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SHORT COMMUNICATION

Cytotoxicity and internalization analysis of silicon nanowires in Buffalo Green Monkey cells: a preliminary study to evaluate the possibility of carrying viruses inside the cells

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SUMMARY

Silicon nanowires (SiNWs) are attractive functional nanomaterials for biomedical applications. The ability to easily tune their size and density, potential biocompatibility, and knowledge of the chemical activation of SiNWs surface make them natural tools to interact with biological materials. We evaluated the possibility of exploiting SiNWs as carriers to introduce organic compounds into cells. The cellular toxicity and the internalization capacity of free-standing and label-free SiNWs were tested on Buffalo Green Monkey cells (BGM). Confocal fluorescent observation of SiNWs conjugated with fluorescein-polyethylene imine (PEI) confirmed the internalization of the NWs into the Buffalo Green Monkey Cells (BGM).

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Inorganic nanowires are emerging as a powerful tool for fundamental biological studies and biomedical applications. High surface-to-volume ratio, easy material functionalisation methods and three-dimensional nanostructured topography, which efficiently interacts with the micro/nanoscale structures of biological systems, make these materials an excellent interface for proteins, DNA, cells, viruses, etc. (Kwak *et al*., 2015; Liu X and Wang S *et al*., 2014). Among them, silicon nanowires (SiNWs) have been shown to be a successful nanoscale platform for a variety of applications, since a wide range of synthesis techniques allow tuning SiNWs properties to incorporate a various set of functionalities, including tailoring of structural morphology and enhancing of nanowires optical and thermal efficiency. This has led to the creation of a large library of SiNW-based tools. As a result, single SiNWs have been integrated in syringe-injectable neuron-nanoelectronics (Liu *et al*., 2015). A disordered array of SiNWs has been exploited for photothermal treatment of cancer as well as for surface enhanced Raman spectroscopy (SERS) probes combined with electrochemical devices (Maiolo *et al*., 2016), and forests of SiNWs have been used as whole-cell biosensor integrating microbes (Mintz-Hemed *et al*., 2017). In addition, vertical SiNWs exhibit optimal cell adhesion properties, making them good for cell proliferation (Convertino *et al*., 2018; Qi *et al*., 2009), or ideal sites for biomolecule immobilization (Shalek *et al*., 2010). Recently, freestanding SiNWs have been found to be internalized in living cells essentially via an endogenous phagocytosis pathway (Zimmerman *et al*., 2016), allowing cellular integration of these materials and opening the way to the exploitation of these structures as carrier of specific substance into cells. Thus, properly functionalized, the nanowires can be exploited as carriers for the virus to infect cells, thus maximizing the number of copies of the viruses in processes in which pathogenic replication is poor.

In particular, there are several viruses, like norovirus, hepatitis C virus, etc., unable to multiply in cell lines because of the absence of permissive cell lines. The possibility to introduce the viruses will offer the chance to study viral replication, the molecular biology of the virus providing the ability to produce large amounts of viruses to prepare vaccines.

In this perspective, we investigated the possibility of exploiting freestanding SiNWs as carriers in transfection processes of viruses in non-permissive cells. SiNWs arrays were grown by plasma enhanced chemical vapour deposition (PECVD) on Si substrates and then removed from the substrate used for the growth. Au catalysed SiNWs

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were produced by PECVD on oxidized Si wafers. To induce NWs growth, a 2 nm thick Au film was evaporated onto the substrate. Growth was performed with SiH_4 as precursor at a total pressure of 1 Torr and at a substrate temperature of 350°C (Cuscanà *et al*., 2010; Convertino *et al*., 2010); a 13.56 MHz radiofrequency source was used with a power of 5 W to ignite the plasma. Growth time was fixed at 10 min.

The morphology of the SiNWs was verified by scanning electron microscopy (SEM) at an accelerating voltage of 5 kV. We obtained a forest of nanostructures with an average length of single nanowires of about 20 um and a diameter of 60 nm.

To prepare samples for testing with cells, SiNWs were sonicated in 2% foetal calf serum (FCS) in LBS1 ultrasonic bath (FALC instruments) for 2 hours, thus obtaining a solution with a SiNWs concentration of about 10^{-4} ug/ml.

Their internalization capability was tested on Buffalo Monkey Green (BGM) cells, which are laboratory cells currently in use in virological research. All the experiments were performed using BGM cell lines cultivated in Dulbecco's modified Eagle's (Sigma) medium with 2% foetal calf serum (Corning) (maintenance medium) or 10% FCS (growing medium), containing 2mM l-glutamine and 100 U/ml of a mixture of antibiotics. The cells were grown in an atmosphere of 5% CO_2 in air at 37°C.

In the preliminary experiments the SiNWs were grown directly on chamber slides with 8 wells (NUNC). After 5-10 min sterilization under UV treatment, $1x10^5$ cells were applied in the single well. The cells were observed for 2-3 days to detect the presence of cytotoxicity of the SiNWs, observed under optical microscope.

The BGM cells, cultivated on SiNWs layers, showed normal and uniform growth. It was possible to observe the edges of the nanowires in black and to assume their presence in the cytoplasm, although the cells showed homogenous morphology, representing their good health status.

In the second set of experiments the cells were plated before growth in 8 well NUNC and overlayed with 250 ul of media containing the right amount of SiNWs per well, in order to evaluate cytotoxicity. Before each test, freshly prepared SiNWs solution was dispersed at the desired concentration of 20 (*Figure 1A*), 30 (*Figure 1B*), 40 and 50 uM by direct ultrasonication in LBS1 ultrasonic bath (FALC Instruments) for 2 hrs in culture medium containing 2% FCS. Here we report only the inoculum at 20 uM (Figure 1A) and 30 uM (*Figure 1B*). The SiNWs were represented as single dots and short filaments outside and inside the cells.

The cells were observed each day for 5 days by reverse optical microscope at 10x50 magnitude. On the fifth day, cells were fixed in alcohol and coloured with haematoxylin (5 min) and eosin (30 sec) (*Figure 1A* and *1B*). The concen-

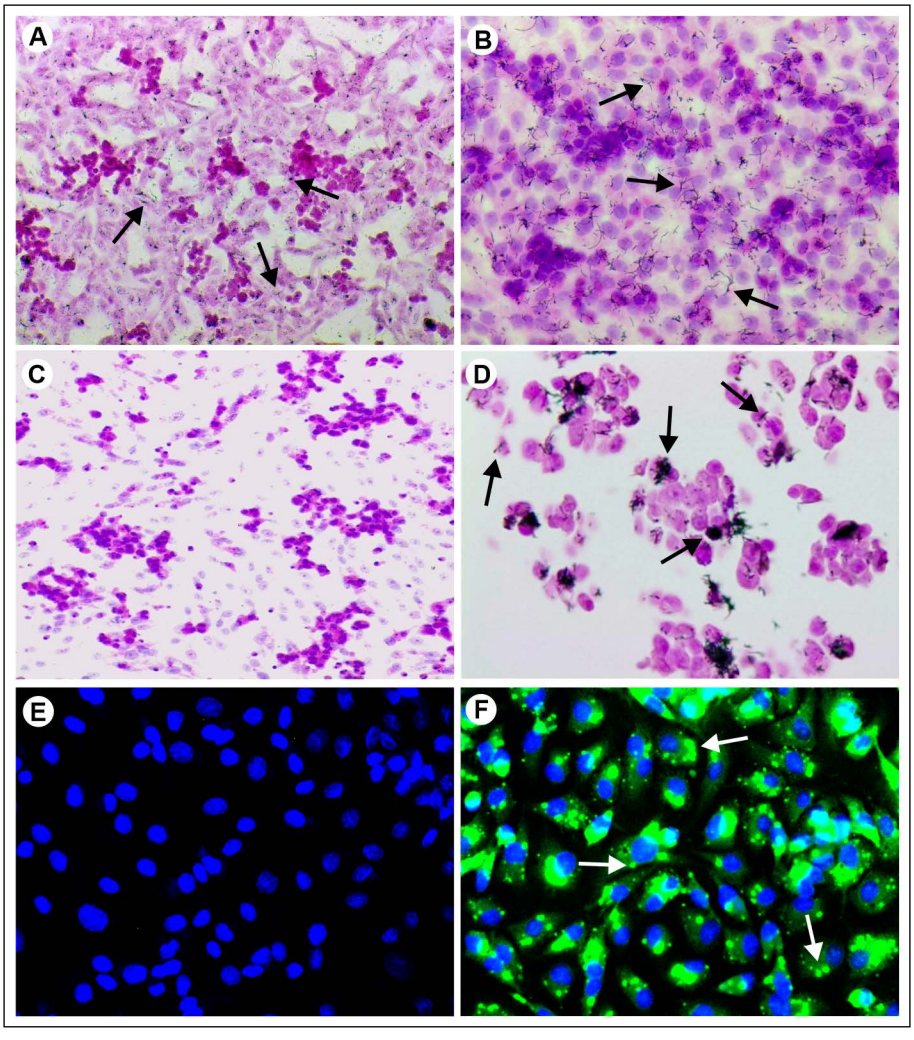

Figure 1 - *Interaction between SiNWs and BGM cells.*
Optical images showing the effects of treatment with SiNWs at 20 (Panel A) and 30 uM (Panel B), respectively. Panels C-D: hematoxylin/eosin staining of BGM cells cultivated in absence (C) or presence (D) of 20 uM SiNWs. Panels E-F: confocal images of control (E) and treated BGM cells (F). Images were acquired at 20X magnification following counterstaining with DAPI. Arrows in panels A-B-D indicate dots and filaments of SiNWs. Arrows in Panel F indicate the fluorescence induced by SiNW-PEI-FITC.

tration of 40 and 50 uM was too high, reducing the ability to correctly observe the cells. For this reason, further tests were performed at a fixed concentration of 20 uM. Nevertheless, no cytotoxicity was observed at any of the different concentrations.

In the second set of experiments, to confirm the presence of SiNWs inside the cells, the BGM cells were seeded in a plate of 8 wells (NUNC) and treated with SiNWs dispersed at the concentration of 20 uM, as in the previous protocol. After three days, the cells were detached and collected at 100xg for 10 min; the pellet was resuspended in formalin 4% and, after centrifugation, dehydrated with ethyl alcohol at 70%, 95% and 100%. The pellet was collected and treated twice with 100% xylol. Finally, the cells were incorporated in paraffin at 60°C for 12 hrs and sectioned with a microtome (Reichert). Every section of 6 um, obtained after dewaxing, was placed on a slide, and after rehydration each of them was finally coloured with haematoxylin (5 min) and eosin (30 sec). These experiments confirmed the absence of cytotoxicity of SiNWs at 20 uM and this confirms the initial hypothesis: the presence of SiNWs in cytoplasm. In fact, silicon nanowires were clearly present around the cells and inside the cells as single and specific black dots (*Figure 1C* and *1D*).

In the last set of experiments the SiNWs at 20 uM were coated with PEI (Polyethylenimine) (Gajardo *et al*., 1991) and FITC (Fluorescein isothiocyanate). The previously synthesized SiNWs (70uM in ethanol) were cleaned from excess of reactants by repeated centrifugation at maximum speed (14,000 rpm for 25 min) in a bench eppendhorf centrifuge (Jouan, BR4i, rotor AB2.14) at room temperature, using isopropanol (Merck) as solvent. The sample (1 ml) was sonicated for 5 min (LBS1 ultrasonic bath, FALC Instruments). After adding 10 ul of a 10 M divinyl sulfone (DVS solution at 97%, Aldrich) (at final 0.1 mmol), the sample was left under stirring for 2 hrs at room temperature and centrifuged as above. The precipitate was then dissolved in 800 ul of isopropanol (Merck) and sonicated for 5 min, and 200 ul of PEI-25 (Gajardo *et al*., 1991) solution 50% (w/w) in H2O (Sigma-Aldrich) were added to the sample. The reaction was sonicated for 5 min and the solution was then left under stirring overnight. The SiNWs were cleaned from excess of reactants by repeated centrifugation (three cycles), then the precipitate was dissolved in absolute ethanol. To synthetize fluorescent SiNWs, 80 ul of a 25 mM FITC ethanol solution (~2 umoles, Fluorescein isothiocyanate isomer I, Sigma) were added to samples (1 ml) of SiNWs dissolved in ethanol, previously wrapped with aluminium foil paper to protect FITC from the bleaching. The samples were stirred for 15 min and washed three times by centrifugation. Then the precipitate was suspended in maintenance medium.

After 3 days post treatment with labelled PEI-FITC-SiNWs at 20 uM and control cells treated con free fluorescein, the cells, washed with complete PBS (Phosphate Buffered salts, Gibco) to eliminate the excess of the fluorescein, were fixed in alcohol at 70% and visualized with confocal microscope at 10 or 20 x 50 magnitude. The analysis confirmed the presence of SiNWs in the cytoplasma of the BGM cells as evident by the amount of fluorescence inside the cells (Figure 1F). In this experiment, the fluorescence was absent outside the cells and in the control cells treated with free fluorescein (*Figure 1E*).

No fluorescence was observed in the control cells (Figure 1E). BGM cells were grown in 8 well chamber slides at $1x10^5$ cell/well. SiNWs, at 20 uM, labelled with PEI-FITC, were applied to the cells. After three days, the cells were counterstained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride) to evaluate the nuclei (*Figure 1E* and *1F*).

The confocal analysis gives an optical imaging technique increasing optical resolution, and the microscope can capture multiple two-dimensional images at different depths in a sample giving a more precise idea of the presence of SiNWs inside the cells. The analysis of the cells showed not only the presence of SiNWs but also the absence of toxicity or apoptotic process.

The confocal observation by DAPI staining further confirms the vitality of the BGM cells, not highlighting any phenomenon of nuclear fragmentation, chromatin condensation and finally the collapse of replication.

The preliminary results obtained from the present *in vitro* study suggest that the investigated nanomaterial is not cytotoxic. Moreover, the SiNWs can efficiently penetrate into the cells. These results open the way to the use of Silicon Nanowires as a carrier in transfection processes of viruses to guarantee more efficient infection of the cell for biological applications, such as vaccine preparation and viral replication.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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