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Biocompatibility of pristine graphene monolayer: Scaffold for fibroblasts

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ABSTRACT

The aim of the present study was to evaluate the cytotoxicity of pristine graphene monolayer and its utility as a scaffold for murine fibroblast L929 cell line. Cell viability, morphology, cytoskeleton architecture (microfilaments and microtubules), cell adhesion and migration into the scratch-wound area were determined using pristine graphene-coated microscopic slides. We found that fibroblasts cultured on pristine graphene monolayer exhibited changes in cell attachment, motility and cytoskeleton organization. Graphene was found to have no cytotoxicity on L929 fibroblasts and increased cell adhesion and proliferation within 24 h of culture. The area of cells growing on graphene was comparable to the area of fibroblasts cultured on glass. Migration of cells on the surface of graphene substrate appeared to be more regular in comparison to uncoated glass surface, however in both control (glass) and experimental (graphene) groups the scratch wound was closed after 48 h of culture. Taken together, our results indicate that pristine graphene monolayer is non-toxic for murine subcutaneous connective tissue fibroblasts and could be beneficial for recovery of damaged tissues after injury. These studies could be helpful in evaluating biocompatibility of graphene, which still remains ambiguous.

1. Introduction

Methods of tissue engineering allow for the development of new therapeutic strategies for tissue injury. There has been tremendous progress in the methodical development of large-scale *in vitro* cell culture and the synthesis of cellular scaffolding materials. Scaffolds used in cellular engineering not only have supporting functions, but also stimulate tissue regeneration at the site of injury. Choosing a suitable material for tissue engineering is crucial for medical applications and achieving therapeutic results. Such material must not be cytotoxic or mutagenic, and must also have adequate physicochemical properties that will ultimately provide a favorable environment for adhesion, proliferation and cell differentiation (Bacakova et al., 2011). So far many materials have been tested. In recent years one such material, graphene, isolated and characterized in 2004, has attracted attention as a promising material in tissue regeneration.

Graphene is a single-atom-thick sheet of sp2-bonded carbon atoms. It is a unique form of carbon in which the atoms are arranged in a hexagon. Graphene has unique physical, chemical, electrical and mechanical properties, which qualify it as a promising nanomaterial in areas such as physics, material science and other technological applications (Park et al., 2009). Some investigations indicate that graphene could have great potential for bacterial inhibition, drug delivery and photo-thermal therapy (Goenka et al., 2014; Akhan and Ghaderi, 2010). Cytotoxicity tests have been widely performed to assess the cytotoxic effect on different biomaterials and to determine biocompatibility. The International Standards Organization (ISO10993-5) classifies the cytotoxicity assay as the first step in the sequence of biocompatibility tests (Poskus et al., 2009). The direct contact method with L929 cells is one of the most frequently used for evaluating the cytotoxicity and may represent a sufficient screening model for *in vitro* evaluation of cytotoxicity (Park et al., 2002). Cytocompatibility of biomaterial can be assessed based on the cell cytoskeleton organization. The cellular cytoskeleton is a flexible and dynamic protein filament system composed of microfilaments, intermediate filaments and microtubules, which together with their associated proteins form a network that connects the cell nucleus, organelles and cytoplasmic membrane. The cytoskeleton determines the shape and movement of organelles throughout the

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whole cell and plays an important role in cell division, transport, apoptosis and proliferation (Mogilner and Keren, 2009; Tschumperlin, 2013; Atherton et al., 2015). It's primary role is to contribute to the spatial organization of the cytoplasm, where it creates a specific scaffold for proteins involved in various intracellular processes. The dynamic reorganization of the actomyosin cytoskeleton in response to various stimuli is at the base of all phenomena related to cellular motility (Lange and Fabry, 2013). Cells creep upon the surface, have morphologically and functionally differentiated front and back region (motion polarization) (Tojkander et al., 2012). This phenomenon is well visible during cell migration in the scratch wound assay. The force required for cell movement is generated by processes occurring in the cortical cytoskeleton, which is located under the cell membrane. The organization of cortical cytoskeleton depends on actin-binding regulatory proteins (Furuhashi et al., 2012). Bunches of contractile actin filaments run parallel to the cell membrane and are attached to the adhesive “bands” by a complex of intracellular linking proteins, such as vinculin (Furuhashi et al., 2012). Vinculin is a protein associated with the cytoplasmic surface of adhesive plaques, which anchors microfilaments containing actin in the cell membrane and attach the cell to the ground (Peng et al., 2011; DeMali et al., 2014; Atherton et al., 2015). The role of vinculin in cell adhesion sites has been described by Atherton et al. (2015). Flat, elongated structures rich in heterodimeric (α and β) cell adhesion receptors produce focal contacts - dynamic macromolecular complexes containing integrins. Cytoplasmatic tails of integrins bind to a wide spectrum of proteins that regulate activation and their interaction with the extracellular matrix. These focal adhesion proteins also send and respond to the extracellular signals and physical properties of the extracellular matrix and provide a physical linkage between the integrins and the cytoskeletal network of actin. Vinculin is recruited into the cytoplasmic tails of β-integrin proteins by interacting with the talin (Burridge and Mangeat, 1984; Yao et al., 2014). A study by Saunders et al. (2006) demonstrated that the presence of vinculin in the focal contacts is crucial for cell adhesion and migration *via* the integrin-actin linkage system. Vinculin reduces cellular motility by affecting cell adhesion: cells lacking vinculin evince less adhesion but faster migration. Conversely, in cells overexpressing vinculin, the number and size of focal contacts increase, whereas motility decreases. Visualization of vinculin is one of the methods of cell adhesion evaluation to various types of substrates (Furuhashi et al., 2012; Tan et al., 2015; Wytrwal et al., 2016). Another part of the cytoskeleton are microtubules. They are made up of polymerised α- and β-tubulin dimers, which build protofilaments and have a distinct polarity. Microtubules play a crucial role during cell migration; they are able to grow and shrink in order to generate force to remodel. Microtubules also control focal adhesion dynamic in migrating cells by modulating Rho GTPase signaling that control actomyosin-based contractility (Stehbens and Wittmann, 2012).

The graphene substrates affect the adhesion and proliferation of various adherent cells (Kalbacova et al., 2010; Tan et al., 2015) and may be used in tissue engineering (Lee et al., 2013; Gurunathan et al., 2014). The effect of graphene on the cytoskeleton of migrating fibroblasts, with particular emphasis on vinculin, has never been studied before. Therefore, in the present study we evaluated the rearrangement and architecture of cellular cytoskeleton (microfilaments and microtubules) and vinculin organization in L929 fibroblasts in response to this substrate produced by the Institute of Electronic Materials Technology (ITME) (Strupinski, 2011; Strupinski et al., 2011; Ciuk et al., 2013). We focused on the basic methods used in material toxicity assessment and the organization of the cellular cytoskeleton with particular emphasis on changes in adhesion and migration of fibroblasts on two different substrates, glass and graphene. Data on structural characteristics of cells will help to understand how fibroblasts move on the graphene substrate and whether graphene is safe for cytoskeletal components. These tests can be used to evaluate the usefulness of graphene as a scaffold for connective tissue cells that could be used as potential “dressings” in the treatment of tissue injury in the future.

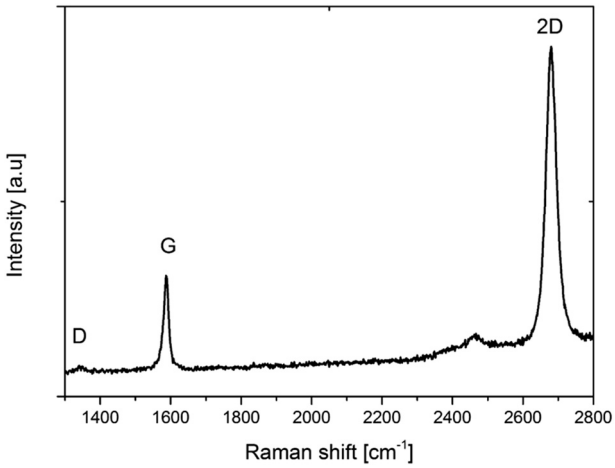

Fig. 1. Raman spectrum of graphene.

2. Materials and methods

2.1. Material

Production of graphene was conducted by chemical vapor deposition (CVD) of volatile phases in a CVD reactor Black Magic Pro 6″. Studies described in this report were carried out with graphene produced on copper substrate, and then transferred onto rounded glass cover slips (1 cm diameter and 0.17 mm of thickness) at the Institute of Electronic Materials Technology (ITME). Electrochemical delamination was used in the transfer process (Ciuk et al., 2013). A sheet of graphene was placed on a cover slide. Cover slides coated with graphene were sterilized by UV (30 min. on the both sides) and placed into wells of a 24-well plate. The quality of each preparation was certified, by measuring Raman spectrum with characteristic peaks marked (Fig. 1), and optical microscope picture of graphene on a cover glass (Fig. 2). Raman spectrum of graphene proves existing of monolayer graphene on glass substrate (Ferrari et al., 2006; Gupta et al., 2006; Graf et al., 2007; Lee et al., 2008). Low Full Width at Half Maximum (FWHM about 34,6 cm^{-1}) of the 2D band for graphene layer presented in Fig. 1 as well as much higher (I_{2D}/I_G = 2,9) intensity of the 2D band by comparison with the intensity of the G band are characteristic of a single layer of graphene. Very low D bands on spectrum is typical of high quality graphene structures (A_D/A_G = 0,09). Additionally, optical microscope image confirmed that graphene is uniform when it comes to its

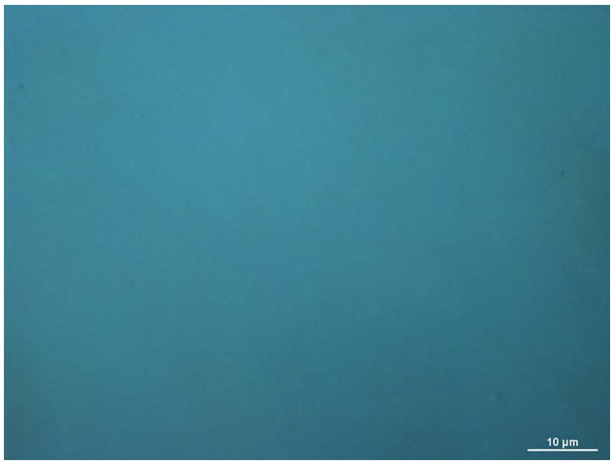

Fig. 2. Optical microscope image of graphene applied on glass cover slide.