by Horvath. Other important factors affecting toxicity to cells and tissues by BNNTs include water solubility, length and purity. As-produced nanotubes have reduced solubility in aqueous solution, which is essential for biological interactions and biocompatibility. Length is a crucial factor as one important study (26) has demonstrated that carbon nanotubes with length >10-15 µm induce the formation of granulomas similar to those caused by asbestos fibres. These problems can be easily solved by dispersing the nanotubes in a polymer solution with the aid of sonication. This produces water dispersions of short nanotubes (1 ± 0.5 µm) with a non-covalent

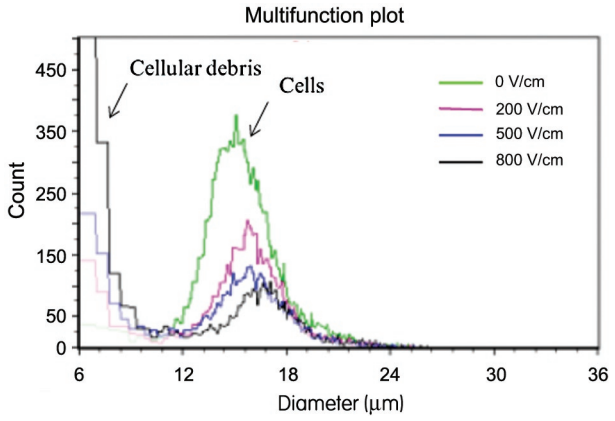

Figure 5: Cytometer analysis.

polymer coating. BNNTs produced in the present work revealed a dose-dependent cytotoxicity (possibly related to the presence of impurity). This was carefully taken into account in the present study as all biological assays were performed at dose below to ED25.

Another important advantage of the morphology of PVC-grown BNNTs in the present study is the high aspect ratio, which renders the tubes very efficient energy nano-transducers. As previously discussed in a previous report (27), the mechanism of BNNT mediated electroporation is related to the dielectric response of BNNTs to static electric fields.

The process of cell electroporation is explained by the Neumann theory (29). Briefly, with static fields or low frequency alternating fields, the potential distribution in the region surrounding a spherical cell with a non-conductive membrane is described by the Laplace equation with appropriate boundary conditions as follows:

$$\Delta\varphi_m = -\frac{3a}{2} \cdot E^{\text{out}} \cdot \cos\gamma$$

where $\Delta\varphi_m$ is the trans-membrane voltage, E^{out} is the external electric field, a is cell radius and γ the polar angle between E^{out} and the site on the cell membrane where $\Delta\varphi_m$ is measured. According to the classical definition of electric field strength as the negative electric potential gradient, the membrane field strength is given by

$$E^m = \Delta\varphi_m / d$$

where d is the membrane thickness. At the poles ($\gamma = 0, \pi$), the trans-membrane electric field E^m is large compared with E^{out}, the amplification β_1 associated with this field concentration is $\beta_1 = E^m / E^{\text{out}} \approx \frac{3}{2}\frac{a}{d}$, corresponding to a 1500 fold amplification of the external electric field (for a cell with $a = 5\,\mu\text{m}$ and $d = 5\,\text{nm}$).

By analysing the response of a dielectric nanotube (diameter D, length l, density ρ, transverse and longitudinal dielectric constants $\varepsilon_\perp$, ε_{II}), the screening factor in the vacuum, defined as the ratio between the external electric field E^{out} and the electric field within the nanotube E^{NT}, is given by (30)

$$E^{\text{out}} / E^{\text{NT}} = \frac{\varepsilon_\perp + 1}{2} = \varepsilon^*$$

This means that a nanotube exposed to an external electric field E^{out} amplifies the field in proximity of its surface by a factor $\beta_2 = \varepsilon^*$.

The value of β_2 depends from many factors, included the dielectric constants of the nanotube and the aspect ratio.

By decreasing the aspect ratio, β_2 approaches 1. In order to enhance β_2, the aspect ratio should be maximised. As already mentioned, the average length of the nanotubes must be below 2 μm in order to address cytocompatibility issues. Thus the small diameter nanotubes of this study are eminently suitable for the proposed application. Another feature that makes our BNNTs excellent candidates is their superior dielectric properties. BNNTs, whether single-walled or multi-walled, or packed in bundles, are excellent semiconductors, with a large band gap that is largely independent of the tube diameter, chirality, and number of walls (31, 32). In contrast, carbon nanotubes exhibit a polarizability tensor highly anisotropic because of their inherent anisotropy (33). Specifically, polarizability for external fields in the longitudinal direction is considerably larger than that in the radial direction. The longitudinal polarizability of SWCNTs scales as the inverse square of the band gap, which depends on the chiral vector *i.e.,* the armchairs SWCNTs are metals while zigzags SWCNTs are semiconductors. In MWCNTs the overall longitudinal polarizability is given by the sum of the polarizabilities of the constituent tubes: a metallic behaviour can be thus assumed. The transverse polarizability of SWCNTs is insensitive to band gaps and chirality and is proportional to the square of the effective radius. In MWCNTs the outer layers dominate the response. The consequence of this anisotropy is that the application of an external electric field generates a torque, which aligns the CNT along the field direction. In conclusion, although CNTs also possess the ability to amplify the external field, the application of two perpendicular fields as described in (34) is required for effective field amplification. This requires a dedicate set-up, which is not compatible with commercial devices already available for biotechnology laboratories and clinics.

BNNTs by virtue of their superior properties compared to CNTs are able to overcome these limitations and are thus the preferred nanomaterial for IRE. Because of the polar nature of the B–N bond (35), BNNTs preferentially form zigzag (rather than armchair or chiral) structures. BNNTs thus exhibit stable electronic properties, with a uniform band gap of 5.5 eV (32). These properties make BNNTs the best nano-probes for enhancing the local electric field at the cell interface. Our experimental results confirmed that BNNTs constitute a very efficient tool for decreasing the energy requirement for irreversible electroporation (Figure 4). The voltage suggested by the supplier for poration of HeLa cells (800 V/cm) was found to induce a cytotoxicity of ~40% but this value rises to ~88% in presence of the BNNTs. We can explain these experimental findings by the following considerations. When cells are placed in a BNNT modified medium, BNNTs interact with cell membrane due to the electrostatic forces. Previous analysis confirmed the interaction of BNNTs with mammalian cells and their subsequent internalization (18). As result of this interaction, the application of the electric field induces an amplification of the field in proximity of the cell membrane of a factor $\beta_1 \cdot \beta_2$, which explains the reduction of voltage required for IRE.

The mechanism through which the cell membrane is permeabilized by IRE is not yet fully understood. According to the electroporation theory, IRE should induce cell death by creating permanent nanopores in the cellular membrane using high voltage direct electrical current. These nanopores disrupt cell membrane. However, it is also reported that the permanent nanopores induced by IRE in the cell membrane disrupt cellular homeostasis, which initiates apoptosis (36).

We found that BNNT mediated IRE induce complete disruption of the plasma membranes, cell swelling, and dilatation of cytoplasmic organelles. Our optical observations were confirmed by cytometer analysis (Figure 5). Experimental data showed that by increasing the electric field applied, cell density decreases. There is also a slight increase of the average cell diameter, which could be related to cell fusion phenomena naturally occurring during the electroporation process (37). Most importantly, the decrease of cell density is concomitant to a parallel increase of the peak at sizes <8 μm, corresponding to the presence of cellular debris.

In conclusion, here, we evaluated first the dose of BNNTs, which does not affect significantly cell viability. Second, we compared BNNT mediated IRE with conventional IRE by using a commercial electroporator. We concluded that BNNTs allow a strong reduction of the electric field required for irreversible electroporation of human HeLa cells. Preliminary experimental data confirmed that the phenomenon of IRE could be further enhanced by increasing BNNT concentration in the medium (does-response behaviour, data not shown). In this regard, we are currently working on the purification and analysis of high purity nanotube. Further work will be devoted to investigate the translation of BNNT mediated IRE into cancer treatment. Many questions remain to be answered, such as how BNNTs will be delivered and distributed intratumorally, whether BNNTs can disperse through the tumor stroma to contact cell membranes, and if electric field enhancement can indeed reduce the required applied voltage for ablation as suggested. The challenge is to develop a clinical compliant protocol of enhanced IRE based on the use of high purity BNNTs with preclinical testing on *in-vivo* human tumour xenograft models in SCID mice.

Conflict of Interest

We certify that regarding this paper, no actual or potential conflicts of interests exist; the work is original, has not been accepted for publication nor is concurrently under consideration elsewhere, and will not be published elsewhere without the permission of the Editor and that all the authors have contributed directly to the planning, execution or analysis of the work reported or to the writing of the paper.

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