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Substrate effect modulates adhesion and proliferation of fibroblast on graphene layer

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ABSTRACT

Graphene is an emerging candidate for biomedical applications, including biosensor, drug delivery and scaffold biomaterials. Cellular functions and behaviors on different graphene-coated substrates, however, still remain elusive to a great extent. This paper explored the functional responses of cells such as adhesion and proliferation, to different kinds of substrates including coverslips, silicone, polydimethylsiloxane (PDMS) with different curing ratios, PDMS treated with oxygen plasma, and their counterparts coated with single layer graphene (SLG). Specifically, adherent cell number, spreading area and cytoskeleton configuration were exploited to characterize cell-substrate adhesion ability, while MTT assay was employed to test the proliferation capability of fibroblasts. Experimental outcome demonstrated graphene coating had excellent cytocompatibility, which could lead to an increase in early adhesion, spreading, proliferation, and remodeling of cytoskeletons of fibroblast cells. Notably, it was found that the underlying substrate effect, e.g., stiffness of substrate materials, could essentially regulate the adhesion and proliferation of cells cultured on graphene. The stiffer the substrates were, the stronger the abilities of adhesion and proliferation of fibroblasts were. This study not only deepens our understanding of substrate-modulated interfacial interactions between live cells and graphene, but also provides a valuable guidance for the design and application of graphene-based biomaterials in biomedical engineering.

1. Introduction

As a new two-dimensional material, graphene has recently been thought of as a perfect candidate for biomedical application due to its unique properties [1–5]. For example, it has been used as a coating material in tissue engineering for its exceptional antibacterial activity and high cytocompatibility [6–8]. Previously, it was found that homogenous graphene substrates played a key role in regulating cell functions and behaviors. For instance, single layer graphene (SLG), which was produced by chemical vapor deposition (CVD), increased the adhesion of osteoblasts and hMSCs (human mesenchymal stem cells) [9]. It has been recognized that micropatterned CVD SLG substrates can not only significantly enhance the adhesion and growth of neuron [10], but also accelerate the osteogenic differentiation of hMSCs [11–13].

It is well known that graphene has to be supported on a substrate for cell culture and tissue engineering application. In fact, the published results of graphene's exceptional cytocompatibility were mostly supported by an underlying SiO_2 or glass substrate, which was also suitable for cell culture. Simultaneously, recent progresses have revealed that the underlying substrate can exert a remarkable impact on the surface properties of graphene. It has been reported that, for instance, the wetting property of graphene was tuned by its underlying substrate [14,15]. The adsorption ability [16,17], morphology [18] and apparent mechanical properties [19] of graphene

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were also closely related to the underlying substrates. So it is reasonable to speculate that graphene-induced cytocompatibility may be also regulated by the properties of its underlying substrates.

In the current work, different substrates, including widely used glass, commercially available silicone sheets, and polydimethylsiloxane (PDMS), with or without SLG-coating were prepared to investigate the effect of the underlying substrate on graphene-induced adhesion and proliferation of NIH-3T3 fibroblasts. Two curing ratios of PDMS substrates with different stiffness were used (stiffer mixture of 10:1 and softer mixture of 50:1, referred as P10 and P50, respectively). Further, in order to investigate the influence of substrate wetting property on graphene-induced cell adhesion and proliferation, P10 was modified by oxygen plasma (hereafter referred as p-P10). Then, the behaviors were investigated, including early adhesion of cell, morphology, cytoskeleton structure and proliferation of fibroblasts. The experimental results demonstrated that graphene improved the substrate cytocompatibility even if the bare substrate was not originally suitable for cell growth. Notably, the fibroblasts adhesion and proliferation were not completely equivalent in all SLG-coated substrates; especially the graphene-induced fibroblasts adhesion and proliferation on softer substrates were lower than that on stiffer substrates significantly. These findings imply that the underlying substrates can exert a conspicuous effect on graphene-induced adhesion and proliferation of fibroblasts. It deepens our understanding of interfacing live cells with graphene, which provides a valuable guidance for the design and application of graphene-based materials in tissue engineering.

2. Materials and methods

2.1. Materials and reagents

SLG on copper foil grown by CVD was purchased from ACS Materials (Massachusetts, USA). Glass coverslips (thickness range: 0.13–0.17 mm) were purchased from VWR (West Chester, USA). PDMS (Sylgard 184) was bought from Dow Corning (Michigan, USA). Commercial silicone sheets (~250 μm in thickness; SM08111321) were purchased from SMI (Michigan, USA). Polymethylmethacrylate powder (PMMA, molecular weight: 996000) was obtained from Sigma-Aldrich (182265, St. Louis, Mo, USA). Ammoniumpersulfate (APS), hydrogen peroxide, sulfuric acid, ethanol and acetone were bought from Beijing Chemical Reagent Company (Beijing, China). All these chemicals were used as received.

2.2. Preparation of bare substrates

Glass substrates were cleaned before used by immersing into piranha solution (hydrogen peroxide (30%)/sulfuric acid 1:3) for 10 min at 120 °C, washed with deionization (DI) water and ethanol, and then dried by blowing nitrogen gas.

PDMS contains two chemical parts, i.e., a PDMS polymer base (A) and a curing agent (B). Different curing ratios of 10:1 and 50:1 were thoroughly mixed and degassed in experiments (referred as P10 and P50, respectively). The PDMS mixture was subsequently poured onto a cover glass and heated at 70 °C for 6 h in an oven to accelerate the polymerization and cross-linking.

Oxygen plasma (FEMTO plasma cleaner, 40 kHz frequency) was used to hydrophilic modification of PDMS substrate with the curing ration of 10:1 (referred as p-P10). The system pressure and oxygen flow rate were kept constant at 100 micro-bars and 20 sccm (standard cubic centimeters per minute), respectively. The plasma power was set at 60 w and lasted for 30 s.

2.3. Graphene transfer

In order to ensure the integrity of graphene during transferring, and simultaneously consider the different properties of the underlying substrates, two different methods were chosen to transfer graphene. The first method was based on directly adhering graphene from copper foil for the flexible and hydrophobic substrates, such as silicon, P10 and P50 used in this study [20,21]. Both sides of as-grown CVD graphene on 25 μm thick copper foils were covered by graphene. In order to remove one side of graphene, a piece of graphene covered copper foil was pressed on a flat substrate gently to make it as possible as flat, PDMS film was then cut into strips and used to seal the copper foil boundary on a flat glass plate. The graphene was destroyed with the treatment of Oxygen plasma of 100 W for 3 min. Then we used PDMS or silicone samples to adhere the other side of the copper foil. To make it adhere firmly, it was necessary to make sure the copper foil was flat. Then relatively dilute APS solution of 0.5 M was adopted to etch the copper. After copper foil was etched completely, the sample was washed with flowing DI water and drying by a weak flow from a nitrogen gun.

The other transfer method was based on widely used PMMA transfer technique for common solid and hydrophilic substrates, for instance glass and p-P10 used in the present experiments [22,23]. In short, a thin protective layer of PMMA was spin coated at 500 rpm for 20 s and 2000 rpm for 30 s on the copper foil, and then heated on a hot plate at 170 °C for 3 mins. The other side of graphene was removed by oxygen plasma under the same conditions as that utilized in the first method. After copper was etched in APS solution and carefully washed, the floating PMMA-coated graphene film was scooped with glass and p-P10 substrate. The samples were left overnight to dry in ambient condition. The top PMMA coating was stripped off in hot acetone of 50 °C for 30 min and followed by dipping in ethanol to rinse off any residue. SLG-coated substrates were referred as silicone-g, P10-g, P50-g, and p-P10-g, respectively.

2.4. Characterization of various substrates

2.4.1. Raman spectra detects graphene

Raman spectra were carried out using LabRAM ARAMIS System (HR800, HORIBA, Paris, France) equipped with a 532 nm laser source. The laser spot has a diameter of 1 μm. To avoid laser-induced heating and damage to the graphene, the laser power was kept below 2 mW during scanning.

2.4.2. Static contact angle measurements

Static contact angle measurements were performed at room temperature (22–25 °C) at a relative humidity of 27–43% using an automatic contact angle meter (OCA 20, DataPhysics, Stuttgart, Germany). A volume of 2 μL DI water drop was used during the static contact angle measurement. Each group contains five parallel samples and 3 test points were randomly chosen for each sample.

2.4.3. Atomic force microscopy and elastic modulus measurement

The surface morphology of transferred graphene on these various substrates was investigated by atomic force microscope (MFP-3D, Asylum Research, USA) utilizing the tapping mode with a silicon cantilever with a spring constant of ~1.2 N/m and resonance frequency of ~75 kHz. The root mean square roughness (rms) of the images was also evaluated using the integrated software. Five positions were chosen with an area of 5 μm × 5 μm for each position. In the experiment, force-distance curves were also collected to obtain the apparent stiffness of these substrates. For the sphere indentation as adopted here, the Young's modulus was finally quantified via the classical Hertz model [24,25].