Graphene substrates promote adherence of human osteoblasts and mesenchymal stromal cells

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ABSTRACT

The biocompatibility of large single layer graphene produced by chemical vapour deposition was investigated using human osteoblasts and mesenchymal stromal cells. The study was focused on cellular adhesion, morphology and the ability to proliferate on graphene substrates. It was found that both of the cell types which were tested adhered and proliferated better when cultured on graphene films than on a SiO2 substrate.

1. Introduction

Graphene is a new two-dimensional material that is attracting a lot of attention due to its unique structural and electronic properties. The exceptional electrical and mechanical characteristics of graphene can be utilised in new electronic devices [1], electromechanical resonators [2] and/or composites [3]. Due to its conductivity, graphene has the potential to be greatly useful in electrical bio-sensing applications [4]. However, biocompatibility is a prerequisite for the use of graphene in biological or medical applications. It is also necessary to evaluate the toxicological and ecological risks of graphene.

Carbon-based materials (carbon nanotubes or nanocrystalline diamonds) are widely tested for both their potential toxicological risks and their possible use in biomedical applications. The results of studies involving carbon nanotubes are still contradictory, showing cytotoxic effects in some cases and improved cell growth in other cases [5]. The toxicity of nanotubes is apparently affected by their degree of dispersion, their level of functionalisation and their length [6–8]. Furthermore, an important issue in biocompatibility tests of carbon nanotubes is their purity. Contaminants, such as amorphous carbon or residuals of catalytic particles, may dramatically affect the living environment. The purification procedures may also introduce cytotoxic substances (e.g. sodiumdodecylsulphate), which may increase the toxicity of the purified sample.

A positive effect on cell adhesion, proliferation and differentiation was observed in the case of osteoblast cultivation on nanocrystalline diamonds. This revealed that diamond modification, which alters the surface hydrophilic/hydrophobic character [9], and the nano-roughness of the surface [10,11] are both important factors which play a significant role in cell cultivation.

In contrast to carbon nanotubes, the topography of single layer graphene is very simple. Furthermore, graphene can be synthesised in a relatively pure form, which allows the biological tests to be less affected by impurities. Therefore, graphene is an ideal model material for experiments with adherent (anchorage-dependent) cells (e.g. osteoblasts, mesenchymal stromal cells (MSCs), etc.).

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Adhesion of osteoblasts is a crucial prerequisite to subsequent cell functions, such as proliferation, synthesis of proteins (e.g., proteins of extracellular matrix (ECM), morphogenetic factors and osteoinductive molecules) and formation of mineral deposits. Adhesion is generally dependent on time, adhesive forces at the cell/material interface, and surface topography [12]. Cell adhesion is primarily mediated by integrins, a widely expressed family of transmembrane adhesion receptors [13]. Upon ligand binding, integrins rapidly associate with the actin cytoskeleton and cluster together to form focal adhesions (FAs), which are discrete complexes that contain structural (e.g., vinculin) and signalling molecules (e.g., focal adhesion kinase) [14]. FAs are central elements in the adhesion process because they function as structural links between the cytoskeleton and ECM to mediate stable adhesion and migration. Furthermore, in combination with growth factor receptors, FAs activate signalling pathways that regulate transcription factor activity and direct cell growth and differentiation [15]. Vinculin is commonly found one of the most prominent residents in FAs [16] and appears to facilitate the assembly of proteins in focal adhesions by cross-linking with various partners.

Human MSCs are mononuclear cell population adherent to tissue culture plastic and have been isolated from adult bone marrow. They are capable of further proliferation as well as differentiation into multiple lineages involved with connective tissue (osteoblasts, adipocytes and chondrocytes) when exposed to various growth factor combinations [17] or substrates with different topography and rigidity [18]). Thus, these cells may serve as a good model for testing the possible increased/accelerated differentiation induced by adhesion onto graphene surfaces.

Recent advances in chemical vapour deposition (CVD) synthesis have allowed the preparation of sufficiently large graphene flakes that are suitable for biological tests. This study is the first to investigate the influence of graphene film synthesised on copper foil by CVD and subsequently transferred to a SiO2 substrate used further for cell experiments. Our results show that the graphene is biocompatible for human osteoblasts as well as for human MSCs. Furthermore, the graphene substrate stimulated the growth of the tested cells. Hence, the graphene can be suggested as a biocompatible, conductive, and patternable material for implant engineering and other medical applications.

2. Experimental

Graphene samples were synthesised using previously published CVD method [19]. Briefly, a thin copper foil (2 x 5 cm) was heated to 1000 °C and annealed for 20 min under H2 gas flowing at 30 sccm (standard cubic centimeters per minute). The film was exposed to H2 and CH4 for 20 min, and finally, the substrate was cooled down from 1000 to 500 °C under H2 and CH4. The synthesised graphene was transferred to a clean SiO2/Si substrate using polymethylmethacrylate (PMMA) according to previously reported procedures [20]. The Raman spectra were recorded by a Labram HR spectrometer (Horiba Jobin Yvon) interfaced to an Olympus BX-41 microscope (objective 50x). The spectrometer was calibrated using the Fg mode of Si at 520.2 cm⁻¹.

Human MSCs were obtained from patients undergoing diagnostic trephine biopsies for suspected haematological disease without tumour-affected bone marrow after giving written consent. MSCs (passage 3) were plated (10,000 cells/cm²) on substrates and incubated at 37 °C in a 5% CO2 atmosphere in AlphaMEM medium supplemented with heat inactivated 10% fetal bovine serum (PAA), penicillin (20 U/ml) and streptomycin (20 μg/ml).

Human osteoblast-like cell line (SAOS-2) was obtained from DSMZ GmbH. Cells were plated (20,000 and 25,000 cells/cm²) on substrates and incubated at 37 °C in 5% CO2 in McCoy's 5A medium without phenol red (BioConcept) supplemented with 15% heat inactivated fetal bovine serum (PAA), penicillin (20 U/ml) and streptomycin (20 μg/ml). These immortalised cells were stable and exhibited features of mature osteoblasts (pronounced alkaline phosphatase activity and mineralisation) [21].

The morphology of the focal adhesions of SAOS-2 and MSC cells was characterised by immunofluorescent staining of vinculin (1:150, Sigma, anti-mouse Alexa 568), actin filaments (Phalloidin-Alexa-488, 1:100, Invitrogen) and nuclei (4',6-diamidino-2-phenylindole (DAPI), 1:1000, Sigma). The epi-fluorescent Nikon E-400 microscope was used (Hg lamp, UV-2A, B-2A and G-2A filter set) and data were recorded by the DS-5M-U1 Color Digital Camera (Nikon). Three-dimensional images of cells were acquired using a Nikon TE2000E microscope equipped with a confocal scanning head (Csi1), oil immersion objective Apo TIRF 60x (N.A. 1.49) and excitation laser 543 nm. Emission of individual fluorophors was detected using a 610/75 band-pass filter. Image sampling density was corrected according to Nyquist criterion. Selected z-stack images were restored (deconvolved) using a maximum likelihood restoration algorithm in the Huygens Professional Software and the measured point spread function. Restored z-stack images were further volume rendered using Imaris Personal software (Bitplane).

In order to count cells, nine size-calibrated fluorescence pictures of DAPI stained cell nuclei from each surface type were obtained using a Nikon E400 microscope with a 4x lens. Areas of 1 mm² were cut out of these calibrated pictures using NIS-Elements software (LIM, Prague). The cell nuclei on these 1 mm² areas were automatically counted after binary masking using C++ scripting for NIS-Elements.

3. Results and discussion

Fig. 1A shows a typical Raman spectrum of a CVD graphene sample on a SiO2/Si substrate excited by 2.33 eV laser energy radiation at an electrode potential of 0 V. The spectrum was dominated by the two features typical for graphene-based materials: the G (TG) band at 1590 cm⁻¹ and the G' (2D) band at about 2688 cm⁻¹. Raman spectroscopy has been frequently used to distinguish a single layer of graphene (1-LG) from multilayer graphene (M-LG). It is generally accepted that the G' band for M-LG is significantly broadened in comparison to the G' band for 1-LG, and the relative intensities of the G band and the G' band change dramatically. The FWHM line-width of the G' band in Fig. 1A is about 30 cm⁻¹, which is a
typical width of single layer graphene. However, it has been shown recently that the broadening of the G’ band may be absent, even in multilayer graphene, if the graphene sheets are misoriented relative to one another [22]. Another signature of single layer graphene is the ratio between the intensity of the G and the G’ band. This approach toward distinguishing between 1-LG and M-LG has been recently criticised because the G’ mode intensity ($I_{G’}$) is strongly dependent on the doping level while the G mode intensity ($I_G$) is not. To avoid the effect of doping, we measured Raman spectra of graphene in the electrochemical cell at a given electrode potential (0 V) using a PAR potentiostat. The electrolyte solution used was 0.1 M LiClO$_4$ dissolved in dry propylene carbonate/PMMA (Aldrich) (Fig. 1A). This ensured that the natural doping was considered and the doping state of the graphene sample was controlled by the externally applied potential. A comparison of the peak intensity of the G’ band and the peak intensity of the G band in Fig. 1A shows that the $I_{G’}/I_G \approx 7$. This confirms that the Raman spectrum corresponds to a single layer graphene.

Fig. 1B shows an optical microscope image of the CVD graphene sample. The thickness of the SiO$_2$ layer was 300 nm, which was convenient for determining the number of graphene layers. The presence of single layer graphene in our sample was confirmed by colour analysis as discussed previously [20].

In order to evaluate the biocompatibility of CVD grown graphene films, we plated human osteoblasts, SAOS-2 cells (immortalised cell line) and human MSCs (primary cells), on the graphene film and on SiO$_2$ which was used as a support material for graphene. In these experiments, SiO$_2$ was used as a biocompatible reference material for cell plating, which was established in a previous study [23].

SAOS-2 cells are widely used among different osteoblastic cell lines for biocompatibility experiments based on their cell anchorage-dependency [6,24,25]. Thus, it is relevant to use
SAOS-2 cells for testing the impact of graphene on this cell line.

After a 48 h incubation, we found osteoblasts homogenously covering the graphene film in a confluent layer (Fig. 2A) but forming separate islands on the SiO2 substrate (Fig. 2B). It can also be concluded from the images that more cells are present on the graphene film than on the SiO2 substrate (more blue-stained nuclei are apparent on the graphene substrate, and the cells already form multi-layers). This was confirmed by counting the cells on both substrates: 46,700 (±9300) cells/cm2 on the graphene film and 36,700 (±5500) cells/cm2 on the SiO2 substrate. In other words, the initially plated osteoblasts (25,000 cells/cm²) almost doubled in density using graphene as the substrate. This result is consistent with the reported doubling time (43 h) of the cell line (DMSZ, GmbH). On the other hand, the amount of cells on the SiO2 substrate increased only by a factor of 1.5 after a 48 h incubation. The observed formation of clusters on the SiO2 substrate is similar to that observed with other cells cultivated on glass substrates [26]. Graphene was shown to be an optimal (suitable) surface for cell growth because of the formation of a confluent layer, the increased number of cells, and the higher proliferation of cells in comparison to the SiO2 substrate.

A well-defined border between the graphene film and the SiO2 substrate was created by high density cell seeding at (25,000 cells/cm²) (Fig. 3). Fig. 3 shows that osteoblasts clearly prefer the graphene substrate. We note that with a lower seeding density (20,000 cell/cm²), which was also tested, the osteoblasts did not reach confluency after 48 h and the border between the graphene film and the SiO2 substrate was not apparent. Hence, it is obvious that cell density is an important factor in observing the different properties of the substrates. Therefore, cell density must be controlled in biological tests for graphene and other substrates. Our result is in agreement with previously observed cell behavior on patterned diamond hydrophobic/hydrophilic arrays [9].

A major area of interest in bone reconstructive surgery is the osteogenic differentiation potential of human MSCs. Therefore, data on the biocompatibility and application utility of graphene film for surface engineering (coating) of implants with human MSCs is highly desired. Fig. 4 shows MSCs on the graphene layer and on a SiO2 substrate after a 48 h incubation showing pattern, similar to osteoblasts (Fig. 2). MSCs cultivated on the graphene layer were homogenously dispersed on the surface and showed spindle-shape morphology, whereas those cultivated on the SiO2 substrate formed distinct islands (clusters) containing more polygonal cells. It has been shown that the MSC morphology regulates biological processes such as proliferation [27] and differentiation [28,29]. Moreover, recent studies suggest that the cell shape, which is dependent on cell plating density, is a key regulator in MSC’s commitment to osteoblast or adipocyte lineages [30]. Thus, the elongated MSC morphology on graphene allowed a higher proliferation than the polygonal MSC morphology on the SiO2 substrate with possible capacity of differentiation towards the osteoblast lineage on graphene substrate. These results are very promising for bone reconstructive surgery.

Fig. 2 – Fluorescent images of human cells incubated on graphene and SiO2 substrate for 48 h: (A) human osteoblasts on graphene, (B) human osteoblasts on SiO2 substrate (actin filaments are stained green and nuclei are stained blue). The scale bar is 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3 – Fluorescent image of SAOS-2 cells forming on the border of the graphene (left) and the SiO2 (right) substrate (actin filaments are stained green and nuclei are stained blue). The dashed line marks the border between graphene and the SiO2. The scale bar is 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
For a more detailed analysis of cellular adhesion on graphene and SiO₂ surfaces, osteoblasts as well as MSCs were immunofluorescently stained for vinculin, a structural protein associated with FA. Vinculin connects other proteins of FA, thus ensuring proper FA formation and turnover (Fig. 5). The 2D images from an epi-fluorescent microscope (Fig. 5A, C, E and G) illustrate that the cells cultivated on graphene films demonstrated a common morphology (fibroblast-like shape or spindle-like shape for osteoblasts and MSCs, respectively) for typical proliferating osteoblasts and MSCs. However, both cell types cultivated on the SiO₂ substrate demonstrated the morphology of non-proliferating cells (round or polygonal cell shape for osteoblasts and MSCs, respectively). Also, the distribution and size of FAs varied depending on the substrate. Both cells (more pronounced in osteoblasts) plated on the SiO₂ substrate formed FAs that were larger and homogenously distributed on the cell periphery resulting in some state of quiescence. Large FAs indicate good anchoring of the cells without the ability to move or proliferate. The FAs in contact with graphene films are smaller, weaker and concentrated to the protruding ends of the cells, which corresponds to an active state of the cells. This indicates possible proliferation of the cells as well as possible motility. These factors are important for the body to accept implants. Single-cell 3D images (Fig. 5B, D, F, H) confirmed and highlighted the already observed cell morphology: proliferating osteoblasts were fibroblast shaped (Fig. 5B), osteoblasts on the SiO₂ substrate were round (Fig. 5D), active MSCs were spindle shaped (Fig. 5F), and MSCs on the SiO₂ substrate were polygonal (Fig. 5H).

We note that the SiO₂ may not be an ideal substrate. However, it is clearly visible from presented pictures that cell morphology is not affected by this substrate and there is a confluent layer of cells which indicates a good proliferation. The hallmarks of apoptosis or necrosis would be visible on our pictures, however we have not found any. Thus we suggest that, the visual effects, such as cell viability and morphology, are good initial screen to decide the value and potential of a biomaterial.

Fig. 4 – Fluorescent images of human MSCs incubated on graphene and on a SiO₂ substrate for 48 h: (A) MSCs on graphene, (B) MSCs on a SiO₂ substrate (actin filaments are stained green). The scale bar is 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5 – Fluorescent images of FAs (staining of vinculin) formed in human cells on different substrates: (A) osteoblasts on graphene (2D image from an epi-fluorescent microscope), (B) osteoblasts on graphene (3D image from a confocal microscope), (C) osteoblasts on SiO₂ (2D image), (D) osteoblasts on SiO₂ (3D image), (E) MSCs on graphene (2D image), (F) MSCs on graphene (3D image), (G) MSCs on SiO₂ (2D image) and (H) MSCs on SiO₂ (3D image). The scale bar is 50 μm.
Graphene as well as other carbon nanostructures are naturally hydrophobic [31], however surface wettability is affected not only by surface chemistry but also by topographical parameters such as roughness and texture. Surface wettability may affect the proliferation of cells because the initial phase of attachment involves the physico-chemical linkages between cells and surfaces through ionic forces or indirectly through an alteration in the adsorption of conditioning molecules e.g. proteins [32]. Hence, more detailed studies are needed to fully understand the interaction between cells and graphene. It would be also interesting to compare the behavior of single and multilayer graphene. Multilayer graphene would flatten the morphology of substrate and thus it will be detected as other type of surface by cells. In addition, electronic structure is dependent on number of graphene layers which may potentially affect the interaction between cells and studied substrate.

4. Conclusions

We demonstrated for the first time that CVD grown graphene is not toxic for human osteoblasts and MSCs. Furthermore, we showed that graphene stimulated their growth. We also showed that cell density was an important factor in observing different surface properties. Therefore, the cell density must be controlled in biological tests of graphene and other materials. The graphene probably has a potential to induce MSC differentiation into the osteoblast lineage (further experiments are necessary), which is promising for bone reconstructive surgery.

Our results indicate that graphene films can be potentially used for engineering of new osteo-conductive/inductive implants or their components. The electrical conductivity of graphene is of particular importance because electricity as well as cocktails of growth factors and substrate properties is able to stimulate cell growth and differentiation. The use of graphene has a potential advantage because it can be patterned on desired areas, which allows engineering of cell growth in specific arrays.

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