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Influence of different types of carbon nanotubes on muscle cell response

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ABSTRACT

The aim of this study was to evaluate the impact of multi-walled carbon nanotubes (MWCNTs), before and after chemical surface functionalization on muscle cell response in vitro and in vivo conditions. Prior to biological tests the surface physicochemical properties of the carbon nanotubes (CNTs) deposited on a polymer membrane were investigated. To 'evaluate microstructure and structure of CNTs scanning electron microscopy (SEM) and Fourier transformation infrared spectroscopy (FTIR) were used. During in vitro study CNTs deposited on polymer membrane were contacted directly with myoblast cells, and after 7 days of culture cytotoxicity of samples was analyzed. Moreover, cell morphology in contact with CNTs was observed using SEM and fluorescence microscopy. The cytotoxicity of CNTs modified in a different way was comparable and significantly lower in comparison with pure polymer membrane. Microscopy analysis of cultured myoblasts confirms intense cell proliferation of all investigated samples with CNTs while for two kinds of CNTs myoblasts' differentiation into myotubes was observed.

Histochemical reactions for the activity of enzymes such as acid phosphatase, cytochrome C oxidase, and non-specific esterase allowed the analysis of the extent of inflammation, degree of regeneration process of the muscle fibers resulting from the presence of the satellite cells and the neuromuscular junction on muscle fibers in contact with CNTs after implantation of CNTs into gluteal muscle of rat.

1. Introduction

Influence of carbon nanotubes (CNTs) on the growth, proliferation and differentiation of various types of cells was studied in several works. Multi- and single walled carbon nanotubes (MWCNTs and SWCNTs) have been used as matrices for culture of various cells including osteoblast, fibroblasts, neuronal cells, and stem cell [1–5]. The interest in these types of nanomaterials in the area of tissue engineering and regeneration results from their unusual properties such as mechanical, electrical, large surface development, and ability to functionalize their surface using growth factors, chemical and biological agents that allow for stimulation and cell differentiation. Furthermore, their diameter, length and the fibrous nature resemble some structures of the extracellular matrix (ECM) such as protein fibers (fibrillar collagen and elastin) that play an important role in the support of cell growth and differentiation.

Potential medical application of carbon nanotubes is skeletal muscle tissue engineering. One of the major diseases affecting skeletal muscle is muscular dystrophy, a genetic hereditary disease, in which patient's muscles are weakened due to loss of muscle-specific proteins, such as dystrophin [6]. Carbon nanotubes are also considered to be used to facilitate muscle regeneration in aged people whose ability to maintain or regrow muscles is greatly reduced and causes the debilitating age-specific muscle wasting, resulting in the lack of mobility [6].

For skeletal muscle formation, several myoblasts fuse to form long and cylindrical multinuclear myofibers. Individual skeletal muscles consist of many myofibers bundled together [7,8]. For proper myofiber development and restoration of muscle function innervations and vascularization of muscle tissue are also important. Therefore, any methods of engineering skeletal muscle must be capable of forming and promoting neuromuscular junctions and stimulating the formation of new blood vessels. The lack of innervation is the main reason of poor restoration of muscle regeneration, resulting in its malfunction and atrophy [6]. There is a lot of information in the literature referring to the effect of carbon nanotubes or carbon nanomaterials on stimulation and regeneration processes of nerve cells [3,4,9].

Materials used as substrates for muscle regeneration should also be characterized by biofunctionality. Appropriate mechanical and electrical properties of synthetic scaffolds are required for mimicking some of the signals forming muscle during repair. Studies have shown that mechanical forces help to recapitulate the natural cellular environment by providing mechanical cues to direct cell orientation. Mechanical stimulation affects metabolic activity, protein localization, and gene regulation as well as enhances myofiber organization and cell proliferation [10,6]. Besides not only mechanical stimulation but also electrical stimulation plays an important role in skeletal muscle differentiation by an increase of cell proliferation and inducing proper orientation of

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myoblasts prior to their fusion into myotubes [11,6]. Electrical impulses generated by a material mimic the signals sent by neurons in natural tissue and enable to activate certain chemical cascading system in the muscle tissue, aiding the muscle tissue's growth and increasing the release of NO which typically activates the regenerative myogenic responses of satellite cells [12]. Very good mechanical properties of CNTs and their high electrical conductivity make them excellent candidates for the regeneration of muscle tissue. Beneficial effect of carbon nanotubes facilitating the unidirectional growth of muscle cells was also described in literature [7].

Despite numerous scientific works dealing with potential application of CNTs alone as well as in the form of films obtained by their deposition on a polymer substrate in tissue engineering, the question arises how their surface structure influences interaction with individual cell, cell culture and in contact with living tissue.

The aim of this work was to study multi-walled carbon nanotubes (MWCNTs) before and after surface functionalization in vitro and in vivo conditions. Nanotubes were added to C2C12 murine myoblast cell culture and implanted into gluteal muscle of rat. The tissue response was analyzed with the aid of histochemical reactions.

2. Materials and methods

Three kinds of pristine and functionalized multi-walled carbon nanotubes (MWCNTs) were used in this work. As-prepared MWCNTs were provided by NanoAmor USA. The MWCNTs synthesized by CVD method with the use of metallic catalyst (Ni) had diameters in the range of 10–30 nm and were 1–2 μm long. ICP-OES analysis of the as-prepared MWCNTs indicated the presence of nickel (1.2 wt.%). The nanotubes were chemically oxidized in a mixture of concentrated H_2SO_4 and HNO_3 acids, according to the procedures described in detail elsewhere [13]. The nanotubes after such a treatment were denoted as MWCNT-F. The aim of this stage was the removal of metallic residues from MWCNTs and receiving their hydrophilic character by the introduction of carboxylic and hydroxyl groups on their surface [14]. The concentration of nickel in CNTs after oxidation (MWCNT-F) is 0.1 wt.%. The oxidation is also required for future functionalization by using ethylenediamine ($C_2H_4(NH_2)_2$). Amino-functionalization of MWCNT-Fs was performed to verify their influence on cell response, especially on nerve cells. As it is described in the literature the functionalization of CNTs significantly reduces their cytotoxicity and enhances cell growth, especially nerve cells [15,16]. The nanotubes prepared in such a way were referred to as MWCNT-NH. The procedure of functionalization was previously described elsewhere [14,17,18].

Multi-walled carbon nanotubes before and after functionalization were investigated with muscle cells in vitro and also this material was implanted into muscle tissue of rat. Histochemical studies allowed for observation of processes connected with muscle fiber regeneration. Muscle fiber function recovery in contact with MWCNTs is associated with the restoration of innervation as a result of production of neuromuscular synapses.

To evaluate direct influence of CNTs on cell response during in vitro study the appropriate sample preparation is required. All kinds of CNTs should be uniformly distributed over the whole surface of the culture dish in which the experiment is conducted. For this purpose, three kinds of MWCNTs were deposited on PTFE membrane filters (Membrane Filters (PTFE supported) Whatman®) using filtration under pressure. These filters have diameter Ø = 47 mm and pore size Ø = 0.2 μm. Before deposition of CNTs on PTFE membrane, each type of carbon nanotubes was sonicated for 5 min using a tip sonicator (PALMER INSTRUMENTS, Model: CP 130 PB, 130 W power, 20 kHz) in ethanol (96% CZDA, CAS: 64-17-5 POCH Co.) in a concentration of CNT 0.4 mg/mL. Four milliliters of each solution was used to obtain nanotubes deposited evenly on the filter surface. The preparation process of PTFE filters with CNTs is schematically shown in Fig. 1.

After the deposition, the membranes with CNTs were dried and cut into disks with a diameter fitting to the size of culture plate wells.

The degree of purification and functionalization of CNTs was estimated using inductively coupled plasma optical emission spectrometry (ICP-OES) (Multiwave 3000, PerkinElmer Co.) and Fourier transformation infrared spectroscopy (FTIR) (Bio-Rad FTS60 V spectrometer), respectively, described in detail in previous articles [13,14]. Morphology and microstructure of CNTs on a membrane were determined using scanning electron microscopy (SEM, Nova NanoSEM 200, FEI). The contact angle of CNTs on membrane was measured by sessile drop method using an automatic drop shape analysis system DSA 10 MK2 (Kruss, Germany). UHQ-water (produced by Purelab UHQ, Elga, Germany) drops of the volume of 0.2 μl were put on each sample and the contact angle was calculated by averaging the results of 10–11 measurements. The surface energy (γ_s) of CNTs was calculated using the Owens–Wendt method [19]. It allows for determining two components of the surface energy, i.e. the dispersive (γ^d_s) and polar (γ^p_s) components. For this purpose, two liquids with known values of γ^d_l and γ^p_l of the components are used. In this work it was water ($\gamma^p = 51$ mJ/m^2 and $\gamma^d = 21.8$ mJ/m^2) and ethylene glycol ($\gamma^p = 19$ mJ/m^2 and $\gamma^d = 29$ mJ/m^2).

2.1. In vitro study

Cell culture

Murine adherent myoblast cell line C2C12 (ATCC, GB), which may differentiate into muscle cells was used. The cells were cultured in 75 cm^2 tissue culture flasks (Nunc, Denmark) in Dulbecco's modified Eagle's medium (ATCC, GB) supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin (Sigma-Aldrich, Germany)) and 10% bovine fetal serum (ATCC, GB). The flasks of cultured cells were incubated at 37 °C in humidified 95% air and 5% CO_2. Cells were routinely processed by harvesting using 5% trypsin-EDTA solution and replicated in tissue culture flasks at a ratio of 1:6.

For in vitro tests membranes with deposited MWCNTs were immersed in 70% ethanol and sterilized with UV radiation for 0.5 h on each side. Sterile disks cut-out from membranes were placed into 48-well culture plates and C2C12 cells were seeded on their surface at a density of 2×10^5/ml per well. The cells were cultured in contact with nanotubes for 7 days.

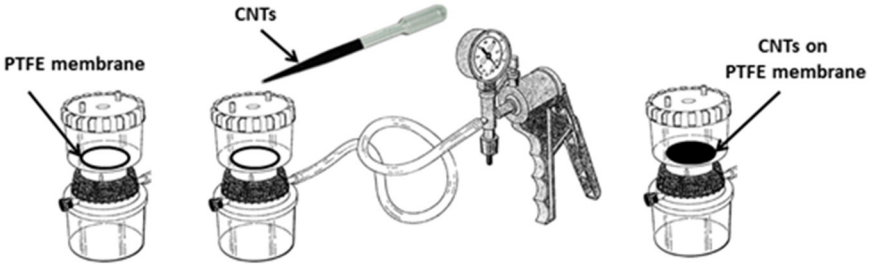

Fig. 1. Scheme showing deposition process of CNTs on PTFE membrane.