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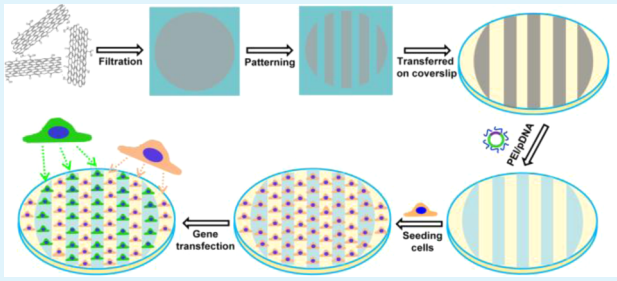
Patterned Substrates of Nano-Graphene Oxide Mediating Highly Localized and Efficient Gene Delivery

Kunyang Li, Liangzhu Feng, Jianwei Shen, Qi Zhang, Zhuang Liu, Shuit-Tong Lee, and Jian Liu*

Institute of Functional Nano & Soft Materials (FUNSOM), Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou, Jiangsu 215123, People's Republic of China

Supporting Information

ABSTRACT: A facile approach was developed to fabricate patterned substrates of nano-graphene oxide, demonstrating highly localized and efficient gene delivery to multiple cell lines in a substrate-mediated manner. The GO substrates served as a valid platform to preconcentrate PEI/pDNA complexes and maintain their gradual releasing for a relatively long period of time. Our approach allowed successful gene delivery in selected groups of cells on the stripe-patterned GO substrates, without transfecting their neighbor cells directly cultured on glass. These GO substrates exhibited excellent biocompatibility and enabled effective gene transfection for various cell lines including stem cells, thus promising important applications in stem cell research and tissue engineering.



KEYWORDS: *localized gene delivery, nano-graphene oxide, patterned substrates, nano−bio interface*

INTRODUCTION

The development of gene delivery techniques has been driven by the merging needs in the stem cell research,[1,2] applications of knock-in/knock-out animal models,[3] and gene therapies,[4] fostering a variety of methods with distinctive characteristics. Among them, the methods using nonviral gene delivery carriers (e.g., cationic lipids, dendrimers, polymers, and inorganic nanoparticles) have drawn attention increasingly, thanks to their versatile properties tunable by synthesis and relatively low bio-safety risks.[5−10] These nonviral carriers can introduce gene transfection in cells from the liquid phase in a conventionally bulky approach,[8,9] or from the surface of the predeposited solid phase in a substrate-mediated manner.[11−13] In the latter method, the cells directly contact the gene vector-loaded carrier materials on the solid substrate. The interactions at their interface (of micro/nano scale in many cases) have been found to play a key role determining the performance of gene delivery, and the gene vectors preadsorbed on the substrate are able to transfect cells in a spatially controllable fashion.[13−16] Although various techniques of gene delivery have been developed in either of these two approaches, it remains a great challenge to simultaneously meet the critical requirements in this field, including high transfection efficiency, improved selectivity, and minimized cytotoxicity.

The unique structure and physical/chemical properties of graphene (G) or graphene oxide (GO) have motivated researchers to explore diversified biological applications,[17] including biosensors,[18,19] gene/drug delivery,[20−22] cancer photothermal therapy,[23] antibacterial materials,[24,25] and tissue engineering.[26,27] Previous reports in the literature showed that, in aqueous suspensions, GO derivatives facilitated delivery of siRNA or plasmid DNA into cells, while protecting DNA from enzymatic cleavage.[28,29] In addition, the cytotoxicity of cationic polyethylenimine (PEI) could be remarkably reduced by complexing or conjugating it with GO.[21,30] On the other hand, G/GO deposited on the solid substrates was demonstrated to promote the adhesion, proliferation, and differentiation of many types of cells, including MSCs and osteoblasts.[26,27,31−33] These substrates showed fascinating capabilities to accelerate stem cell renewal and differentiation, which might be derived from one or several factors collectively, such as surface roughness induced stress,[33] topographic cues by 3D structures,[31] and differential molecular interactions by chemical inducers[26] at the interface between the cells and the substrates. However, it has not been reported yet to perform gene transfection on GO substrates in the literature.

In this work, we have developed a facile approach to fabricate the substrates of nano-graphene oxide with patterns for gene transfection in cells. It represents a new initiative of highly localized and efficient gene delivery mediated by GO substrates. Our experiments suggest that the as-prepared GO substrates can adsorb the complexes of polyethylenimine (PEI) and plasmid DNA effectively and then maintain their gradual releasing for a relatively long period of time. Therefore, they serve as a valid platform to preconcentrate PEI/pDNA complexes for enhanced gene delivery. It significantly promotes efficiency of gene delivery to cells cultured on the patterned GO substrates, featured with

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highly spatial selectivity. In the present study, we have demonstrated that GO substrates exhibit excellent biocompatibility and enable successful gene transfection for various cell lines including stem cells, thus promising important applications in genetic manipulations of cells, stem cell research, and tissue engineering.

EXPERIMENTAL SECTION

Materials. Branched polyethylenimine (PEI, 25 KDa), graphite powder, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DAPI were obtained from Sigma-Aldrich. Calcein-AM and PI were purchased from Dojindo Laboratories (Kumamoto, Japan). Cellulose acetate membrane (0.22 μm) was purchased from JinTeng Experiment Equipment Co., Ltd (Tianjin). Cell medium was obtained from Hyclone, and fetal bovine serum (FBS) was purchased from GIBCO. The EGFP plasmid DNA (pDNA) was amplified according to the standard protocol and purified using an endotoxin-free plasmid kit (TianGEN, TianGEN Biotech CO. LTD) according to the manufacturer's protocol. The concentration of plasmid DNA was quantified by UV spectral measurements at 260 nm with an extinction coefficient of 50 μL ng^{-1} cm^{-1}.

Preparation of Patterned GO Substrates. Graphene oxide (GO) nanosheets were synthesized according to the previously reported procedure of a modified Hummer's method.[34] Then, 15 mL of GO suspension (5 μg/mL) was gradually dropped on the cellulose acetate membrane under the filtration for 0.5 min, assisted by vacuum suction. A PDMS device containing a predesigned pattern on its surface was used as a micro stamp, and pressed on the GO film. When the PDMS stamp was removed, it left the complementarily-patterned GO film on the cellulose acetate membrane. The as-prepared GO film with the support of the cellulose acetate membrane was flipped over and in physical contact of a piece of precleaned glass coverslip, and then pressed by a heavy metal unit. The weight was kept for several hours or overnight at the room temperature to ensure transferring the patterned GO on the glass coverslip. This procedure was straightforward and robust, allowing patterned GO film with features as small as 100 μm transferred onto glass (or silicon wafer). The as-prepared GO substrates were stable at room temperature with a long shelf time. They were sterilized by the UV lamp irradiation before the cell experiments.

Characterizations of GO Suspensions and GO Substrates. The size distribution of GO in suspensions was measured by the method of dynamic light scattering (Zetasizer, Malvern), suggesting the diameter of nearly 200 nm on average (Figure S1a, Supporting Information). The absorption spectra of GO (Figure S1b, Supporting Information) were measured by a PerkinElmer Lambda 750 UV−visible spectrophotometer. The atomic force microscopy (AFM) images of GO on a silicon wafer were recorded using a Multi-Mode V AFM (Veeco). The thickness of GO nanosheets was 1 nm approximately, indicating that the as-prepared GO were mostly monolayer (Figure S1c, Supporting Information). The scanning electron microscopy (SEM) images of the GO film were measured by a FEI Quanta 200F scanning electron microscope. The contact angles of water on the as-prepared GO film or other control substrates were imaged and measured by a DataPhysics OCA.

Adsorption and Desorption of PEI/pDNA Complexes on/from GO Film. A fluorescent method was developed to quantify PEI/pDNA complexes in aqueous solutions, which enabled indirect calibration of the adsorption efficiency of pDNA on GO substrates. The standard curve of fluorescence vs pDNA concentrations was obtained by the titration experiments using a fluorescence spectrometer (FLWOROMAX-4, HORIBA). In addition, the adsorption of PEI/pDNA complexes on GO substrates was also examined by a UV−vis spectrometer (PerkinElmer Lambda 750) and X-ray photoelectron spectroscopy (XPS) (Shimadzu, ULTRA DLD). For UV−vis experiments, GO was transferred onto quartz glass. For XPS experiments, GO was transferred onto a silicon wafer.

Cell Culture and Cytotoxicity Assays of the GO Substrates. HEK293T cells and HeLa cells were cultured in high glucose DMEM medium. hMSC cells were cultured in F-12 DMEM medium. The medium contained 10% fetal bovine serum (FBS), streptomycin (100 U mL^{-1}), and penicillin (100 U mL^{-1}). Cell culture was performed at 37 °C in a humidified atmosphere containing 5% CO_2 .

The cell viabilities of HeLa cells and HEK293T cells cultured on the GO film were examined using the standard methyl thiazolyl tetrazolium (MTT) assay. The cell samples cultured on the tissue culture microplate were assayed in parallel for normalization. The cell viability cultured on the different substrates was also evaluated by dual-color staining (calcein AM and PI) for live/dead cells according to the manufacturer's protocol. The bright-field images and fluorescent images were taken by a fluorescence microscope (Olympus) configured with Nuance CCD.

Localized Gene Transfection on the Patterned GO Substrates. The PEI/pDNA complexes at different N/P ratios were incubated with the as-prepared GO substrates overnight. After the supernatant was discarded, the GO substrates were washed twice with PBS before cell seeding. The cell samples of different cell lines (HEK293T and HeLa) were seeded on the GO substrates and cultured for 48 h (if not otherwise specified). hMSCs were typically cultured for 72 h. After localized gene transfection was performed with GO substrate mediation, the cell samples were examined for the fluorescence of GFP expression with a confocal microscope (TCS SP5, Leica). In the control experiments, the conventional method of gene transfection was performed according to a standard protocol. We used GO-coated glass coverslips, but without PEI/pDNA preadsorption in cell culture for fair comparison. An N/P ratio of 10 (if not otherwise specified) was typically administrated for the controls. At lower N/P ratios, the efficiency of GFP transfection was significantly decreased. In addition, the cells after transfection were trypsinized to prepare the cell suspensions, followed by a flow cytometry assay (BD FACS Calibur) to measure the percentages of the cells with GFP expression.

Luciferase Reporter Assay. HEK293T cells were transfected with PLG-3 plasmid with various N/P ratios of 10, 20, and 40. After 48 h transfection, cells were harvested and lysed, and the luciferase activities were measured by a Microplate System (Thermo) using a Luciferase Reporter Gene Assay Kit of Beyotime (RG005). Each experiment was performed in triplicate.

RESULTS AND DISCUSION

In our approach, preparation of patterned GO substrates primarily included two steps of transferring GO nanosheets and one step of patterning. As schemed in Figure 1a, we started with transferring nano-GO from the aqueous suspension onto the acetate membrane by the method of filtration assisted with vacuum. The thickness of GO film deposited on the filter acetate membrane was controlled by the concentration and total volume of the GO suspension. Afterwards, a polydimethylsilcone (PDMS) stamp was pressed on the top of the GO film/acetate membrane and then removed, leaving the complementary pattern of the PDMS stamp on the GO film. Last, the patterned GO film/acetate membrane was reversely attached onto the new substrate, such as a glass coverslip, and pressed mechanically with a heavy metal unit for overnight. Then, the metal unit and the acetate membrane were removed, and the patterned GO film was successfully transferred onto the glass coverslip.

The optical micrograph (Figure 1b) showed the as-prepared substrates including different patterns of GO film on the glass coverslip. We demonstrated successful transferring of GO film onto glass with features as small as 100 μm, which were defined by the width of the microchannels on the PDMS stamp surface (Figure S2, Supporting Information). The surface roughness of GO film after transferring was characterized by SEM, showing that it was overall flat, but with many tiny wrinkles in the length of sub-microns (Figure 1c). The quality of GO film after the steps of transferring described as above was verified by Raman spectrometry (I_D/I_G ratio: 1.15, as shown in Figure 1d). The measurements of contact angle indicated that the as-prepared GO substrates possessed medium hydrophilic/hydrophobic surface

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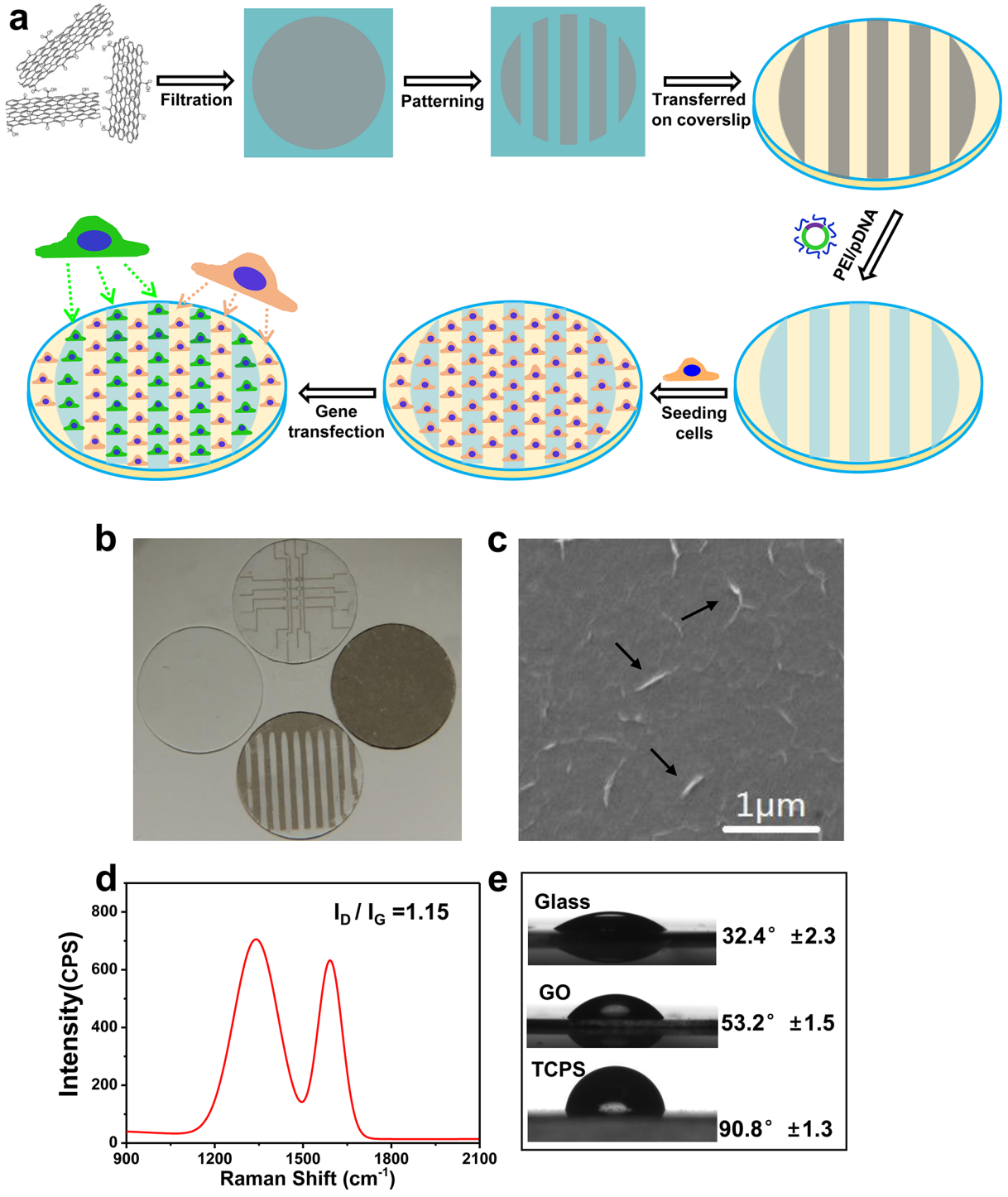

Figure 1. (a) Schematic illustration of preparing patterned GO substrates and the subsequent reverse gene transfection in cells. (b) Optical micrograph showing a panel of patterned GO substrates. (c) SEM image of GO substrates, black arrows indicating the wrinkles on the surface of GO. (d) Raman spectrum of GO substrates. (e) Contact angle of GO substrate, compared with glass and tissue culture plate.

property, compared with the bare glass and the regular cell culture microplate (Figure 1e). The surface roughness and hydrophilic/hydrophobic property may benefit the adhesion of cells on the GO substrate surface and loading of the complex of plasmid DNA samples for gene delivery.

We developed a robust method to study the adsorption and desorption of PEI/pDNA on the as-prepared GO substrates fluorescently (details available in the Supporting Information and Figure S3). Figure 2a shows distinctively different profiles of fluorescent change after incubation with the GO substrates and bare glass coverslips (as the control substrate). After the samples of PEI/pDNA complexes incubated with the GO substrates (blue curve), the fluorescence of the supernatants decreased quickly within 2 h. Further incubation till 6 h allowed most PEI/pDNA complexes to be adsorbed on the GO substrates, thus leading to minimized residue fluorescence in the supernatant (close to the blank control buffer solution). When the pDNA without PEI incubated with the GO substrates (red

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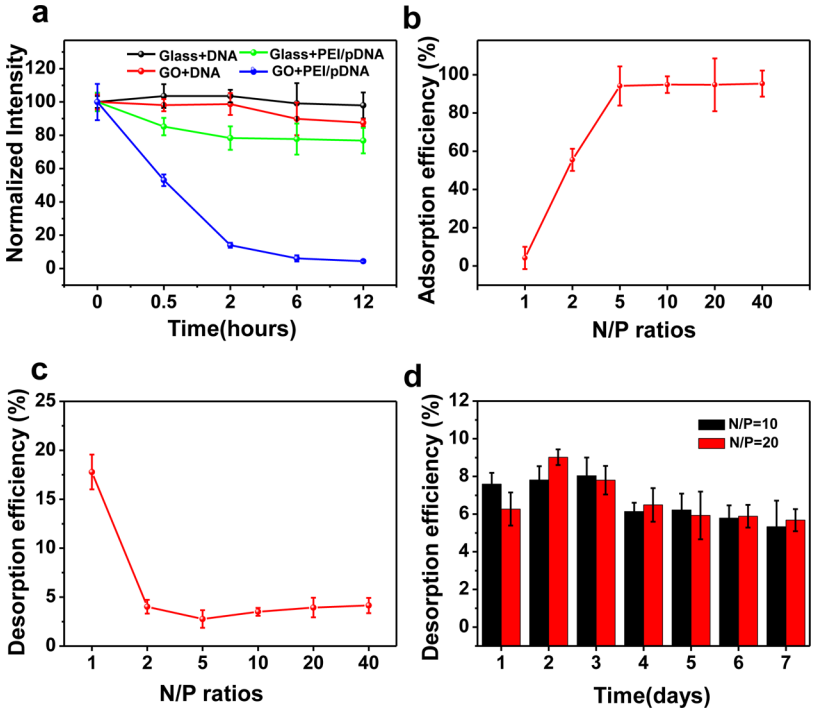

Figure 2. Adsorption and desorption efficiency of PEI/pDNA complexes on the GO substrates. (a) Time course of adsorption efficiency of pDNA or PEI/pDNA complexes incubated with GO substrates and glass as controls. (b) Adsorption efficiency of PEI/pDNA on GO substrates at different N/P ratios. (c) Desorption efficiency of PEI/pDNA at different N/P ratios from GO substrates. (d) Releasing profiles of PEI/pDNA from GO substrates continuously monitored in 7 days. Mean values and standard deviation are calculated from three independent experiments.

curve), the fluorescence of the supernatant did not decrease much, suggesting low adsorption of bare pDNA on the GO film. This phenomenon in our experiments was consistent with previous reports in the literature:[35] although GO displayed high adsorption/affinity for short and single-stranded DNA, the adsorption of long or double-stranded DNA on GO was much lower. In our case, at a neutral pH value, the carboxylic acid groups on the GO surface were deprotonated; therefore, the affinity between bare pDNA (polyanion) and GO was governed by the repellence of negative electrostatic interactions. [35] When pDNA was complexed with PEI (polycation), the electrostatic repulsion by negative charges was significantly reduced. Thus, the hydrophobic interactions and $\pi-\pi$ stacking dominated high adsorption of PEI/pDNA on GO substrates. In the control experiments using the substrates of precleaned glass coverslips, the adsorption of pDNA with (green curve) or without (black curve) PEI was much lower than their counterparts on GO, suggesting most of the nucleic acids still stayed in the supernatant away from the glass substrates. Figure 2b provides additional evidence on the importance of electrostatic interactions between PEI/pDNA and GO substrates by suggesting nearly complete adsorption of the complexes at higher N/P ratio (less electrostatic repulsion). In addition, we attempted, but failed, to directly acquire fluorescent images of PEI/pDNA on GO substrates, possibly because the fluorescent quenching effect by GO was dominant in this format. Alternatively, we succeeded in detecting the characteristic peak of DNA on GO substrates by UV−vis spectrometry and X-ray photoelectron spectroscopy (XPS) (Figure S4, Supporting Information). Therefore, the combination of all the evidence as above suggested that the GO substrates can serve as a valid platform to preconcentrate PEI/pDNA at the appropriate N/P ratios for gene delivery with a potential of high efficiency.

Figure 2c displays the desorption behaviors of PEI/pDNA complexes from the as-prepared GO substrates at different N/P ratios. The experiments were performed by the fluorescent measurements of the supernatants after we removed the GO substrates incubated with PEI/pDNA complexes for 12 h to new wells of the microplate. The results were individually normalized with the total amounts of adsorbed PEI/pDNA at different N/P ratios. The relatively fast desorption at lower N/P ratios suggested weak affinity between these PEI/pDNA and GO substrates that resulted from the residue electrostatic repulsion. The desorption of PEI/pDNA from GO became much slower at higher N/P ratios. We further monitored the desorption of PEI/pDNA (N/P = 10 and 20) for a relatively long term by periodically changing fresh buffer solutions. As shown in Figure 2d, the releasing behavior of PEI/pDNA from the GO substrates was overall quite stable in the monitoring period (7 days), with a very slow slope of decay. It indicated that our approach allowed a maintainable releasing profile of PEI/pDNA, which should be beneficial to enhanced efficiency of gene delivery.

Biocompatibility of the as-prepared GO substrates was evaluated using HEK293T and HeLa cell lines. Graphene and graphene oxide have been widely examined in the literature, suggesting good biocompatibility. Our approach was featured by the facile and mild conditions of transferring GO to the substrates by physical interactions; therefore, we expected that these GO substrates should present good compatibility in cell experiments. As shown in Figure 3, the cell samples cultured using GO substrates, glass coverslips, and regular cell culture microplates were performed with MTT assays to compare their relative viability. These two cell lines (HeLa and HEK293T) displayed similar trends of viability on these substrates. The cell viability on GO substrates was comparable with that on regular tissue culture microplates. Interestingly, the toxicity of PEI to

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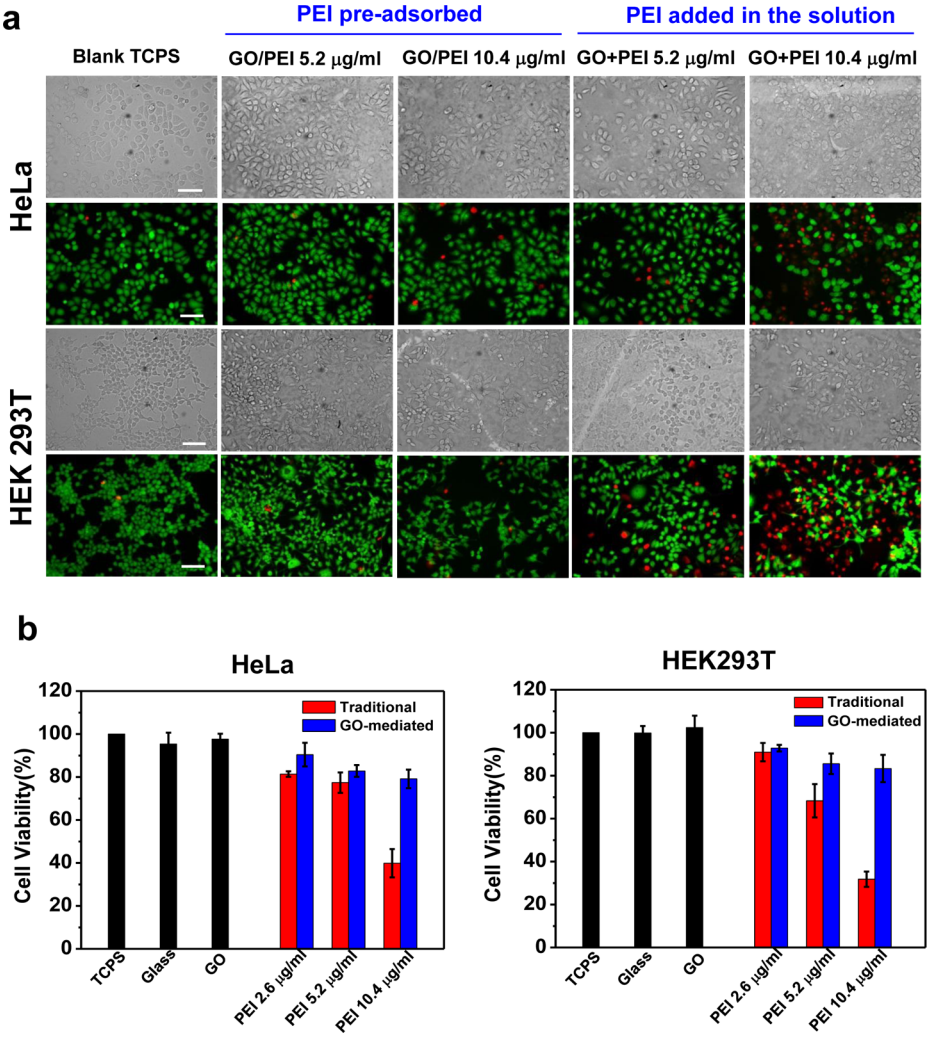

Figure 3. Assays of cell viability cultured on the GO substrates for 48 h. (a) The bright-field images and fluorescence images, live/dead cells stained by calcein-AM (green) and PI (red), PEI preadsorbed on GO or added in the solution as specified. The cells were cultured on GO substrates for fair comparison. Scale bar: 100 μm. (b) The MTT assays of HeLa cells (left) and HEK293T cells (right); the control experiments included the GO substrates without PEI, and bare glass, normalized by the cells cultured on the regular culture microplate. Mean values and standard deviation are calculated from three independent experiments.

cells exhibited a dramatic decrease when PEI was preadsorbed on the GO substrates. In contrast, when PEI was directly added into the bulky solution of the cell culture medium (traditional method), we observed a loss of nearly half cell viability in the relatively higher concentration. For the incubation of 24, 48, and 72 h, the cells on the GO substrate kept more than 90% of viability, better than the results using glass coverslips (Figure S5, Supporting Information). In addition, the dual-color staining experiments with dye molecules of calcein-AM and PI (Figure 3a) also suggested negligible cytotoxicity on cells with our approach, thus supporting better biocompatibility of the GO substrates than the traditional method in the presence of PEI.

We demonstrated that GO substrates can mediate gene delivery for a variety of cell lines with high transfection efficiency. Gene transfection of cells was performed with the complexes of PEI/pDNA$_{GFP}$ at a series of N/P ratios. As shown in Figure 4a, GFP expression of HEK 293T cells (bright green) suggested successful gene transfection with our approach. When the N/P ratio increased from 10 to 40, we observed enhanced transfection efficiency by the approach of GO substrate-mediated gene delivery (the top row in Figure 4a). As the control experiments with the conventional method, PEI/pDNA complexes were added in the bulky solutions after the step of cell seeding (the bottom row in Figure 4a). In the parallel comparison, our new approach achieved higher efficiency of gene transfection than the conventional method. Noteworthy, the conventional method suffered from increased cytotoxicity by the usage of larger amounts of PEI at higher N/P ratios, thus resulting in obviously more cell detachment and loss (sparse distribution of transfected cells). However, in our approach with PEI/pDNA preadsorbed on GO substrates, the cells showed significantly enhanced resistance to cytotoxicity of PEI at identical N/P ratios. The data using flow cytometry (Figure S6, Supporting Information) supported efficient gene transfection and minimal cytotoxicity on cells with our approach of GO substrate mediation. Additionally, we performed gene transfection with PLG-3 plasmid and then measured luciferase activities for further quantitative comparison between our approach and the traditional method. It produced consistent results with the flow cytometry data. Highly localized gene delivery was demonstrated using GO substrates containing a stripe-patterned geometry. As shown in the bright-field images of Figure 4b, the dark stripes (GO) and gray stripes (glass) were

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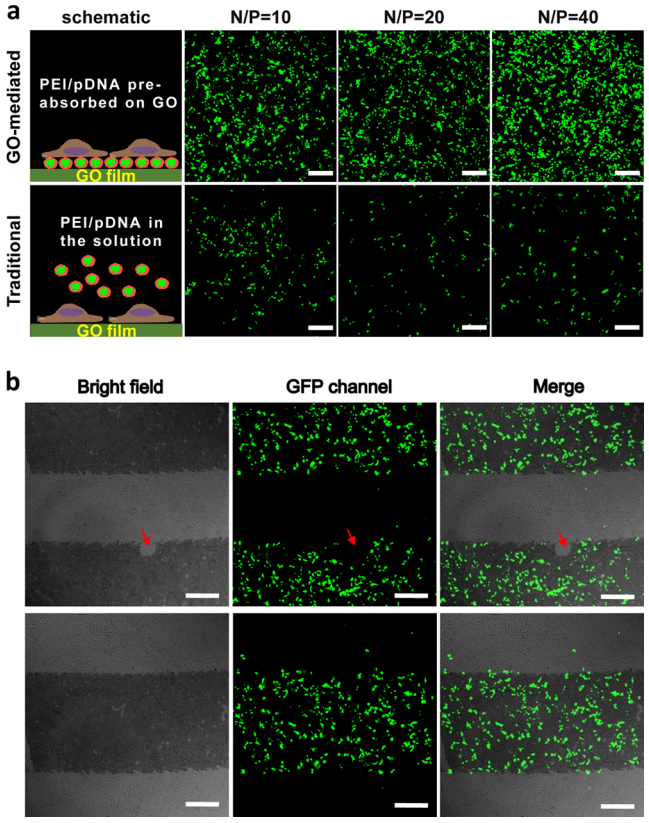

Figure 4. (a) A panel of fluorescent images of HEK293T cells expressing GFP with our GO substrate-mediated approach, compared with the traditional method of adding gene vectors in the bulky solution at different N/P ratios. Scale bar: 250 μm. (b) Highly localized gene transfection of HEK293T cells defined by the patterns of GO substrates. The dark stripes in the bright field indicate GO, the gray stripes indicate bare glass without GO. Note that the cells on gray regions scarely expressed GFP. Red arrow: semicircle defect of GO film. Scale bar: 250 μm.

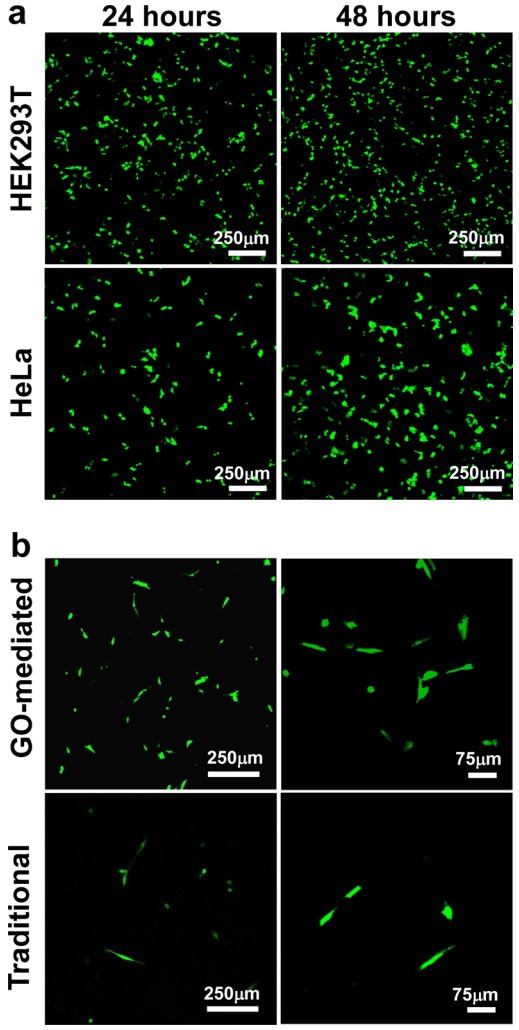

Figure 5. (a) The GO substrate-mediated gene transfection after 24 and 48 h, respectively, difference cell lines including HEK293T and HeLa. (b) More efficient gene transfection of hMSC cells with our approach than the traditional method.

alternatively spaced with each other, defined by the patterns of microfabrication. Because of their dramatic difference in adsorption on these two types of substrates (Figure 2a), the PEI/pDNA complexes were mainly enriched on the surface of GO stripes after the washing steps. Therefore, the cells cultured on GO stripes were effectively transfected by GFP plasmids, showing the color of bright green. In contrast, there was barely any GFP expression in the cells cultured on glass (Figure 4b). Interestingly, there was a small defect on the GO stripe with a shape of a semicircle (red arrow). The cells inside the semicircle (on the glass) did not express GFP, but the adjacent cells around the semicircle on GO were successfully transfected. Overall, the panel of images displayed clear boundaries differentiating the cells with or without GFP transfection, defined by stripe-patterned GO substrates. It confirmed effective gene delivery mediated by GO substrates in a highly localized manner. This unique feature of our approach can be applied to prepare genetically different cell patterns, thus offering a new platform to study cell−cell interactions.[36,37]

Other cell lines, including HeLa and human mesenchymal stem cells (hMSC), were examined with our approach of GO substrates. Figure 5a presents the fluorescent images of HEK293T and HeLa cells after GFP gene transfection for 24 and 48 h. The results suggested that our approach enabled sufficient gene delivery for both, even though the transfection efficiency for HeLa cells was slightly lower than that for HEK293T cells as expected because HeLa cells were relatively more difficult for gene transfection. Incubation (such as 48 h) or even longer (up to 96 h, Figure S7, Supporting Information) of the cells on GO substrates enhanced GFP transfection, consistent with our previous results that GO substrates can effectively preconcentrate and maintain the releasing of PEI/pDNA complexes. Gene delivery of hMSC mediated by GO substrates was evaluated with a comparison of the traditional method (Figure 5b). Our approach achieved an efficiency of GFP transfection several fold higher than the traditional method in bulky solutions. It is well-known that human mesenchymal stem cells hold great potential in regenerative medicine and tissue engineering.[38] Yet the technical challenge remains standing to prepare genetically engineered hMSC by the nonviral methods. With the enhanced efficiency of transfection, our approach may provide a new tool of facilitating manipulations of cell renewal and differentiation in the genetic level.

CONCLUSIONS

In summary, we have developed an approach of preparing patterned GO substrates for gene transfection. It is featured with imprinting by mechanical pressure and does not require

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additional chemical reagents, thus being straightforward and robust. Varieties of GO patterns on the substrates can be achieved using PDMS stamps conveniently. PEI/pDNA complexes can selectively be enriched on GO stripes, and then released in a sustainable manner. Therefore, the as-prepared GO substrates effectively preconcentrate PEI/pDNA based on the pattern designs. We have demonstrated that the patterned GO substrates exhibit excellent biocompatibility and work well with multiple cell lines by delivering genes efficiently with a highly localized feature. Only the cells cultured on the GO stripes can be transfected with GFP, displaying clear boundaries with groups of cells cultured on glass adjacently. These unique features in gene delivery mediated by our patterned GO substrates are important for a broad range of applications, including stem cell research, tissue engineering, and the development of gene therapies.

■ ASSOCIATED CONTENT

Supporting Information

Characterization of GO in suspensions, fluorescent measurements in the titration experiments, images showing the feature size of GO substrate, MTT assays for different incubation times, quantitative gene transfection including flow cytometry and luciferase activity assays, and gene transfection for a longer period of time. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jliu@suda.edu.cn. Tel: (86) 512 65884565. Fax: (86) 512 65888020.

Notes

The authors declare no competing financial interest.

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