Application of Graphene as Candidate Biomaterial for Synthetic Keratoprosthesis Skirt

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Purpose. Synthetic keratoprostheses are required for visual rehabilitation in patients with end-stage corneal blindness. This study aimed to assess the biocompatibility of graphene material and its potential as a novel synthetic keratoprosthesis skirt material for corneal tissue engineering.

Methods. Human corneal stromal fibroblasts were cultured on material surfaces including pristine graphene film, graphene foam, pristine titanium (Ti) discs, and tissue culture plastic surface (TCPS). Cell attachment was assayed by immunostaining of paxillin and vinculin. Cell viability and proliferation were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Click iT 5-ethynyl-2′-deoxyuridine (EdU) assays. The growth of fibroblasts on three-dimensional graphene foam was examined by scanning electron microscopy, and cytokine release was analyzed by enzyme-linked immunosorbent assay. Graphene films were implanted into rabbit corneal stromal pockets and examined by slit-lamp biomicroscopy, anterior segment optical coherence tomography, and histology.

Results. Pristine graphene demonstrated good biocompatibility with human stromal fibroblasts in terms of cell adhesion, viability, and proliferation. Cells on graphene films showed higher number than on TCPS control. Cells grown on graphene had 10% more proliferation than on Ti. The expression levels of IL-6 and IL-8 were reduced when cells were seeded on graphene foam compared to Ti and graphene film. Implantation of graphene film into rabbit stroma (n = 6) did not show any signs of infection, neovascularization, or inflammation.

Conclusions. Graphene displayed excellent short-term biocompatibility with corneal cells and tissue. This demonstrates that graphene can be developed as a tissue engineering material for use in cornea.

Keywords: synthetic keratoprosthesis, graphene, biocompatibility, corneal tissue engineering

Conventional corneal transplantation has poor outcomes in patients with end-stage corneal diseases. In such circumstances, the use of an artificial cornea, or keratoprosthesis (Kpro), plays a significant role in providing visual rehabilitation. Many Kpro devices have been developed over the past decades, including AlphaCor,2,3 Boston Keratoprosthesis (Boston Kpro),4 osteo-odontot keratoprosthesis (OOKP),5 and the Moscow Eye Microsurgery Complex in Russia (MICOF) keratoprosthesis.6,7 The Boston Kpro and OOKP, the most commonly used Kpros, have been used to treat patients with multiple graft failures, aniridia, chemical injuries, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and thermal burns as well as other conditions with high risk of corneal graft rejection and failure.8

The differentiation in use of the two most commonly used Kpros is in the condition of the ocular surface before treatment. The Boston type 1 Kpro is the best choice in patients with moderate ocular surface disease with adequate tear film; however, in patients with severe dry eyes, an OOKP or a Boston type 2 Kpro is required.9–12 The differentiation between a type 1 and type 2 Boston Kpro is the protrusion of the optic through the lid in a type 2 device. Despite the successful use of OOKP in patients with severe ocular surface disease,13–14 there are limitations in its application: The surgery is complex, involving a two-stage procedure that needs a large group of surgeons and extensive resources; device extrusion secondary to lamina resorption remains a problem; this procedure is not possible in children, due to small tooth dimensions and a higher risk of tooth/bone resorption; OOKP remains limited to adult patients with healthy canine teeth; and the optic size is restricted by the size of the tooth root available in the patient.12 To circumvent these limitations, novel biomaterials combined with new designs of synthetic artificial corneal devices are needed.

Bioactive and bioinert materials have been studied for use in the Kpro skirt with various in vitro cell culture models, animal implantation testing, and preclinical/clinical applications.9,15 Bioactive materials, including glass and hydroxyapatite (HA) ceramics, have shown good tissue integration.16,17 However, serious complications such as aqueous leakage, retroprosthetic membrane formation, and endophthalmitis are frequently
observed due to the degradation of these bioactive materials,9,15 bioinert materials, such as titanium (Ti) or titanium alloys, aluminum, or their modified compounds have also been used as Kpro skirt materials.10 We have previously shown that sintered titanium dioxide (TiO2) exhibited excellent biocompatibility and corrosion resistance properties in an in vitro simulated ocular environment and in vivo animal model.16,17 To further improve the biocompatibility and bactericidal properties of the implants, we functionalized the surface of TiO2 with antimicrobial peptide coating.19,20 The nonoxidized, metallic Ti has been used in the Boston KPro, due to its better compatibility compared to PMMA,21 and the decreased formation of retroprosthetic membranes when Ti was used as a backing plate.22 Functionalized Ti substrates have displayed excellent antimicrobial properties that effectively alleviated postsurgery implant-associated infection.19,20 These studies have expanded the current strategies to improve the biocompatibility and survival rate of implanted synthetic KPros and demonstrated the excellent biocompatibility of Ti as a biomaterial for synthetic KPro. However, a solid Ti Kpro may be heavy, and Ti has poor fretting fatigue and tribologic behavior, and 3D G-foam (thickness: 1.2 mm, density: 320 mg/cm3, pore size: 580 μm) were purchased from Graphene Supermarket (www.graphene-supermarket.com, in the public domain). Graphene films were punched into small discs using a trephine of 4-mm diameter and used as implants for animal studies. Titanium discs (thickness: 0.5 mm, diameter: 8 mm) were purchased from Goodfellow (Cambridge, UK). All test materials were sterilized by autoclaving prior to use.

Human Corneal Fibroblast Isolation and Culture

Human corneal stroma was collected from donor corneal tissue with approval from the Institutional Review Board of SingHealth, Singapore. After the surgical removal of corneal epithelium and endothelium, the stroma was cut into small pieces and digested overnight in 0.2% type I collagenase (Worthington, Lakewood, NJ, USA) in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) at 37°C for 6 to 8 hours. Single-cell suspension was collected, washed with phosphate-buffered saline (PBS; Invitrogen), and cultivated in DMEM containing 10% fetal calf serum (Gibco, Carlsbad, CA, USA). The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 balanced with air. The culture medium was changed every 2 to 3 days. Fibroblasts at passages 5 to 7 were trypsinized, reseded, and cultured on the sterile material surface (1.5 × 104 cells/cm2) with normal culture medium.

Immunostaining

After 24 hours of culture, cells were fixed with freshly prepared neutral buffered 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO, USA) in PBS. After blocking, they were incubated with primary antibody (mouse anti-vinculin, Sigma-Aldrich Corp.; mouse anti-paxillin, Abcam, Cambridge, MA, USA) and phallolidin-rhodamine (2 μg/mL; Life Technologies, Carlsbad, CA, USA). The secondary antibody was FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotech, Santa Cruz, CA, USA). After washes, samples were mounted in Fluoroshield with DAPI (4’,6-diamidino-2-phenylindole; Santa Cruz Biotech) and viewed under fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

Cell Proliferation and Cytotoxicity by MTT and Click-IT 5-ethyl-2’-deoxyuridine (EdU) Assays

After 1 and 7 days of culture on material surface, the cell number was assayed by MTT reduction. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent, was added to the culture at a dose of 0.5 mg/mL for 4 hours, followed by addition of dimethyl sulfoxide (DMSO) to a final concentration of 5% to dissolve the formazan crystals. The supernatant was collected and the optical density was measured at 560 nm (excitation) with a reference filter of 620 nm using a microplate reader (INFINITY 200; Tecan, Männedorf, Switzerland). The relative cell number was expressed as the ratio of optical density of cells on graphene or Ti materials to that of cells on regular culture plastic surface. Cell proliferation was detected by Click-IT EdU Assay (Life Technologies). In brief, cells were incubated with 10 μM EdU for 18 hours and fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with 5% bovine serum albumin (Sigma-Aldrich Corp.), Click-IT reaction solution (Life Technologies) was added to cells for 30 minutes. Samples were mounted in Fluoroshield with Hoechst 33342 (5 μg/mL) and viewed under fluorescence microscopy (Carl Zeiss). The EdU-labeled nuclei were quantified to obtain the cell proliferation index.

Materials and Methods

Materials

Two-dimensional multilayer G-film (thickness: 25 μm, density: 2 g/cm3) and 3D G-foam (thickness: 1.2 mm, density: 320 mg/cm3, pore size: 580 μm) were purchased from Graphene Supermarket (www.graphene-supermarket.com, in the public domain). Graphene films were punched into small discs using a trephine of 4-mm diameter and used as implants for animal studies. Titanium discs (thickness: 0.5 mm, diameter: 8 mm) were purchased from Goodfellow (Cambridge, UK). All test materials were sterilized by autoclaving prior to use.

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Graphene Implantation Into Rabbit Corneas

The animal handling procedures adhered to the ARVO Statement for Use of Animals in Ophthalmic Vision and Research, and the experimental protocol was approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at SingHealth, Singapore.

New Zealand white rabbits (n = 6), aged 1 to 2 months and weighing 2 to 2.5 kg, were used in this study of corneal stromal implantation of graphene. They were anesthetized by intramuscular injection of ketamine hydrochloride (35 mg/kg; Parnell Laboratories, Alexandria, NSW, Australia) and xylazine hydrochloride (5 mg/kg; Troy Laboratories, Smithfield, NSW, Australia). The right eyes received corneal stromal pocket creation at midstromal level and implanted with G-film (trephined to 4-mm diameter) as described in our previous work, while the left eyes were the untreated control.

Ocular Imaging

Rabbits were monitored daily for their general behavior, and ocular examination was performed weekly after surgery. Slit-lamp photographs were taken with a Zoom Slit Lamp NS-2D (Righton, Tokyo, Japan). Anterior segment optical coherence tomography (AS-OCT) was performed with Visante (Carl Zeiss Meditec, Dublin, CA, USA) under normal room lighting. An anterior segment scan centered over the pupil was taken along the horizontal axis (between 0° and 180°). The scan direction was aligned until a full corneal reflex was achieved (indicated by an interference flare on the axis of anterior chamber). The fixation angle was adjusted to obtain a horizontal image.

In vivo confocal microscopy was performed with Heidelberg Retina Tomograph II Rostock Corneal Module (RCM; Heidelberg Engineering GmBH, Dossenheim, Germany). Visco-tears (Carbomer 980, 0.2%; Novartis, North Ryde, NSW, Australia) was used as a coupling agent between the applanation lens cap and cornea. The full thickness of the central cornea (2-mm diameter) was scanned using the video mode that captured sections at 2-μm intervals, up to 20 μm from the initial desired depth. Any blurred or nontangential images were excluded. Images were taken at sufficient depth to image both anterior and posterior to the implant.

Histology

After euthanasia, rabbit corneas were fixed in 4% paraformaldehyde and processed for paraffin embedding. Thin sections (5-μm thickness) were stained with hematoxylin and eosin (H&E, Sigma-Aldrich Corp.), mounted, and imaged by light microscopy (Carl Zeiss).

Statistical Analysis

Data are presented as mean ± standard deviation where statistical analysis was used. P values were determined using one-way ANOVA tests or Student’s t tests. Data were considered to be statistically significant when the P value was less than 0.05.

RESULTS

Graphene Films Supported the Adhesion of Corneal Stromal Fibroblasts

To study the adherence of cells on the substrate materials, we seeded human corneal stromal fibroblasts on Ti and G-film and examined the formation of focal adhesion complexes. Within 24 hours after seeding, the cells adhered to the surface of substrate materials with extension of cell processes. By immunofluorescence, the expression of two focal adhesion proteins, paxillin and vinculin, was similar for cells cultured on Ti and G-film, respectively. The signals were predominantly at the cell periphery with intense association with the cytoplasm. The expression of paxillin and vinculin was greater than that on tissue culture plastic (P < 0.05) but less than that on Ti surface (P < 0.001). The expression of paxillin and vinculin was greater than that on tissue culture plastic (P < 0.05) but less than that on Ti surface (P < 0.001). This indicated that G-film had no obvious cytotoxicity and could facilitate cell adhesion when compared to Ti.

Graphene Film Facilitated Corneal Stromal Cell Survival and Proliferation

Human corneal stromal fibroblasts were seeded at a density of 1.5 × 10³ cells/cm² on G-film, Ti disc, and tissue culture plastic surface (TCPs) (reference control) for 1 and 7 days, respectively. At 1 and 7 days, the number of cells on materials was determined by MTT assay. At day 1, the cell numbers were similar among the three cultures (P = 0.48 for TCPs versus G-film) (Fig. 2). At Day 7, the cell number on G-film was 2.4 times greater than that on Ti surface (P = 0.03), and slightly more than that on tissue culture plastic (P = 0.39). This indicated that G-film had no obvious cytotoxicity and could facilitate cell survival.
Cell proliferation on Ti and G-films was examined by Click-iT EdU assay. EdU-labeled cells were quantified on random images. At 24 hours after cell seeding, there were significantly more proliferating cells on G-film (43 ± 3%) than on Ti surface (33 ± 2%) (P < 0.05, one-way ANOVA) (Figs. 3A, 3B).

**Adhesion of Corneal Stromal Fibroblasts to 3D Graphene Foam**

A 3D form of graphene, G-foam, was also used to assess the growth of stromal fibroblasts on graphenic materials. Due to the difficulty in calculating the surface area of this highly porous material, we could not directly compare cell density and growth rate with 2D materials as described above. Instead, we studied the cell attachment and growth by SEM. After 8 and 24 hours of culture, stromal fibroblasts were adhered on the wall of G-foam. The attached fibroblasts showed spindle-shaped morphology and formation of bridge-like processes (up to 10–100 μm) across the pores of G-foam (Fig. 4A).

**Cytokine Production by Corneal Stromal Fibroblasts on Graphene Film and Foam**

Conditioned medium was collected from the culture of stromal fibroblasts on different materials at 8 and 24 hours of LPS treatment. By ELISA, it was observed that IL-8 levels were higher than IL-6 levels at 8 hours after induction, while IL-6 levels were much higher than IL-8 at 24 hours in the medium of each material. This can be attributed to the earlier secretion of IL-8 compared to IL-6 by human corneal fibroblasts upon the induction with LPS. The cytokine levels in conditioned medium collected from G-foam were significantly reduced as compared to cytokines levels present in the conditioned medium collected from Ti and G-film (Fig. 5B, P = 0.01). At 8 hours after induction, IL-6 was present in media from cells grown on G-foam at 50 ± 20 pg/mL, which was significantly lower than that on G-film (190 ± 30 pg/mL) (P = 0.005, two-tailed Student’s t-test assuming unequal variance) and Ti (320 ± 60 pg/mL) (P = 0.002, same t-test conditions). The concentration of IL-8 at 8 hours on G-foam was 0 ± 0 pg/mL also significantly lower than that on G-film (200 ± 50 pg/mL) (P = 0.05) and Ti (500 ± 200 pg/mL) (P = 0.05). At 24 hours after induction, IL-6 expression level on Ti was 300 ± 20 pg/mL, which was significantly lower that on G-film (200 ± 50 pg/mL) and G-foam (500 ± 200 pg/mL) (P = 0.005 versus G-foam, two-tailed Student’s t-test assuming unequal variance), and 600 ± 20 pg/mL on Ti (P = 0.09 versus G-foam, two-tailed Student’s t-test assuming unequal variance). Interleukin-8 concentrations were 4 ± 4 pg/mL on G-foam, 200 ± 20 pg/mL on G-film (P = 0.004 versus G-foam, two-tailed Student’s t-test assuming unequal variance), and 90 ± 8 pg/mL on Ti at 24 hours (P = 0.001 versus G-foam and P = 0.01 versus G-film, two-tailed Student’s t-test assuming unequal variance). Adsorption of cytokines onto G-films and G-foams over 24 hours was examined without cells to determine if the changes in cytokine levels represented a change in expression of inflammatory proteins, or if the graphene materials were binding the proteins and removing them from the media. Results are presented in Supplementary Figure S1; they showed a maximum absorption of ≈50 pg on G-foam (48 and 49 pg...
In Vivo Assessment of the Biocompatibility of Graphene Film

We performed an in vivo study to examine the effect of G-film implantation into rabbit corneal stromal pocket. The rabbits behaved normally, though one eye had blocked vision. Four of them showed anterior corneal melting at the third week after surgery, one at the fourth week, and the last one at the fifth week. When examined before corneal melting, corneas remained clear in graphene-implanted eyes under slit-lamp biomicroscopy. There were no signs of corneal infection, inflammation, or neovascularization (Figs. 5A1, 5B1). Similar observation was made in untreated control corneas. In AS-OCT scanned images, the graphene implant appeared as a white line, indicating the implant location. The central corneal thickness measuring from the plane of implant to corneal surface was maintained at 60 to 100 μm before corneal melting. The corneal surface curvature of the implanted eye appeared to be mildly irregular (Figs. 5A2, 5B2). In vivo confocal microscopy further illustrated the intact corneal epithelium and regular cell sizes and cell–cell contacts (Figs. 5A3, 5B3). The light reflectivity and keratocyte densities were similar between control (Fig. 5A4) and graphene-implanted stroma (at the site next to the graphene implant) (Fig. 5B4). There was no stromal inflammation caused by graphene implantation.

Histology

Hematoxylin and eosin histochemistry showed that there was no observable stromal structural difference between control and graphene-implanted corneas at 3 weeks after surgery (Fig. 6). The empty stromal space represents the location of graphene implant, which was removed before sample processing and microtome sectioning. In graphene-implanted stroma, neovascularization was not observed; however, there were more infiltrated mononuclear cells.

Discussion

Recent studies have begun to recognize the importance of GBMs for their application in tissue engineering. Biocompat-

![Graphene as Biomaterial for Keratoprosthesis Skirt](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934564/)

**Figure 5.** In vivo imaging of (A) control rabbit cornea and (B) graphene-implanted cornea. (A1, B1) Slit-lamp biomicroscopy showing the clarity of control (A1) and graphene-implanted (B1) corneas. (A2, B2) AS-OCT scanning images showing corneal topography of control (A2) and graphene-implanted (B2) corneas. (A3, A4, B3, B4) In vivo confocal images of corneal epithelium (A3, B3) and stroma at the level of graphene implant (A4, B4). Asterisk indicates the location of the graphene implant.

![Hematoxylin and eosin (H&E)-stained images of rabbit corneas: (A) control and (B) graphene implanted. Scale bars: 50 μm.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934564/)

**Figure 6.** Hematoxylin and eosin (H&E)-stained images of rabbit corneas: (A) control and (B) graphene implanted. Scale bars: 50 μm.
proliferation, which may account for the decreased compatibility of the cells compared to graphene. Our results indicated that graphene material supported both stromal cell adhesion and proliferation. When cells were cultured in 3D G-foam matrix, cell attachment and cell process extension were observed under SEM.

In addition to facilitating the growth of corneal stromal fibroblasts, G-film and the 3D G-foam were shown to reduce the cellular production and release of inflammatory cytokines (IL-6 and IL-8) when compared to Ti surface. Interleukin-6 and -8 are produced and released by human corneal fibroblasts in response to inflammation and infection. This cellular activity could be altered when cells are grown under a suboptimal condition such as the substrate surface. The secretion of IL-6 and IL-8, in response to interaction with materials, could therefore be used as an indication of the inflammatory potential of the substrate material. In the current study, the culture medium collected from fibroblasts seeded on 3D multilayer G-foam displayed the lowest level of cytokines compared with Ti and G-film. The lower cytokine levels for the G-foam samples may have resulted from two mechanisms—lower expression of cytokines by cells, or by adsorption of the inflammatory signal molecules by the graphene materials. Media with 65 or 200 pg/mL IL-6 exposed to the G-film showed 24% or 19% reduction of IL-6 from the media, respectively, while exposure to the G-foam showed a reduction of 72% and 24%, in both cases adsorbing approximately 50 pg of the cytokine over the 4-mm disc (Supplementary Fig. S1). This indicated that while there was likely some protein adsorption to the graphene surface, it is unlikely to account for the large decrease in cytokines detected in the fibrinoblast experiments (Fig. 4B). A potential anti-inflammatory biomaterial would be useful as a skirt material in patients with chronic inflammatory (e.g., Stevens-Johnson syndrome).49

In our study conducted with a rabbit corneal stromal implantation model, slit-lamp biomicroscopy did not show any corneal haze, or any sign of neovascularization or inflammation. The AS-OCT imaging showed that an intact G-film did not lead to any changes in corneal stroma in the short term, and confocal imaging showed that the epithelium was intact with normal cell–cell interactions. Keratocyte morphology was normal in all areas and keratocytes were not activated, even those in intimate contact with the G-film. No sign of any inflammatory response was observed by in vivo confocal microscopy, and histologic examination of the tissue largely agreed, revealing only very minor infiltration of mononuclear cells in the section of the cornea anterior to the implanted G-film. This finding suggests a minimal inflammatory response to graphene implantation in the cornea; however, the surgical insult and implantation of a relatively large and stiff material are likely to have contributed to this, rather than a specific response to the graphene material itself. In our previous work, eyes that had surgical treatment without introduction of any foreign materials (sham-implanted eyes) have also shown the presence of some mononuclear cells around the cornea. The rabbit corneal surface appeared uneven on AS-OCT, which could be due to the stiffness of G-film. The study of graphene implantation into the cornea allowed us to establish the acute responses of the eye to the implanted material. Long-term studies are not possible with solid films due to attenuated nutrient supply to the anterior corneal layers after implantation of G-film, which might restrict nutrient flow between corneal layers causing melting as seen in our rabbits. Further studies with modified G-film (such as film with perforation of different sizes and densities) will allow a longer follow-up period to test the long-term effects of intracorneal implantation. In addition, investigation of different forms of graphene (e.g., graphene oxide, reduced graphene oxide, and graphene-decorated flexible surfaces) with modified mechanical properties, as well as long-term in vivo behaviors of different graphene materials, are warranted to obtain a better understanding of its potential in clinical application.51,52 Our initial findings have demonstrated great promise for GBMs in the development of ophthalmologic biomaterials.

To conclude, in summary our study compared the potential biomaterials to be used as a skirt of next-generation Kpro, with respect to cellular adhesion/proliferation and in vivo short-term biocompatibility testing. The GBMs encouraged human corneal stromal fibroblast adhesion/proliferation as well as a reduction of expression in, and absorption of, excessive proinflammatory IL-6 and IL-8 cytokine mediators that were produced. Moreover, there was minimal inflammatory response after implantation into a rabbit corneal stromal pocket. The G-film showed excellent biocompatibility with human corneal stromal fibroblasts and acute biocompatibility with rabbit corneal tissue, demonstrating its potential as a candidate biomaterial for synthetic keratoprosthesis skirt.

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