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Research paper

Investigating the effects of electrical stimulation via gold nanoparticles on in vitro neurite outgrowth: Perspective to nerve regeneration

Moein Adel [a,b,c], Masoumeh Zahmatkeshan [a,d], Behrooz Johari [e], Sharmin Kharrazi [a], Mehdi Mehdizadeh [f], Bahram Bolouri [a,g,*], Seyed Mahdi Rezayat [a]

[a] Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[b] Deputy of Research and Technology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[c] Department of Medical Nanotechnology, School of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[d] Neuroscience Research Center (NRC), Iran University of Medical Sciences, Tehran, Iran
[e] Department of Medical Biotechnology, School of Allied Medicine, Tehran University of Medical Sciences, Tehran, Iran
[f] Cellular and Molecular Research Center, School of Advanced Technologies in Medicine, Department of Anatomical Sciences, Iran University of Medical Sciences, Tehran, Iran
[g] Department of Medical Physics, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Neural tissue engineering research field has been progressed by using different approaches especially for repairing of damaged neural cells. In addition, it is known that electrical stimulation can be used for neurite growth and nerve regeneration. In this study conductive properties of gold nanoparticles (GNPs, 39 nm) and their contribution to the enhancement of electrical stimulation to nerve cells have been conducted. In experimental section, polyethyleneimine (PEI) polymer coated cover glasses was used to create a positively charged glass surface and adsorption of GNPs was used in conjugation with this polymer coated substrate. Subsequently, PC12 cells were cultured on the modified glass surface and pulsed electric field of 1.5 V, 20 Hz was applied as electrical stimulation for 55 min duration. Images from FESEM showed a uniform distribution of GNPs on glasses surface. In addition, enhanced neurite outgrowth (120 μm) using electrical stimulation was determined by inverted phase contrast microscopy images. Altogether, synergist combination of GNPs together with pulsed electrical stimulation can be used for enhanced nerve regeneration.

1. Introduction

Following the injury of nervous tissue, in particular, spinal cord injuries, axons do not regenerate appreciably in their native environment; thus, medical treatment approaches are needed. The current clinical approach to treating damaged nerves includes using nerve cells or nerve autograft transfer. However, this strategy remains a major issue due to donor site complications, limitations of graft nerves, and in result inefficient functional recovery [1–4]. Many studies have been conducted in recent years to find novel ways to removing these limitations and decrease distance between ends of injured nerve. In regard to electrical stimulation (ES), various attempts have shown its important role in neurite outgrowth in vitro and in result in vivo damaged nerve ends regeneration [5–7]. Studies reported various cellular responses to an electrical stimulation that are different for constant and alternative potential. Constant potential to mammalian cells cultured on an electrode regulate proliferation rate, and the alternative potential electrically activates the gene expression of nerve growth factor (NGF) [8].

There has also been great interest to study the development of electrically conductive materials since their significant properties for induction of functional actions in stimulated cells and tissues by enhancement of electrical stimulation [3,9,10]. Traditionally, gold nanomaterials are one the most appropriate conductive materials especially its potential for enhanced electrical shock transferring in electrical stimulation of cell and tissue cultures [11,12].

On the other hand, surface modifications have been shown to be an effective method in improvement the interactions of cell surface interactions and also an increase of cellular functions on substrates. Among the surface modifications, nanoparticles (NPs) are used to the fabrication of thin films and employment of coated devices in cell culture applications [13,14]. Layer-by-layer assembly (LBL), a new approach of thin film deposition, has been widely utilized to construct homogeneous films, which works based on oppositely charged materials. In this method, charged NPs (negative or positive) are used for adsorption on charged layers of polyionic complexes with opposite charge [14–16]. The great advantages of LBL assembly for the fabrication of NP-based

* Corresponding author at: Department of Medical Physics, School of Medicine, Iran University of Medical Sciences, Crossing of Hemmat & Chamran PKWY, Tehran, Iran.
E-mail address: bbolouri@iums.ac.ir (B. Bolouri).

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materials over other techniques of surface modification include controlled adsorption with nanometer precision and the spatially controlled construction of conductor layers [14].

Here we aimed to evaluate the deposition of gold nanoparticles (GNPs) onto polyethyleneimine (PEI) pre-coated surfaces and fabrication of an electrical device to enable the induction of neurite extension in culture and nerve cell differentiation. GNPs, with their unique optical, electrical, and molecular-recognition properties and their easy synthesis and biocompatibility, are the subject of substantial research and application in biology and medicine [17–20] [6,7]. Moreover, conductive gold films and GNPs are used extensively to provide a platform for proliferation promotion and enhanced differentiation of various adhered cell types [7,11,21–23]. Therefore, by a pulsed potential application, we investigated the potential of GNPs in inducing cell differentiation of PC12 cells.

2. Methods

2.1. Fabrication of gold nanoparticle coated glass

First, the cleaned glasses were modified by putting them in positively charged PEI (Acros Organics [24]) (Mw 1800). Polymer dissolution was carried out in its native pH (7.4), without salt, which allowed the PEI to extensively protonation of its amines side chain. Then cover glasses immersed in PEI solution (1 mg/mL) for 12 h and rinsed three times with distilled water. Subsequently, the product dipped in GNP solution[1] in which they underwent gentle shaking for 24 h. Finally, the GNP-coated substrates washed by distilled water for four times.

2.2. Cell culture

Rat pheochromocytoma-derived PC-12 cell lines (Pasteur Institute; Tehran, Iran [25]) were cultured in RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated horse serum (HS), 5% heat-inactivated fetal bovine serum (FBS) (all from Gibco [26]), and 1% penicillin-streptomycin (Sigma [27]). Then, cells cultured and grown in standard cell culture set up (at 37 °C humidified air containing 5% CO_2) and were changed approximately once every week.

2.3. Electrical stimulation of PC12

The GNP-coated surfaces in wells were seeded with 5 × 104 PC12 cells/3 cm^2. Cells were allowed to attach to the substrate for 24 h in the culture at 37 °C and 5% CO2. After 24 h, the PC12 cells exposed to a pulsed potential of 100 mV/mm for 55 min in a CO_2 incubator. The GNP-coated substrates acted as the anode in electrical stimulation and a gold (Au) wire affixed at the opposite end (along with the length) of the well acted as the cathode, and a silver (Ag) wire acted as a quasi-reference electrode. The potential application was performed with a power generator (Searle, England) for stimulation. Following electrical stimulation, incubation of cells was conducted for the cells for an additional 48 h (3 days total, assigned as ES 3 d). During the experiment, the medium was refreshed every day. For positive controls, 100 ng/mL of NGF (Gibco [26]) was added in differentiation medium without electrical stimulation. Images of the cells were obtained both before and after subjecting them to a pulsed potential. PC12 cells cultured at the same seeding density (5×10^4 cells/3 cm^2) on the control group substrates, on the GNP-coated surfaces without electrical stimulation, on PEI-coated surfaces and on the Petri dish. The neurite length of the cells subjected to stimulation was compared with those of the controls to approximate the extent of differentiation. The cells were grown on GNP-coated surfaces and were exposed to a pulsed potential (100 mV/mm) (through the medium, not the gold film) to test the potential effect of convective ion transport or oxidation-reduction products on neurite outgrowth. The procedure was performed using two Au wires that put on opposite ends of the cell well (as the anode and cathode). A gold wire positioned on a third side of the well that acted as a quasi-reference.

2.4. Neurite length measurements

Thin GNP-coated surfaces can be studied using optical microscopy and quantitative image analysis for detailed investigation of the cell-material interactions. Cells were observed using an inverted phase contrast microscope (Motic AE31 [28]), with a 20× objective. Image J (NIH) and AutoCAD software were used to measure the lengths of the individual neurites for each cell. The length of the cells described by the straight-line distance between the neurite tip and the cell body-neurite base junction. In addition, all cell analyses were performed on different days for three times.

2.5. Cell viability assay

Viability was assessed by both trypan blue dye exclusion and 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay (MTT-assay), before and 24 h after application of the electrical charge.

For the trypan blue exclusion test, detached PC12 cells of test surfaces were washed twice with 10% FCS-supplemented RPMI medium and re-suspended in 1 mL FCS-supplemented RPMI medium. Then 200 μL of each cell suspension mixed with the 200 μL of 0.4% (w/v) trypan blue solution prepared in PBS and then incubated for 5 min. In each cell suspension, viable and nonviable cells were counted 3 times, and the experiment was conducted three times.

2.6. MTT assay

The attached cells were exposed to an MTT reduction assay (MTT-assay), followed by the addition of 200 μL MTT (5 mg/mL in PBS) (Sigma Chemicals, St. Louis, USA [27]) solution to each well with 2 mL cell culture medium. Then the tissue culture plates incubated for 4 h at 37 °C and then the supernatant was removed by aspiration, and 2 mL dimethyl sulfoxide (DMSO; Merck [29]) was added to each well and mixed thoroughly which allowed the crystalline formazan produced by live cells to completely dissolve. Ultimately, 200 μL (in triplicate) of a solution of each well was added to a 96 flat-bottom well plate. Then the optical density was read at a wavelength of 545 nm (test) and 630 nm (reference) using the Stat-Fax 2100 microplate reader (Awareness Technology Inc. [30]). The dye intensity is proportional to the number of living cells.

2.7. Statistical analysis

All tests of this study were repeated for three times on different days. The average and standard error of the mean (S.E.M) were calculated for all data. One-way ANOVA was used to analysis the differences between experimental groups with unpaired two-tailed *t*-tests. p value < 0.05 was considered statistically significant. In addition, one-way analysis of variance followed by Tukey's test was used for calculation of statistical significance of neurite length for multiple comparisons ($p < 0.05$).

3. Results

3.1. SEM images of the nanoparticle-coated surface

Fig. 1 shows the GNPs deposited on glass slides pre-coated with PEI in order to electrically stimulate PC12 cells. It is observed that the distribution of the GNPs on the glass slides pre-coated with PEI was constant

[1] A kind gift from Dr. Sharmin Kharrazi.

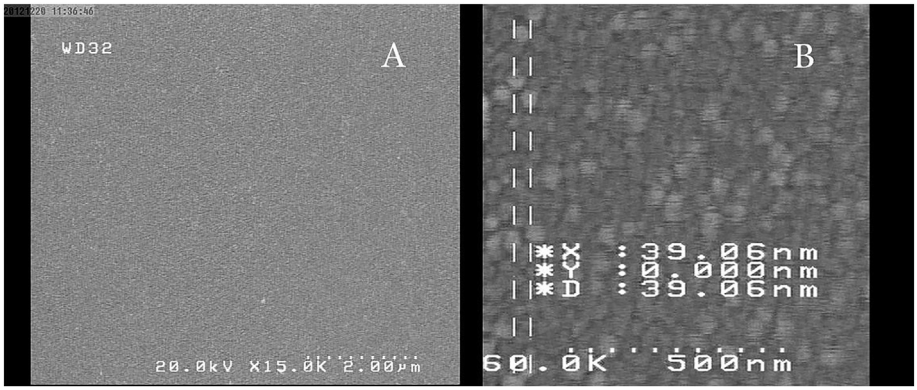

Fig. 1. FESEM images of the AuNP films on PEI-modified glass slide. (A) 15,000× (B) 60,000×.

and uniform in size. Mean particle size formed on the PEI-modified substrate was 39 nm.

3.2. Cell viability

The results of cell viability tests, trypan blue exclusion test, and MTT assay indicated that nearly 90% of the PC12 cells exposed to pulsed stimulation survived (Fig. 2). These results illustrate that these substrates supported cell adhesion and proliferation and didn't have any toxic effect on the PC12 cells.

3.3. Neurite outgrowth measurement

The result of the neurite outgrowth measurement of PC12 cells showed that the pulsed electrical stimulation induced more neurite outgrowth in cells adhered to the GNP after 3 days than those after one day, those not stimulated, or those that were positive controls, as shown in Fig. 2. Thus, GNPs as a conductive material deposited on cover glass can be a promising method for studying the neurite elongation of PC12 cells. Fig. 3 shows a schematic of neurite outgrowth of PC12 cells on GNPs affected by electrical stimulation.

3.4. Contrast phase microscopic analysis

Fig. 4 illustrates the contrast phase microscopic images of stimulated PC12 cells on the GNP-coated surfaces after 3 days of culture. The cells adhered proliferated to the GNP-coated surfaces, thereby forming the cell-matrix interaction. In addition, as mentioned above, the time course plays an important role in the neurite extension of the PC12 cells. Finally, these results reveal that uniform distribution of GNP on surfaces increases the passing of the electrical stimulation and in result neurite outgrowth of the PC12 cells.

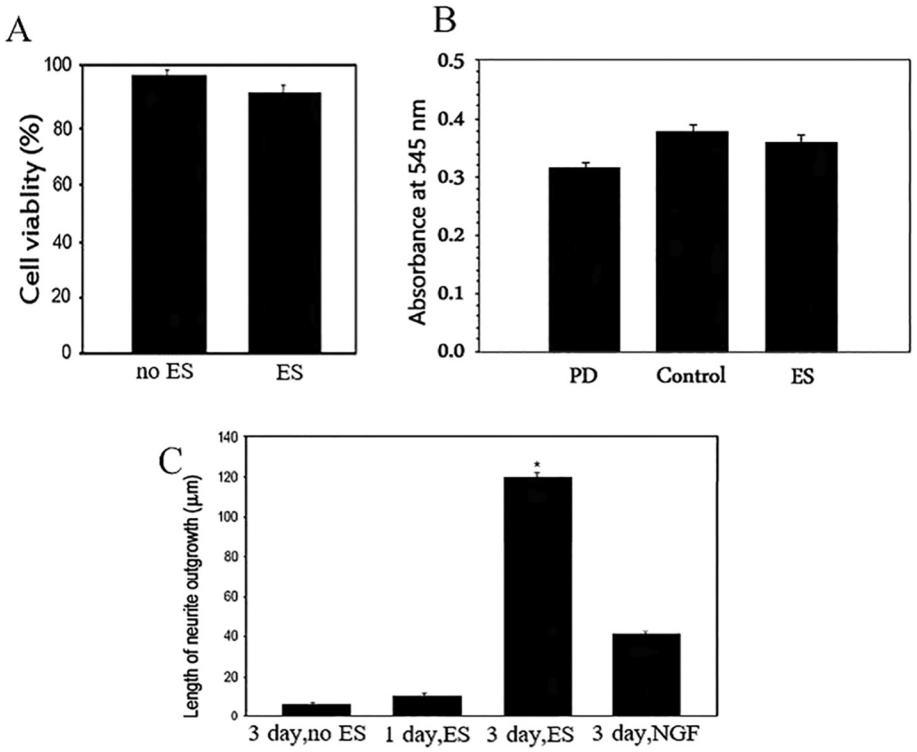

Fig. 2. (A) Percentage of PC12 cell viability on gold nanoparticle-coated glass substrate in response to pulsed electrical stimulation: (no ES) no stimulation, (ES) following pulsed electrical stimulation. (B) PC12 cell viability on tissue culture Petri dish (PD), gold nanoparticle-coated glass substrate with or without ES (ES and control groups) after 24 h of culture, obtained by MTT assay. (C) Quantitative analysis of neurite length for PC12 cells cultured on gold-nanoparticle coated substrate. (3 days, no ES) no stimulation, (1 day, ES) after one day of stimulation, (3 day, ES) after 3 days of stimulation, and (3 days, NGF) positive control. Values are presented as mean ± standard error of the mean ($n = 30$). Error bars at each time-point indicate standard error of the mean (*: $P < 0.001$).

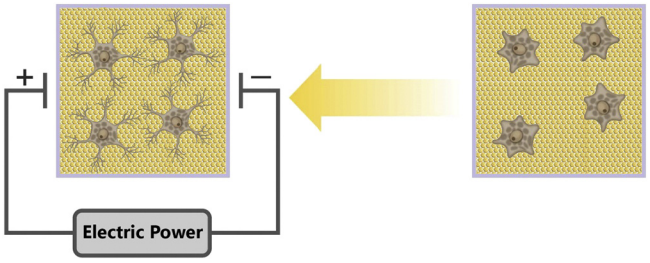

Fig. 3. Schematic of electrical stimulation of PC12 cells cultured on the gold nanoparticle-coated surfaces to neurite outgrowth.

4. Discussion

The impact of GNP-coated surfaces as conductive materials and electrical stimulation on neuronal differentiation was analyzed using exposing of PC12 cells grown on these substrates to a pulsed stimulation of 100 mV/mm for 55 min after 1 and 3 days (Fig. 2). The GNP-coated surfaces were confirmed by images of FESEM in Fig. 1. The images display that NPs covered almost the entire coated surface which indicates that the GNPs were physically entrapped by the PEI film.

Our study also showed that approximately 90% of electrically exposed PC12 cells survived. This finding is in agreement with Park et al.'s results which showed the alternating current stimulation treated PC12 cells tended to display greater viability compared to those treated with constant current stimulation [5]. Shi et al.'s findings also showed that the electrical stimulation induced surprisingly greater cell viability compared to the non-stimulated fibroblasts cultured in the tissue culture Petri dishes [7].

In addition, in nerve cell migration and neuroregeneration, neurite extension is an important phenomenon and requires multiple cues (biophysical, biochemical, and bioelectrical) [31]. Electrical stimulation can be used as physical stimuli and also can act as a highly efficient option for regulating cellular functions. It is well known that bioelectricity is a normal part of body actions and plays as an intrinsic factor in maintaining normal biological processes including nervous system signaling, contractions, and wound healing [32]. Recently, researchers have extensively begun to explore specific neural cellular interactions to materials and implants.

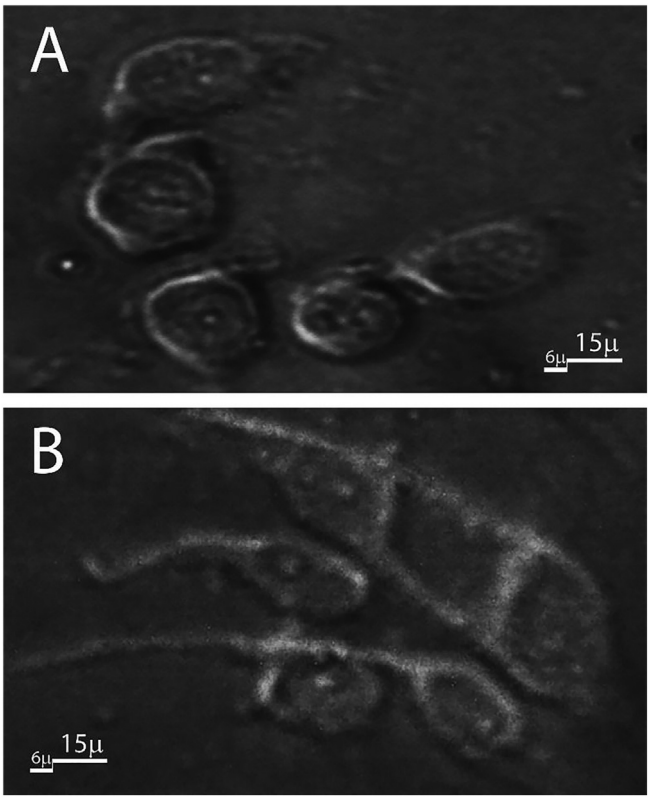

Fig. 4. Phase-contrast optical images of PC12 cells cultured on AuNP films without (A) and with (B) electrical stimulation.

Based on the above results, when cells cultured on the GNP-coated surface without any potential application, proliferation was normal, and no cell differentiation or critical effects in the morphology of PC12 cells were observed, as shown in Fig. 4. This suggests no effects of the GNP coated surface on proliferation and differentiation. When cells treated with pulsed stimulation (100 mV/mm) by the frequency of 20 Hz for 55 min and incubation in 3 days, sprouted long neuritis; indicating the neurite outgrowth of PC12 cells through the electrical stimulation.

Image analysis software was used to quantify the length of the neurite outgrowth and results showed 120 ± 4 μm for average neurite length of cells cultured on the GNP-coated surface after stimulation. In comparison neurite length of no electrical treated cells was 15 ± 3 μm (statistically different for $p < 0.001$). Results of neurite length differences of our experiment are significantly higher than those developed on conductive polymers including: PCL/Gelatin/PANi: 31 μm [33], PLLA/PANi: 28 μm [34], PPy-NGF: 17 μm [35], heparin/polypyrrole (PPy) composite: 77.5 μm [36]. However, the application of electrical stimulation by polymers might be a combination of some challenges. These polymers may release residual chemicals during stimulation that can be a barrier in the induction of its effects. For example, the non-uniform distribution of PPy particles on the membrane surface causes the heterogeneous nature of the electrical stimulation [7] and poor polymer-cell interaction [37]. However, to overcome these questions in this study, GNPs were used due to their well-known biocompatibility and significant electrical properties. In addition, their uniform distribution, physical action in cells, and capability of incorporating different dopant ions into their surface via ionic complex used to the fabrication of various surface characteristics [5,31].

Furthermore, several studies in agreement with the current study strengthen the use of electrical stimulus as a potential stimulus for neurite outgrowth, even without the presence of other traditional non-biological and biological stimuli such as differentiation growth factors [8,34,37,38]. The precise mechanism of impact of electric stimulation on neural regeneration is unclear. One hypothesis is that the repeated potential shift in the vicinity of a cellular membrane could cause an oscillation of charged molecules in the membrane and in result induction of activation of modulating components [8]. Moreover, several reports suggest that depolarization-induced neurite outgrowth without NGF required extracellular calcium influx through L-type channels as a stretch-activated (SA) channel [39], and a calcium-dependent potassium ion channel [40]. Specifically, a study reported that activation of Src or EGF receptors or tyrosine kinases may happen by calcium influx in the vicinity of cellular membrane [41,42]. Therefore, extensive researches in this field suggest that induction of mechanical stress in result to electrical stimulation may take part in activation of intracellular protein tyrosine kinases via fluctuations of the cellular membrane.

Finally, our study showed that pulsed current stimulation induced neurite outgrowth of PC12 cells adhered to the GNPs coated surfaces. Furthermore, average neurite outgrowth was 120 μm in one round of electrical stimulation. Thus, GNPs as an electrically conductive matrix will be a promising material for nerve regeneration. Our future works will direct towards optimizing properties of NPs and stimulation parameters for in vivo nerve regeneration and do a comparative study with other material including silk and carbon materials.

Conflict of interest

The authors declare that there are no conflicts of interest.

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