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Abstract

Osseointegration in orthopedic surgery plays an important role for bone implantation success. Traditional treatment of implant surface aimed at improved osseointegration has limited capability for its poor performance in supporting cell growth and proliferation. Polydimethylsiloxane (PDMS) is a widely used silicon-based organic polymer material with properties that are useful in cosmetics, domestic applications and mechanical engineering. In addition, the biocompatibility of PDMS, in part due to the high solubility of oxygen, makes it an ideal material for cell-based implants. Notwithstanding its potential, a property that can inhibit PDMS bioactivity is the high hydrophobicity, limiting its use to date in tissue engineering. Here, we describe an efficient approach to produce porous, durable and cytocompatible PDMS-based 3D structures, coated with reduced graphene oxide (RGO). The RGO/PDMS scaffold has good mechanical strength and with pore sizes ranging from 10 to 600 μm . Importantly, the scaffold is able to support growth and differentiation of human adipose stem cells (ADSCs) to an osteogenic cell lineage, indicative of its potential as a transition structure of an osseointegrated implant.

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Development of porous 3D graphene-PDMS scaffolds for improved osseointegration

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Abstract

Osseointegration in orthopedic surgery plays an important role for bone implantation success. Traditional treatment of implant surface aimed at improved osseointegration has limited capability for its poor performance in supporting cell growth and proliferation. Polydimethylsiloxane (PDMS) is a widely used silicon-based organic polymer material with properties that are useful in cosmetics, domestic applications and mechanical engineering. In addition, the biocompatibility of PDMS, in part due to the high solubility of oxygen, makes it an ideal material for cell-based implants. Notwithstanding its potential, a property that can inhibit PDMS bioactivity is the high hydrophobicity, limiting its use to date in tissue engineering. Here, we describe an efficient approach to produce porous, durable and cytocompatible PDMS-based 3D structures, coated with reduced graphene oxide (RGO). The RGO/PDMS scaffold has good mechanical strength and with pore sizes ranging from 10-600 μm . Importantly, the scaffold is able to support growth and differentiation of human adipose stem cells (ADSCs) to an osteogenic cell lineage, indicative of its potential as a transition structure of an osseointegrated implant.

Introduction

Orthopedic implants have been widely used to repair bone damage caused by trauma or disease. Metal and its alloys are widely used for implant fabrication due to their mechanical properties, excellent corrosion resistance and biocompatibility [1]. However, metal-based orthopedic implants show limited osseointegration resulting in bone resorption and post-implant loosening [2, 3]. Ways to improve the interface between implant surface and

surrounding tissue have been developed, including increasing surface roughness, chemical surface modification, or coating materials [2, 4-8]. However, since osseointegration largely relies on two-dimensional (2D) structure manipulation, the majority of approaches show poor efficacy. An alternative and perhaps better approach would be to develop a 3D porous scaffold for enhanced osseointegration and post-surgical recovery.

As one of the most extensively utilised synthetic materials for biomedical devices and implants, polydimethylsiloxane (PDMS) has been widely used in medicine for over 30 years [9]. A number of valuable bulk properties, including elasticity, chemical and biological inertness, permeability to oxygen, and ease of fabrication make PDMS a promising candidate for long-term interfacial coating for orthopedic implants [10]. To encourage incorporation of newly formed bone through a PDMS scaffold coating, it is necessary to introduce interconnected micro and macro pores. This should lead to stable integration of the orthopedic implant with surrounding tissue [2]. However, notwithstanding its appeal, PDMS scaffolds do not support cell adhesion and proliferation well due to the inherent hydrophobicity and biofouling tendency [11]. It is therefore critical to render PDMS surfaces hydrophilic for their use as cell supporting scaffolds.

Graphene has attracted much attention since its rediscovery [12] in 2004, due to its innate strength, ease of isolation, thermal and electrical properties as well as good biocompatibility [13, 14]. Several studies have demonstrated that various graphene based materials, such as graphene coating and stand-alone graphene hydrogel, improve cell adhesion and proliferation [14], and support stem cell differentiation into various lineages, including osteoblasts, neurons, and myocytes [15-17]. In addition, graphene has been approved to support bone regeneration *in vivo* [18]. Consequently, strategies based on graphene coating have potential for generating PDMS scaffolds with enhanced cytocompatibility. Furthermore, there is a

growing interest in delivering stem cells with appropriate scaffolds to enhance bone formation in critical-sized bone defects [19, 20]. Human ADSCs represent excellent candidates being accessible for autologous transplantation, and able to be differentiation to osteogenic lineage for bone regeneration [21].

Here, we propose a way to fabricate a porous, durable and cytocompatible interfacial scaffolds via the coating of RGO onto porous PDMS. The fabrication process is simple, scalable and efficient. The structure has applicable mechanical strength with interconnected pores facilitating vascularization and accessibility of nutrients, liquid, and gas for cell support. Moreover, the scaffold supports human ADSC proliferation and differentiation to osteogenic lineage demonstrating the potentially application for interfacial transition material for improved osseointegration.

2. Experimental section

Materials

Natural graphite flakes, sodium nitrate (NaNO_3) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (USA). 98% sulfuric acid (H_2SO_4), potassium permanganate (KMnO_4), 32% hydrochloric acid (HCl), 30% hydrogen peroxide (H_2O_2), absolute ethanol (EtOH) and 95% n-hexane were purchased from Chem-Supply (Australia). L-ascorbic acid was purchased from BDH Chemicals (Australia). SYLGARD®184 silicone elastomer kit (PDMS) was purchased from Dow Corning (USA) and used as received.

Synthesis of soluble RGO

Graphene oxide (GO) was synthesized by modified Hummers method as previously described [22, 23]. Briefly, 1 g natural graphite flakes was oxidized by mixing with oxidizing agents (50 ml 98% H_2SO_4 , 1 g NaNO_3 , 6 g KMnO_4). The reaction was terminated by addition of 30% H_2O_2 . The resulting mixture was washed with 1 M HCl followed by distilled water. The obtained graphite oxide was exfoliated into GO by 5 hr ultrasonication (Unisonics cleaner, Australia). GO was reduced by 50 mM L-ascorbic acid solution overnight [24]. Impurities in the RGO product were removed by washing with distilled water, followed by centrifugation (Eppendorf 5702 Centrifuge; Germany) until the pH was almost neutral. As-synthesized RGO was subsequently dispersed in water to form a stable 1 mg/ml suspension by 1 hr ultrasonication.

Synthesis of porous RGO/PDMS structure

Fig. 1. Schematic of the fabrication and use for tissue engineering of porous RGO/PDMS scaffold.

As shown in Fig. 1, 30 g NaCl was added into a 90 mm diameter petri dish followed by mixing with 10 ml 70% EtOH. The NaCl solution was heated at 95°C for 30 min with further

removal of moisture at 60 °C for 10 hr. Base (10 g) and curing (1 g) agents of PDMS were mixed thoroughly and 2.02 ml hexane was used to dilute the PDMS mixture. Then 5.5 g diluted PDMS mixture was combined with 4 °C NaCl scaffold in a petri dish and maintained at 4 °C for 12 hr followed by curing of the PDMS at 60 °C for 5 hr and leaching of sacrificial NaCl scaffold in 50 - 60 °C water for 3 days. Dried porous PDMS scaffold was dipped in 1 mg/ml RGO solution and dried at 60 °C for 1 hr, with the process repeated twice more, followed by 2 hr drying. After final drying, the scaffold was cut into a desired shape for further use.

Variation of coating times (up to 6 times) of RGO was conducted for optimization. After a 3rd coating of RGO, the conductivity of the RGO/PDMS scaffold reached peak value and the morphology of the coated RGO layer was uniform throughout the whole scaffold (Fig. S1). Therefore, 3 coatings of RGO were deemed optimal and utilized for the subsequent fabrication of RGO/PDMS scaffolds.

Scanning electron microscopy

The morphology of synthesized RGO and structural features of RGO/PDMS scaffold were characterized by using JEOL JSM-7500FA Scanning Electron Microscope (SEM) and JEOL JSM-6490LA SEM respectively. For characterization of the scaffold with cells, freeze-dried samples were coated with platinum (15 nm) using an Edwards sputter coater and then assessed with the JEOL JSM-6490LA SEM.

Porosity analysis

One piece of cube-shaped PDMS scaffold (weight: M_1 (g), volume: V (cm³)) was soaked in water and squeezed by tweezers until all the pores in the structure filled with water (total weight: M_2 (g), water density: ρ (g/ml)). Porosity of the scaffold was defined as:

$$\varphi = \left[\frac{M_2 - M_1}{\rho} \right] / V$$

Raman analysis

Raman spectroscopy of synthesized RGO and 3D RGO/PDMS scaffolds was performed using a Jobin Yvon Horiba HR800 Raman spectrometer with a 632.8 nm excitation laser and 300-lines mm⁻¹ grating.

Mechanical analysis

Tensile and compression tests were conducted with a universal mechanical tester (Shimadzu EZ, Japan). Tensile testing was performed on a cuboid sample (gauge length: 10.0 mm, width: 10.0 mm, thickness: 3.5 mm). Compression testing was similarly performed on cuboid samples (gauge length: 10.0 mm, width: 10.0 mm, thickness: 3.5 mm; Fig. S2).

Electrical analysis

Measurements of sheet resistance for the samples were conducted according to published methods and on cuboid samples (length: 20.0 mm, width: 10.0 mm, thickness: 3.5 mm) [25, 26]. Briefly, two rectangular aluminium electrodes [length: L (m)] were placed in parallel on the sample surface with a separation distance of D (m) and a multimeter was used to measure the resistance [R (Ω)] between them. Sheet resistance [ρ_{SR} (Ω /sq)] of the tested sample was defined as:

$$\rho_{SR} = R * L/D$$

1 **ADSC culture**

2 Human ADSCs were purchased from Lonza, Australia. Gibco Dulbecco's Modified Eagle
3 Medium (DMEM), foetal bovine serum (FBS), 100x penicillin-streptomycin, 100x Non-
4 Essential Amino Acids Solution (NEAA), basic fibroblast growth factor (bFGF) were
5 obtained from Thermo Fisher (Australia).

6 ADSCs were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin,
7 1% NEAA and 1ng/ml bFGF in a humidified incubator at 37 °C with 5 % CO₂ atmosphere.
8 The initial cell seeding density was 2x10⁴ cells per cm².

9 **ADSC differentiation**

10 Osteogenic differentiation of ADSC was induced by culture medium supplemented with 50
11 µM ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate [27]. ADSCs were
12 seeded on the top of 2D and 3D samples (Dimensions of 2D rectangular sample: 10.0 mm ×
13 10.0 mm, dimensions of 3D cuboid sample: 10.0 mm × 10.0 mm × 3.5 mm) at 5 × 10⁴ cells
14 per scaffold in culture medium for 24 h, followed by differentiation medium. For cell seeding
15 of 3D scaffolds, each scaffold was squeezed several times using tweezers for homogenous
16 cell distribution and loading throughout a construct. Medium was then changed every 2 days
17 for 3 weeks. Samples were subsequently fixed in 3.7 % paraformaldehyde/PBS solution for
18 30 min. After being rinsed in Milli-Q water, the samples were stained with 0.6 % Alizarin
19 Red-S solution at pH 4.2 for 20 min at room temperature followed by extensive washing with
20 water. The stained samples were eluted in water with 20% methanol and 10% acetic acid for

30 min, and 200 µl eluted solution of each sample was transferred to the well of a 96-well plate, screened with a microplate reader (POLARstar Omega) at 405 nm for absorbance.

ADSC proliferation and live/dead cell analyses

ADSC proliferation was studied using PrestoBlue (Thermo Fisher, Australia) cell viability reagent in accordance with the manufacturer's protocol. Cell seeding number for 2D and 3D scaffolds was the same, both were 0.025 million per scaffold. Cell seeding procedure and scaffold dimensions were identical to differentiation samples. Cells were evaluated 1, 3, 5, and 7 days after cell seeding with samples studied in triplicate for each time point using a microplate reader (POLARstar Omega) at 544 nm for fluorescence intensity. Following screening, samples were transferred to basal culture medium for further characterisation.

Calcein AM (5 µg/ml; Thermo Fisher, Australia) and propidium iodide (PI; 1 µg/ml; Thermo Fisher, Australia) were used as live and dead cells assay respectively, as per manufacturer's instructions. Briefly, samples were incubated with Calcein AM and PI at 37 °C for 5 min, followed by a media change. AxioImager microscope (Zeiss, Germany) was used for assessment and image acquisition.

Alkaline phosphatase assay

ADSCs were seeded onto 2D and 3D scaffolds (dimensions of 2D rectangular sample: 5.0 mm × 5.0 mm, dimensions of 3D cuboid sample: 5.0 mm × 5.0 mm × 3.5 mm.) at 10⁴ cells per scaffold and bathed with osteogenic differentiation medium. Medium was changed every 2 days of cell culture. Following 1, 3, 5, 7 days of differentiation, alkaline phosphatase (AP) activity was evaluated by using an AP activity assay in accordance with the manufacturer's instructions (Biovision, USA). Briefly, 3D and 2D scaffolds were incubated in 600 µl and

300 μ l Assay Buffer respectively for 1 hr. For each sample, the supernatant of the lysis solution was mixed with 0.5 mM substrate solution for 30 min at 25 °C in a light-proof environment. The formation of fluorometric substrate was measured at Ex/Em = 360nm/440nm by using a microplate reader (POLARstar Omega).

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD) unless specified. A two-way ANOVA with Bonferroni post hoc test was used for comparison, and a p-value of less than 0.05 was considered to be statistically significant.

3. Results and discussion

Material characterisation

Fig. 2. Characterization of synthesized RGO. (A, B) SEM images of RGO at low and high magnification, respectively. (C) Raman spectra of RGO film.

RGO aqueous dispersion was obtained according to the experimental protocol detailed above, and the dispersion was found to be stable over a period of at least 4 weeks. The RGO dispersion was dried on a glass slide for SEM imaging. Graphene layered structures were

observed by SEM with the lateral size of graphene being more than 6 μm (Fig. 2A and B). Raman spectrum shows a typical D band ($\sim 1330\text{ cm}^{-1}$) and G band ($\sim 1590\text{ cm}^{-1}$) attributed to RGO, whereby D band relates to aromatic structure and G band relates to the degree of graphitization [28]. The ratio of D to G band (I_D/I_G) was 1.36, indicating a defect induced in the synthesis process comparable with previous reports [28, 29].

Fig. 3. Porosity of PDMS and RGO/PDMS scaffolds. (A, B) Photomicrographs of porous PDMS and RGO/PDMS structures, respectively. (C, D) Low and high magnification SEM images of graphene coated porous PDMS scaffold, respectively.

RGO/PDMS scaffolds were mechanically robust and porous structures (Fig. 3A and C) with uniform coatings of RGO (Fig. 3B and D). Pore sizes varied from 10 - 600 μm and the porosity of the structure was calculated to be $63 \pm 7\%$. I_D/I_G value for 3D RGO/PDMS scaffold was 1.30, which indicates negligible defects induced during the fabrication process.

Fig. 4. Mechanical testing of 3D RGO/PDMS scaffold at 10 mm/min under ambient conditions. (A) Mechanical response over time of specimen for 100 times compression. (B) Mechanical response over strain of specimen for 10 times compression (inset: mechanical response over time for 10 times compression). (C) Mechanical response over strain of specimen for tensile testing.

Both RGO/PDMS and PDMS scaffolds showed good resistance to compression and tensile stress. The RGO/PDMS scaffold was stable for over $100 \times$ compressions for a strain in excess of 93 % (Fig. 4A and B), whereby the peak stress value was three times higher than that of PDMS scaffold with same compression strain (Fig. S3A). RGO/PDMS scaffold also has a maximum elongation ratio of 0.85-0.90 with tensile strength of 81.05 kPa (Fig. 4C). The peak stress value was 11% higher than that of PDMS scaffold with similar elongation ratio (Fig. S3B). Hence, RGO coating increased the mechanical strength of the PDMS scaffold. The relative lower mechanical strength compared with metal/metal alloy implants

and bones supports the scaffolds role as a robust cushion layer between implant and surrounding tissue [3]. Importantly, the PDMS substrate was still completely covered with RGO layer after $100 \times$ compressions, indicating strong and durable absorption of RGO on the PDMS substrate.

Electrical sheet resistance of the RGO/PDMS scaffold was about $300 \text{ k}\Omega/\text{sq}$, which may have resulted from the mild reduction environment for RGO, complicated inner porous architecture of the 3D PDMS substrate, and high contact resistance between graphene layers [30, 31].

ADSC culture and differentiation

Fig. 5. (A, B) Fluorescence microscope images of live (Calcein AM; green), and dead (propidium iodide; red) ADSCs cultured on porous PDMS and RGO/PDMS structures,

respectively following 10 days culture. (C) SEM images of ADSCs on RGO/PDMS scaffold surface, and (D) inside the scaffold after less than 24 hr culture. Cells are indicated by yellow arrows while filopodia are circled red.

The cytocompatibility of PDMS and RGO/PDMS scaffolds was initially investigated by assessing ADSC viability. Calcein-AM and propidium iodide staining indicated high cell viability following 10 days culture on PDMS and RGO/PDMS scaffolds (Fig. 5A and B). Cytocompatibility of the RGO/PDMS scaffolds is consistent with previous reports of graphene cell support [32, 33]. Cell adhesion was indicated by an abundance of filopodia across the surface and into pores of RGO/PDMS compared to PDMS scaffolds (Fig. 5C and D).

Fig. 6. Average percentage increase of fluorescence intensity (relative to Day 1 average value of 3D RGO/PDMS samples) over time as an indicator of cell proliferation for cells on different 3D scaffolds and 2D culture (control). Mean $\pm$ standard deviation, $n=3$, * $p<0.05$.

RGO/PDMS film was fabricated according to a reported method [34] and used to compare cell proliferation for 2D planar cell culture verses the 3D scaffolds. As shown in Fig. 6, 3D scaffolds enhanced cell proliferation between days 3 and 5 of culture, with the rate of increase diminishing rapidly thereafter for 3D PDMS but more slowly for 3D RGO/PDMS. Cell numbers for days 5 and 7 were significantly higher than days 1 and 3 ($P<0.05$; Fig. 6). Overall, these data demonstrate that 3D RGO/PDMS scaffolds are biocompatible and able to support ADSC growth. The efficacy of 3D RGO/PDMS may be due to a combination of 3D culture more closely resembling the natural niche and mildly reduced graphene oxide being relatively hydrophilic [35].

Fig. 7. Alizarin Red staining of calcium deposition for ADSCs differentiated for 3 weeks on (A) RGO/PDMS porous scaffold and (B) PDMS scaffold in osteogenic induction medium. (C) Quantification of calcium deposition using Alizarin Red S staining. Mean $\pm$ standard deviation, $n=3$, $*p<0.05$.

Higher calcium deposition was measured for cells differentiated for 3 weeks on 3D RGO/PDMS scaffold, with low to negligible levels expressed by cells on 3D PDMS, 2D RGO/PDMS and negative controls (Fig. 7A, B and C). For differentiation samples in osteogenic medium, calcium deposition on 3D RGO/PDMS scaffold was significantly higher than 3D PDMS and 2D RGO/PDMS ($P<0.05$; Fig. 7 C). Samples cultured in cell osteogenic medium have significantly higher calcium deposition compared to cell growth medium ($P<0.05$; Fig. 7 C). 3D RGO/PDMS scaffold was able to facilitate stem cell induction to osteogenic lineage without additional chemical inducers, consistent with previous reports of graphene and 3D biomaterial scaffolds being conducive to bone engineering [36, 37]. Presently, the higher calcium content of 3D RGO/PDMS may be due to the synergistic effect of graphene and 3D culture supporting osteogenic differentiation. Importantly, after 3 weeks of differentiation, RGO/PDMS scaffolds remained intact and without deformation, which is necessary for longer-term cell support and tissue formation *in vitro* or *in vivo* [38]. Scaffolds developed here were more durable than previously reported biodegradable porous scaffolds used for osseointegration [2].

Fig. 8. Upregulation of the AP activity of ADSCs in the 3D RGO/PDMS, 3D PDMS and 2D RGO/PDMS for 1, 3, 5, 7 days. Mean $\pm$ standard deviation, n=3, *p<0.05.

Expression of early osteogenic differentiation cell marker AP was significantly higher for 3D scaffolds than 2D RGO/PDMS film, with the 3D RGO/PDMS associated with the highest AP expression compared to 3D PDMS scaffold (Fig. 8). Upregulation of AP peaked at Day 3 for 3D RGO/PDMS scaffold, and day 5 for 3D PDMS scaffold. The peak value of AP expression for 3D RGO/PDMS scaffold was 0.13 fold and 2.1 fold higher than 3D PDMS and 2D RGO/PDMS respectively. Again, our findings are consistent with previous reports of RGO and 3D cell culture being beneficial to osteogenic differentiation of ADSC [39, 40].

Conclusion

We have developed a scalable, simple and efficient method of fabricating durable, biocompatible, porous RGO/PDMS scaffolds. The approach includes the use of salt porogen and subsequent dip-coating methods. The scaffold was highly porous while being mechanically robust. Moreover, we have demonstrated scaffold cytocompatibility, extending to ADSC culture and subsequent osteogenic differentiation. Taking the conductivity of 3D RGO/PDMS into consideration, there is the potential to electrically stimulate cells via the scaffold. By enabling 3D support of ADSC culture and differentiation within a mechanically robust and flexible structure, the scaffold has the potential to be used as a transition layer for improved osseointegration in orthopedic surgery. In addition, it may be adapted for synthesis of various other tissue types *in vitro* and *in vivo* for both research and clinical use.

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Supporting information

Fig. S1. After 3 times coating, RGO was uniformly deposited throughout the whole scaffold.

Fig. S2. Mechanical testing system setups for (A) tensile test; (B) compression test.

Fig. S3. Mechanical testing of pristine 3D PDMS scaffold at 10 mm/min under ambient conditions. (A) Mechanical response over time of specimen for 100 times cyclic compression. (B) Mechanical response over strain of specimen for tensile testing.