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博士論文

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(酸化グラフェンと還元型酸化グラフェンコーティングによる
コラーゲンスキャフォールドの生体活性)

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Title**A comparative study of the bioactivities of collagen scaffold coated with graphene oxide and reduced graphene oxide.**

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Running title

The bioactivities of collagen scaffold coated with GO and RGO.

Key words

Graphene oxide (GO), reduced graphene oxide (RGO), tissue engineering, regenerative scaffold, cell ingrowth.

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Abstract

Graphene oxide (GO) is a single layer carbon sheet with a thickness of less than 1 nm. GO has good dispersibility due to surface modifications with many functional groups. Reduced graphene oxide (RGO) is produced by the reduction of GO. We examined the bioactivity of GO and RGO films, and collagen scaffold coated with GO and RGO.

GO films were fabricated on a culture dish. Some GO films were chemically reduced with ascorbic acid or sodium hydrosulfite solution. The biological properties of each film were evaluated by SEM, DNA content and alkaline phosphatase (ALP) activity following seeding with MC3T3-E1 cells. The adsorption of calcium on each film was also assessed. Subsequently, GO- and RGO-coated collagen scaffolds were prepared and characterized by SEM observation and compression tests. Each scaffold was implanted into the subcutaneous tissue of the back of rats. Measurements of DNA content and cell ingrowth area of the implanted scaffolds were performed 10 days post-surgery.

The results show that GO and RGO possess different biological properties. ALP activity and calcium adsorption were strongly enhanced by RGO, suggesting that RGO is effective for osteogenic differentiation. SEM showed that RGO-modified collagen scaffolds have rough irregular surfaces. The compressive strength of the RGO-coated scaffolds were greater than that of the GO-coated and non-coated scaffolds. Ingrowth of cells was frequently observed in the RGO-coated scaffolds. Taken together, these results suggest that GO and RGO possess different biological properties, and that RGO is more effective for biomedical applications than GO.

Introduction

Tissue engineering requires three major elements: cells, signaling molecules and scaffolds. Natural and artificial scaffolds have been developed for the repair and regeneration of various tissues^{1, 2)}. Scaffolds provide the environment and space for repopulation and specialization of stem cells, blood vessels and extracellular matrices³⁾. The surface morphology of the scaffold often has a profound effect on the attachment of surrounding cells and tissues after implantation⁴⁾. Many investigators have reported that surface modification of scaffolds with nano-sized materials stimulate their bioactivity, such as cell proliferation, tissue compatibility and degradability^{4, 5)}, indicating that effective modification of the scaffold surface may play an important role in facilitating tissue engineering.

Recently, carbon-based nanomaterials such as carbon nanotubes (CNT)⁶⁾, carbon nanohorn⁷⁾, fullerene⁸⁾ and graphene⁹⁾ have been extensively studied for biomedical applications. Graphene oxide (GO), a monolayer of carbon, holds particular promise as a tissue engineering substrate due to its unique physicochemical properties, including large surface area, high dispersibility, and hydrophilicity^{10, 11)}. The good dispersion of GO is attributed to many oxygen-containing functional groups on the surface of a GO nanosheet^{11, 12)}. Several investigators have reported that GO can serve as a carrier for drugs and other biomolecules^{13, 14)}. In addition, GO up-regulates the degree of proliferation and differentiation of cultured cells, suggesting that GO is a biocompatible substrate^{15, 16)}. Therefore, surface modification of a scaffold using GO nanosheets as a coating should promote biological responses and tissue reforming activities.

Reduced graphene oxide (RGO) is chemically fabricated by the reduction of GO with a reducing agent¹⁰⁾. GO and RGO have different physical and chemical natures: RGO tends to coagulate in solution and show electrical conductivity¹⁰⁾, in contrast to GO. RGO have been reported to affect cell behavior, protein loading capacity and antibacterial activity in different ways¹⁷⁻¹⁹⁾. Therefore, we hypothesized that GO and RGO biomodification of a scaffold would provide novel properties to the scaffold and enhance the tissue-reforming process. However, the different effects of these scaffolds have not yet been investigated. Accordingly, the aim of this study was to assess the biological effects of GO and chemically synthesized RGO film, and then modified a bio-safe collagen scaffold with GO and RGO following *in vitro*. The bioactivities of each scaffold were evaluated in rat by *in vivo* comparative analyses to assess the biocompatibility and tissue ingrowth rate after implantation into connective tissue.

Methods

Fabrication of GO and RGO Films.

GO aqueous dispersion (1 wt%, nano GRAX®, Mitsubishi Gas Chemical Company, Tokyo, Japan) was prepared as described previously²⁰. The single layer graphene oxide was characterized by atomic force microscopy (AFM, JEOL, Tokyo, Japan; Figure 1). The GO monolayer was ~ 1 nm thick with an average width of about 20 μm ²⁰.

The GO film was fabricated on a culture dish (Techno Plastic Products, Trasadingen, Switzerland). One ml of dilute GO solution (0.1 wt%) was used for a 40 mm diameter dish. The solution was left to dry and the film was rinsed well with phosphate buffered saline (PBS). Some of the GO films were chemically reduced with either 10% ascorbic acid or 2% sodium hydrosulfite solution for 1 h at 70°C. The morphology of the GO and RGO films was observed with a scanning electron microscope (SEM, S-4000, Hitachi, Tokyo, Japan) at an accelerating voltage of 10 kV after coating with a thin layer of Pt-PD. Each film was also characterized by X-ray diffraction (XRD, RINT2000, Rigaku, Tokyo, Japan) using Cu K α radiation at 40 kV and 40 mA. Diffractograms were obtained from $2\theta = 10^\circ$ to 40° at increments of 0.02° at a scanning speed of $4^\circ/\text{minute}$. The electrical resistivity of the GO and RGO films was measured using a resistivity meter (MCP-HT450, Mitsubishi Chemical Analytic, Yokkaichi, Japan). The hydrophilicity of the films was investigated by sessile drop method using a contact angle meter (DMs-200, Kyowa Electronic Instruments, Tokyo, Japan).

Cell morphology on the GO and RGO films.

1×10^4 mouse osteoblastic MC3T3-E1 cells (RIKEN Bioresource Center, Tsukuba, Japan) were seeded on GO and RGO films and cultured in humidified 5% CO₂ at 37°C using medium (MEM alpha-GlutaMAX-I, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Qualified, Life Technologies) and 1% antibiotics (Pen Strep, Life Technologies). A culture plate without GO was used as a control. After 24 h of culture, samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min and rinsed in cacodylate buffer solution. Films were then dehydrated in increasing concentrations of ethanol. Following critical point drying, samples were analyzed by SEM.

Measurement of DNA content and ALP activity on films.

GO and RGO films were made on a 24-well culture plate (Tissue culture testplate 24, Techno Plastic Products, Trasadingen, Switzerland) using the fabrication method described above. Ascorbic acid was used as the reducing agent. Each film was seeded

with 2.2×10^4 MC3T3-E1 cells and cultured for 7 or 14 days in humidified 5% CO₂ at 37°C using medium supplemented with 10% FBS and 1% antibiotics. A culture plate without GO was used as a control. After homogenization of the samples, the DNA in the cell suspension was measured using a DNA quantity kit (Prymarycell, Sapporo, Japan) according to the manufacturer's instructions using a fluorescence spectrophotometer (F-3000, HITACHI, Tokyo, Japan) equipped with a 356 nm excitation filter and a 458 nm emission filter. The cell suspension was also used to determine alkaline phosphatase (ALP) activity using a LabAssay ALP kit (Wako Pure Chemical Industries, Osaka, Japan). Optical density was measured using a microplate reader (ETY-300, Toyo Sokki, Yokohama, Japan) using an absorbance of 405 nm. ALP activity was calculated based on the amount of DNA in the sample.

Adsorption of calcium on films.

Two ml of culture medium supplemented with 10% FBS and 1% antibiotics was infused into each well coated with GO or RGO film. At 1, 3 and 7 days, specimens were rinsed using PBS, and acetic acid (0.5 M) was added for calcium determination. The calcium content of the acetic acid supernatant was determined colorimetrically by the ortho-cresolphthalein complexone method²¹). Optical density was measured at 575 nm.

Preparation of collagen scaffold coated with GO and RGO.

GO was dispersed in water:1-methyl-2-pyrrolidinone (1:9) solution at a concentration of 0.1 wt%. One hundred microliters of GO solution was injected into collagen scaffolds (6 × 6 × 3 mm, average porosity of 97.3%, Terudermis®, Olympus Terumo Biomaterials, Tokyo, Japan). Some samples were reduced by immersion in sodium hydrosulfite solution for 3 min. Then, the scaffolds were rinsed well with ethanol and air-dried (Figure 7A). The porosity of the scaffolds was calculated according to the following equation: porosity = $100 \times (1 - \rho_1 / \rho_2)$, where ρ_1 = bulk density and ρ_2 = theoretical density of the scaffold. Subsequently, each scaffold was characterized by SEM and a compression test. The compressive strength was measured using a universal testing machine (EZ-S, Shimadzu, Kyoto, Japan). The cross-head loading speed was set at 0.5 mm/min.

Surgical procedure.

The experimental protocol followed the institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No. 13-76). Seven 10-week-old male Wistar rats weighing 190 to 210 g were

given general anesthesia by an intraperitoneal injection of 0.6 ml/kg sodium pentobarbital (Somnopenhyl, Kyoritsu Seiyaku, Tokyo, Japan), as well as a local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine (Xylocaine Cartridge for Dental Use, Dentsply-Sankin K.K., Tokyo, Japan).

After a skin incision was made, each scaffold was implanted into the subcutaneous tissue of the back of each rat. Collagen scaffold without coating was applied as a control. Skin flaps were sutured (Softretch 4-0, GC, Tokyo, Japan) and tetracycline hydrochloride ointment (Achromycin Ointment, POLA Pharma, Tokyo, Japan) was applied to the wound.

DNA content of the implanted scaffolds.

Rats were euthanized using an overdose of sodium pentobarbital (2.0 ml/kg) at 10 days post-surgery. Several specimens extracted from the wound were freeze-dried. Following pulverization, the DNA content in each scaffold was examined using a DNA quantity kit.

Histological observation and measurement.

Several specimens were collected from the rats for histological observation. The tissue blocks, including the surrounding soft tissue, were fixed in 10% buffered formalin, embedded in paraffin wax, and cut into 6 μ m sections. Sections were stained with hematoxylin-eosin (HE). The samples were examined using light microscopy. Three stained sections were taken: one from the center of the scaffold and one 1 mm to either side of the center. Histomorphometric measurements of the rate of tissue ingrowth and the number of giant cells were performed using a software package (Image J 1.41, National Institute of Health, Bethesda, MD, USA).

Statistical analysis.

The means and standard deviations of each parameter were calculated for each group. Statistical analysis was performed using the Scheffé test for each measurement. P-values <0.05 were considered statistically significant. All statistical procedures were performed using a software package (DR. SPSS 11.0, SPSS Japan, Tokyo, Japan).

Results

Characterization of GO and RGO films.

RGO film was obtained by the reaction of reducing agents; 10% ascorbic acid or 2% sodium hydrosulfite solution, resulting in a dark colored film. (Figure 2A-C). SEM showed that GO and RGO films were wrinkled. The rough surface of RGO film was more defined than that of GO film (Figure 2D, E). RGO film exhibited higher electrical conductivity than GO film (Figure 2F). Electrical resistivity of RGO film treated with each reducing agent was equivalent. XRD showed a strong peak at 8-9° in the GO pattern that was absent in the RGO pattern (Figure 3). Contact angle measurements revealed the higher hydrophilic nature of GO: the contact angle of the GO film was significantly lower than that of RGO (Figure 4).

Cell morphology.

SEM showed cells attached to the GO and RGO films (Figure 5). Cell spreading and cell elongation was inhibited on the GO and RGO film compared to the control film.—

Measurement of DNA content, ALP activity and calcium content.

The amount of DNA and the ALP activity are presented in Figure 6A and B. MC3T3-E1 cells proliferated on GO and RGO films, but the DNA content of these films was significantly lower than that of the control at 7 days and similar tendency was observed at 14 days. The ALP activity of cells attached to GO films was decreased compared to the control at 14 days, whereas cells attached to RGO displayed the highest ALP activity, suggesting that reduction of graphene oxide modulated the differentiation of attached cells. The amount of calcium in the various samples is presented in Figure 6C. The RGO film significantly adsorbed calcium throughout the experimental period compared to the GO film. No time dependent increase in calcium was found for both GO and RGO films. In contrast, calcium was not detected on the surface of the control dish.

Characteristics of GO and RGO coated collagen scaffold.

SEM showed that GO-modified scaffolds contain interconnective spaces and have an average porosity of 96.9%. Higher magnification showed that GO film coated the surface of the collagen fibers of the scaffold. After reducing treatment, the scaffold had rough irregular surfaces, suggesting aggregation of GO on the surface caused by the reducing agent. The nature of this nano biomodified surface was confirmed by cross sectioning the material (Figure 7B-E). The compressive strength of GO- and

RGO-coated collagen scaffold was approximately two- and three-fold greater, respectively, than that of non-coated scaffold; these differences were statistically significant (Figure 7F).

Histological observation and measurement.

Evidence of tissue ingrowth was frequently observed in the nanocarbon-coated scaffolds (Figure 8A, C). Inflammatory response involving the accumulation of leukocytes and lymphocytes was rarely seen around the residual scaffold, indicating that the material was highly tissue-compatible. Macrophage-like giant cells associated with resorption of scaffold was often found in the scaffolds (Figure 8B and D). Black discoloration of giant cell was observed. In contrast, in the control group receiving non-modified collagen scaffold, cell and tissue ingrowth was rarely demonstrated in the implanted material (Figure 8E, F).

Significant differences were found in DNA content and area of tissue ingrowth between nanocarbon-modified scaffolds and unmodified scaffolds (Figure 9A and B). RGO samples showed an overall higher tissue ingrowth compared to GO. In particular, giant cells were significantly more prevalent in the RGO-coated scaffolds (Figure 9C). These results indicate that GO and RGO are likely to exert various biological functions in the body.

Discussion

The examination of DNA content and ALP activity revealed that RGO film enhances cell activities such as cell proliferation and differentiation compared to GO film. We speculated that these results are associated with the physicochemical properties of GO and RGO. Films of GO and RGO demonstrated high sorption of calcium in culture medium with FBS. Previous reports showed the efficacy of GO for rapid removal of some materials from contaminated water^{22, 23}. GO has an affinity for Ca^{2+} because the surface of GO is covered with epoxy, hydroxyl and carboxyl groups, which are well-suited for interacting with cations and anions²². We also speculated that calcium adsorption on the RGO film was enhanced by graphite intercalation, (i.e., the insertion of calcium between the graphite layers)^{22, 24}. Therefore, it seemed likely that GO and RGO films adsorbed calcium. Furthermore, it was reported that the addition of Ca^{2+} stimulated ALP activity and matrix mineralization of mouse osteoblasts in three dimensional culture²⁵. In this study, high Ca^{2+} accumulation on the RGO film might provide an environment for osteogenic cell differentiation.

In SEM observation, Cell spreading was suppressed on the GO and RGO film compared to the control. In addition, inhibition of cell proliferation was found on GO film. Since cells can attach to hydrophobic culture surfaces via the cell membrane²⁶, the hydrophilic nature of GO films should trigger adverse biological effects. On the other hand, antibacterial activity of GO-based materials has been recently described²⁷. GO and RGO induced significant production of superoxide anion radical and proved to be effective bactericidal agents²⁸. Oxidative stress also stimulated the inhibition of cell viability and provided a cytotoxic effect^{29, 30}. Chang et al. demonstrated that cells cultured on GO showed similar cyto-structures to the control (non-treated) cells, but reactive oxygen species (ROS) produced by a high dose of GO induced a slight decrease in cell viability³¹. Furthermore, the level of ROS in GO-treated cells was higher than in RGO-treated cells²⁸. Therefore, GO film would suppress both DNA content and ALP activity of MC3T3-E1 cell.

In animal tests, scaffolds with GO and RGO distinctly stimulated tissue response, and especially tissue-ingrowth into RGO-coated scaffolds. Ordinarily, the mechanical properties of the regenerative scaffold play a facilitative role in maintaining the space for ingrowth of cells and blood vessels from the surrounding tissue. In the present examination, the GO and RGO coating significantly increased the compressive strength of the collagen scaffold, likely due to the GO nanosheet assembling on the strut surface of the collagen scaffold and adding to its elasticity. Furthermore, the compressive strength of the RGO-coated collagen scaffold was approximately two-fold greater than

that of the GO scaffold. GO is aggregated by the reducing process, resulting in a tough coating on the scaffold. GO and RGO coated scaffold would maintain the regenerative space in the body in tissue engineering approach. In general, regenerative scaffolds are designed to provide a highly porous structure for tissue-ingrowth; however, higher porosity generally causes lower mechanical strength ³²⁾. SEM images of RGO-coated materials, however, show that the collagen sponge foam coated with nanocarbon retained its porous structure. Additionally, SEM showed a rough surface on the RGO-coated scaffold, while the GO film had a relatively smooth surface. The interfacial morphology of cells strongly affects the induction of cell reactions ^{33, 34)}. Many investigators have demonstrated that nano-/micro scale structures on bio-based materials provide advantages for tissue engineering processes ^{35, 36)}. Thus, nano-modification of RGO could similarly provide advantages, such as porosity and surface structure, for tissue engineering of different cell types and of multi-cellular organisms.

Inflammatory cells such as neutrophils and lymphocytes were rarely seen around the nano-modified samples in this study. In addition, collagen scaffold consisting of atelocollagen is recognized as a biocompatible material ^{37, 38)}, suggesting that GO- and RGO-coated scaffolds have good biocompatibility. However, histological samples following exposure to RGO frequently showed numerous macrophage-like cells in the scaffold, indicating the clinical need for biomedical procedures which prevent incomplete healing due to macrophage assembly. Furthermore, GO and RGO appeared to agglomerate by phagocytosis of macrophage-like cells, because black discoloration of giant cell was found. GO accumulations have been reported following phagocytosis and remain as a residue in cell lysosomes ³⁹⁾. In contrast, carbon nanotubes were gradually biodegraded in macrophage lysosomes over two years post-implantation ⁴⁰⁾. Therefore, degradation of GO-based nanomaterials is an important point to be considered in the future.

Conclusion

This study revealed that GO and RGO possess different biological properties, and that RGO is more effective for biomedical applications than GO. The bioactivity of nanocarbon-modified materials could serve as suitable biomaterials for tissue engineering.

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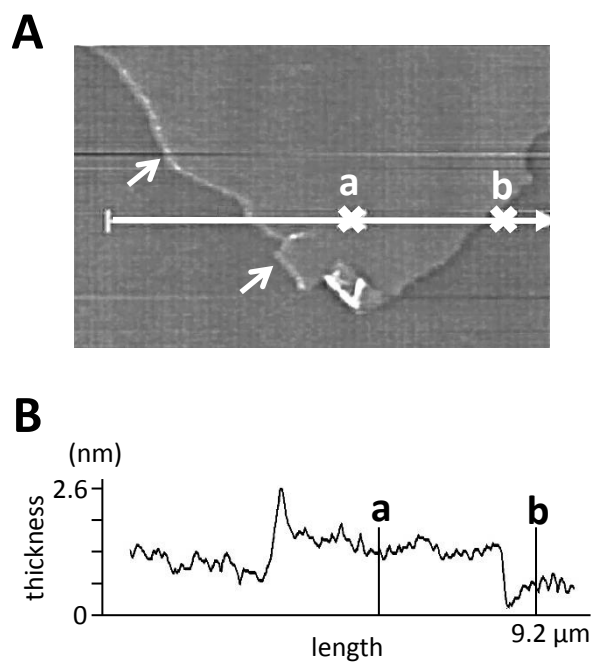


Fig.1

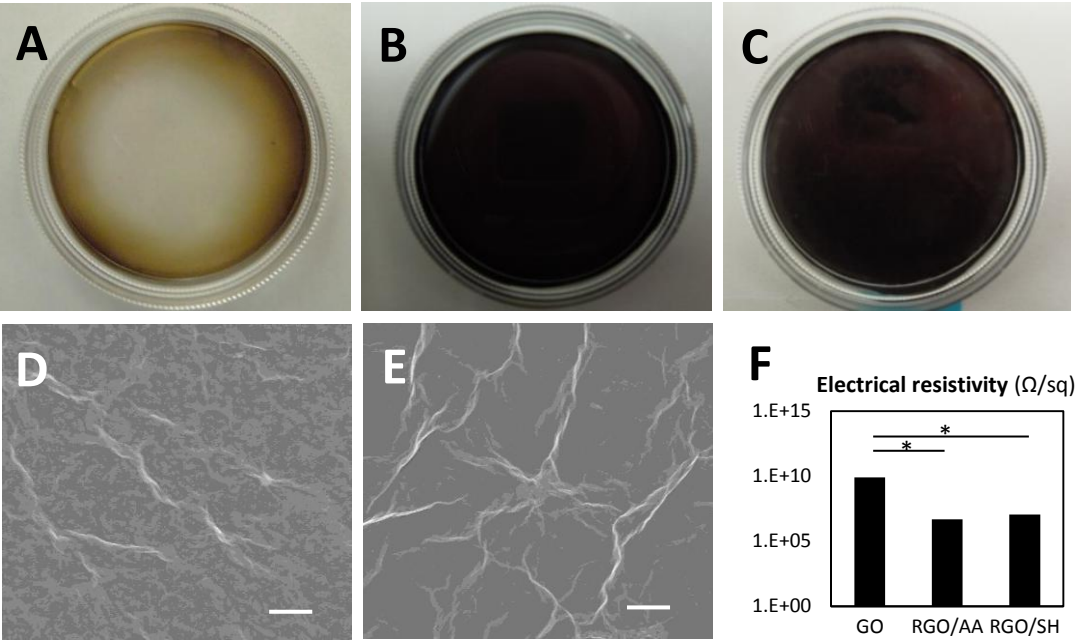


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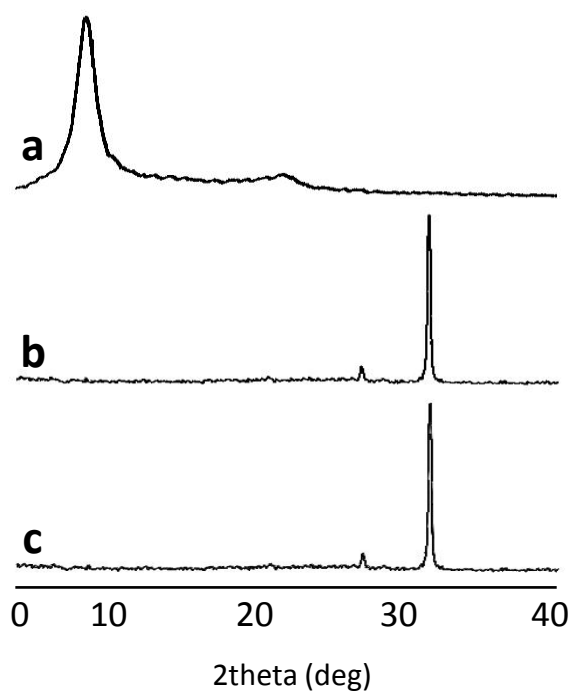


Fig.3

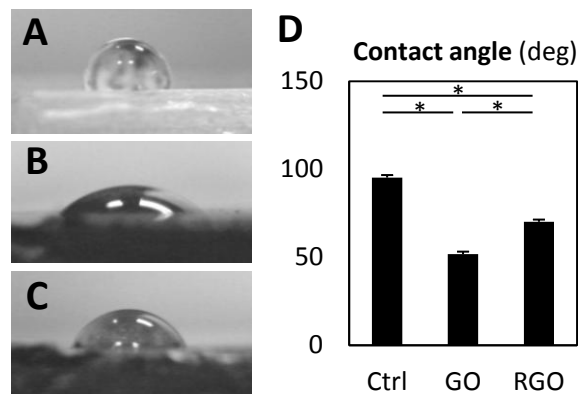


Fig.4

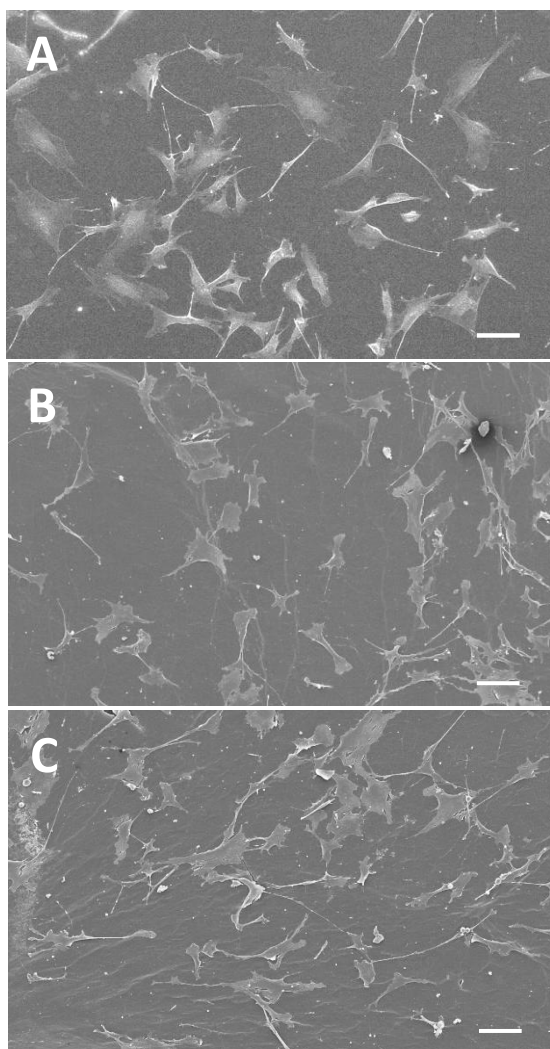


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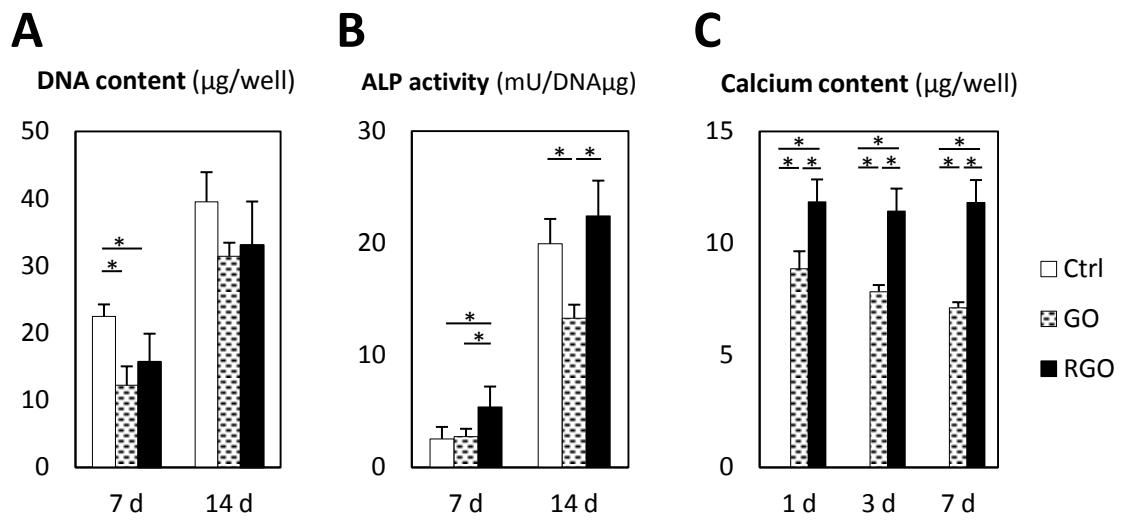


Fig.6

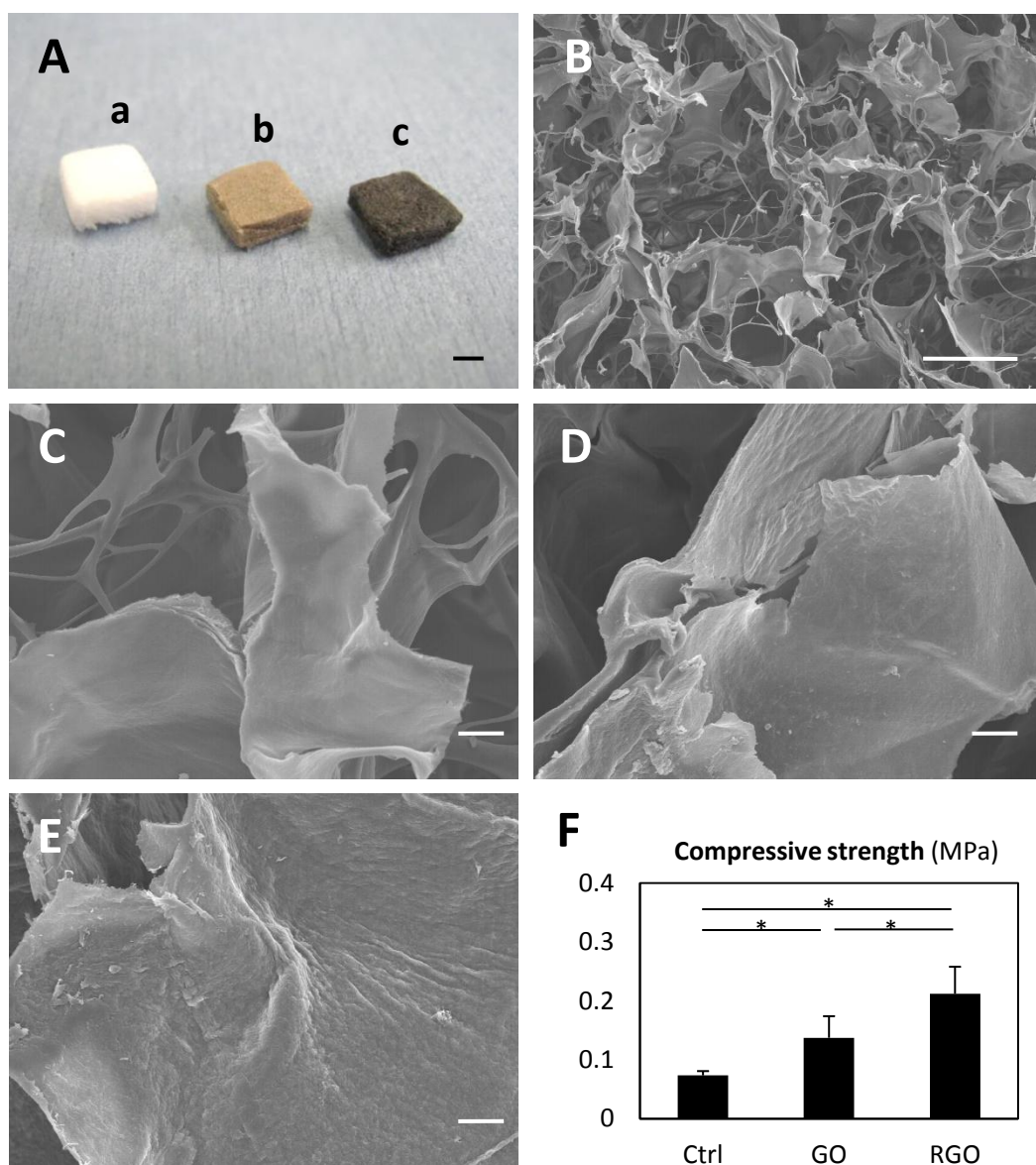


Fig.7

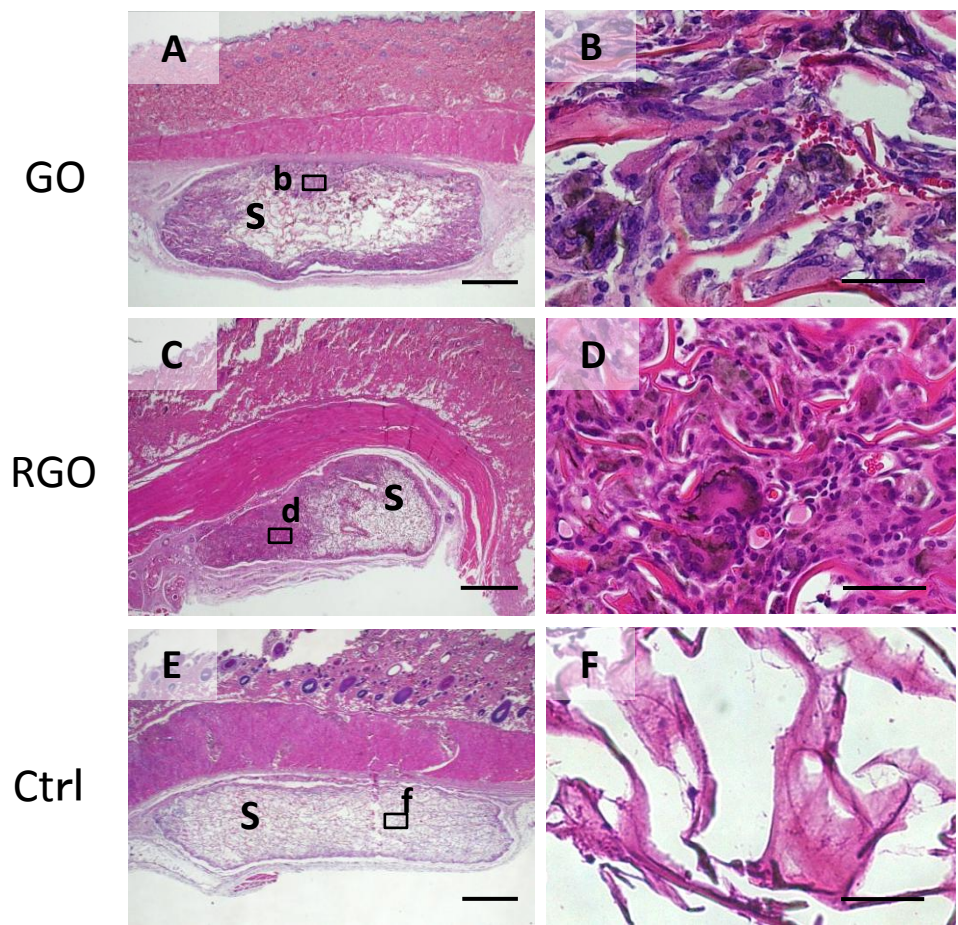


Fig.8

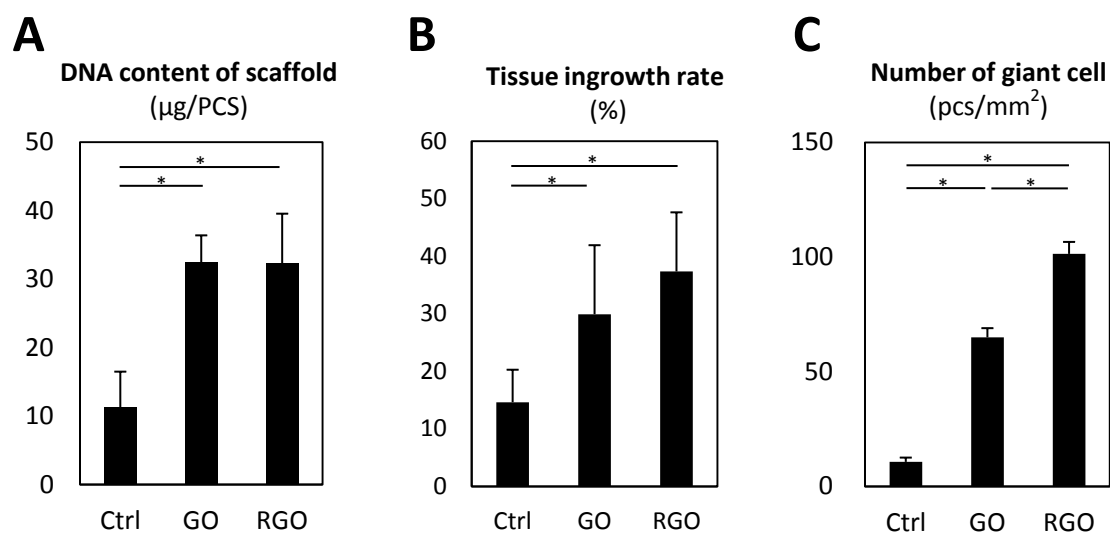


Fig.9

Figure legends

Figure 1

(A) AFM image of a monolayer of GO sheet (arrows). (B) Height profile for the white line in (A). Scanning points (a) and (b) indicate the presence and absence of the GO sheet, respectively.

Figure 2

(A) GO film. (B) GO film reduced with ascorbic acid. (C) GO film reduced with sodium hydrosulfite. (D) SEM micrograph of the GO film. (E) SEM micrograph of the RGO film. (F) Electrical resistivity measurement ($N = 5$, mean $\pm$ SD) of the GO film (GO), GO film reduced with ascorbic acid (RGO/AA) and GO film reduced with sodium hydrosulfite (RGO/SH). * : $P < 0.05$. Scale bar represents 10 μm (D, E).

Figure 3

XRD patterns of GO film (a), GO film reduced with ascorbic acid (b) and GO film reduced with sodium hydrosulfite (c).

Figure 4

Contact angle image. (A) Culture dish. (B) GO film. (C) RGO film. (D) Summary of the contact angle ($N = 6$, mean $\pm$ SD). * : $P < 0.05$.

Figure 5

SEM micrograph of cell morphology on (A) culture dish, (B) GO film and (C) RGO film. Scale bars represent 100 μm .

Figure 6

In vitro assessment of each film ($N = 6$, mean $\pm$ SD). (A) DNA content. (B) ALP activity. (C) Calcium content. * : $P < 0.05$.

Figure 7

(A) Collagen scaffold (a), GO-coated collagen scaffold (b) and RGO-coated collagen scaffold (c). (B) SEM micrograph of the inner surface of the collagen scaffold. (C) Higher magnification of the collagen scaffold, showing the smooth surface. (D) Higher magnification of the GO-coated collagen scaffold, showing its irregular surface. (E) Higher magnification of the RGO-coated collagen scaffold. A rough surface was frequently produced by chemical reduction. (F) Compressive strength of

each scaffold. * : $P < 0.05$. Scale bars represent 2 mm (A), 100 μm (B) and 10 μm (C, D, E).

Figure 8

Histological findings at 10 days. (A) Specimen implanted with GO-coated scaffold. (B) Higher magnification of the framed area in (b). Macrophage-like giant cells appeared in the GO-coated scaffold. (C) Specimen implanted with RGO-coated scaffold. (D) Higher magnification of the framed area in (c). (E) Specimen implanted with collagen scaffold. (F) Higher magnification of the framed area in (f). Abbreviations: S, scaffold. Scale bars represents 1 mm (A, C, E) and 50 μm (B, D, F). Staining: HE.

Figure 9

The assessment of each scaffold ($N = 6$, mean $\pm$ SD). (A) DNA content of the scaffold. (B) Tissue ingrowth rate. (C) Number of giant cells. * : $P < 0.05$.

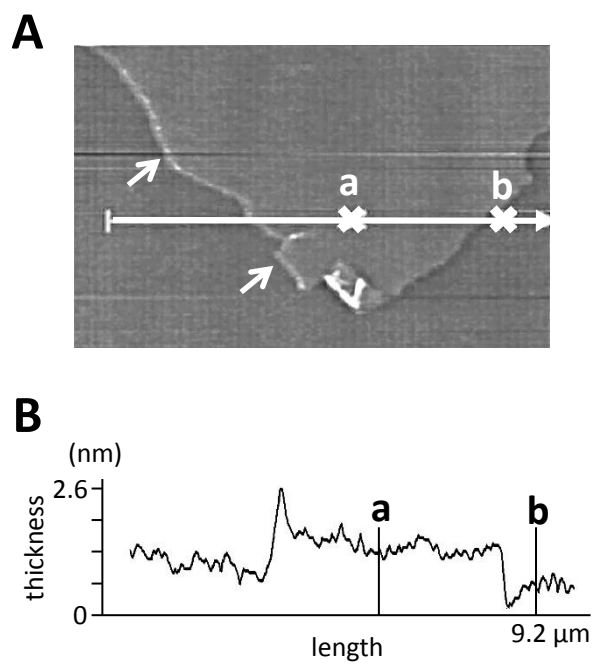


Fig.1

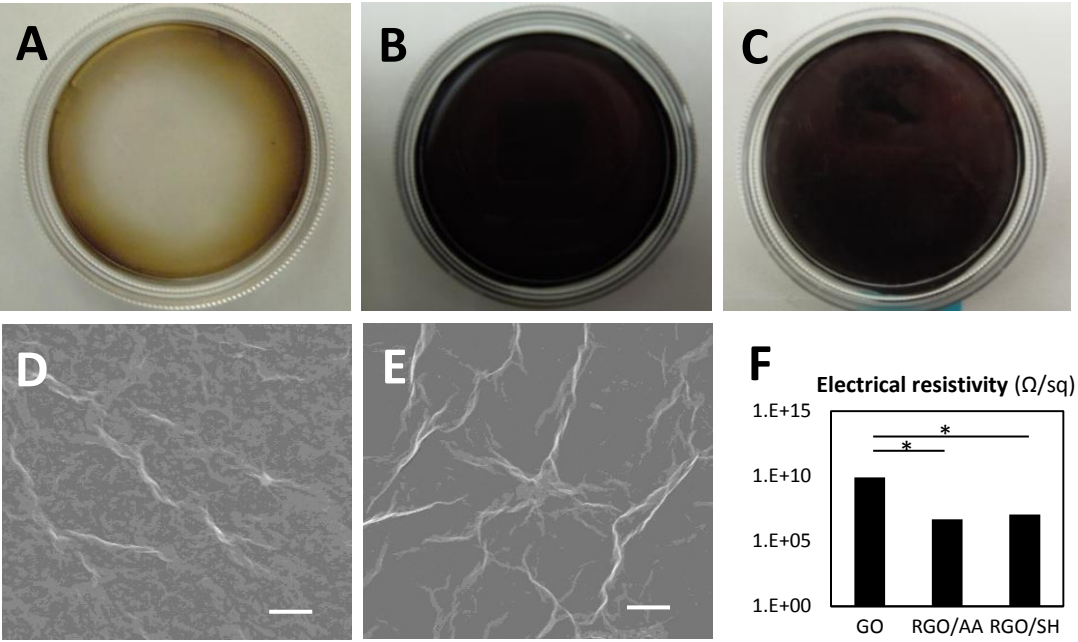


Fig.2

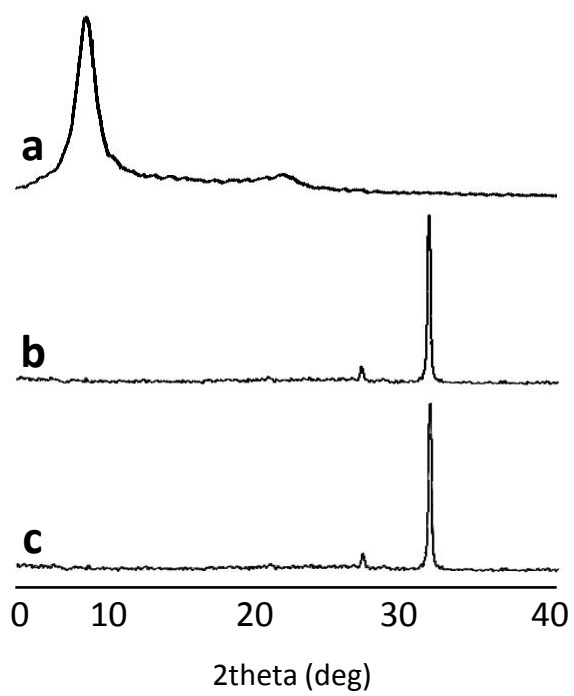


Fig.3

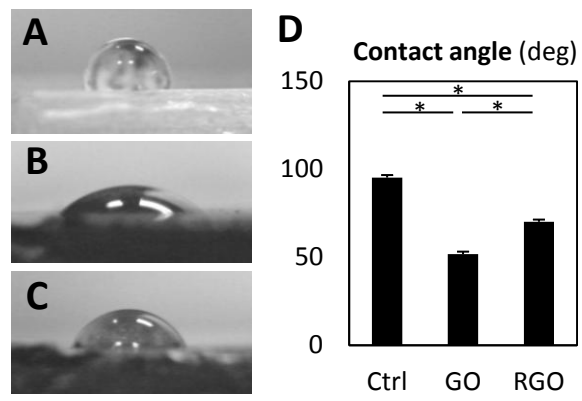


Fig.4

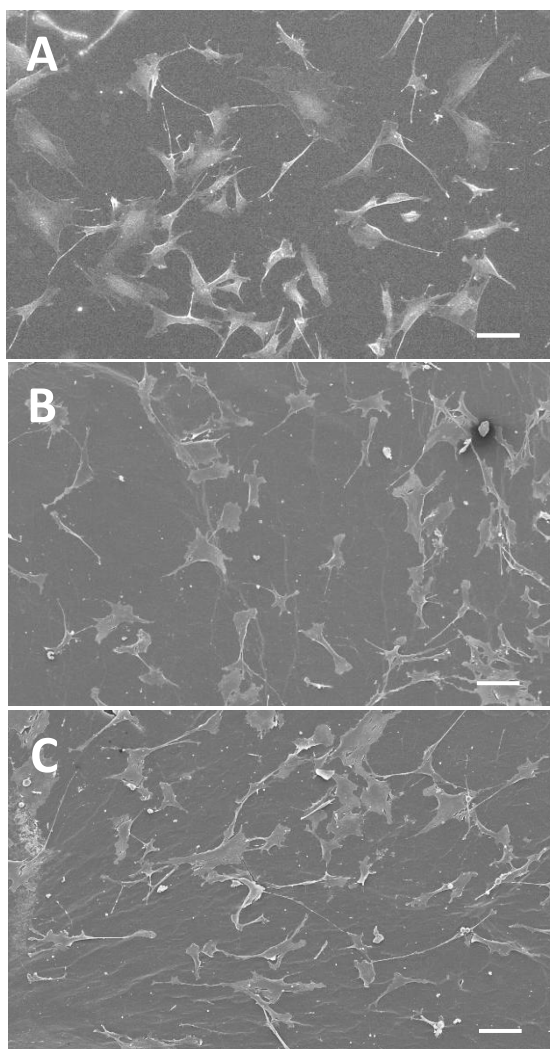


Fig.5

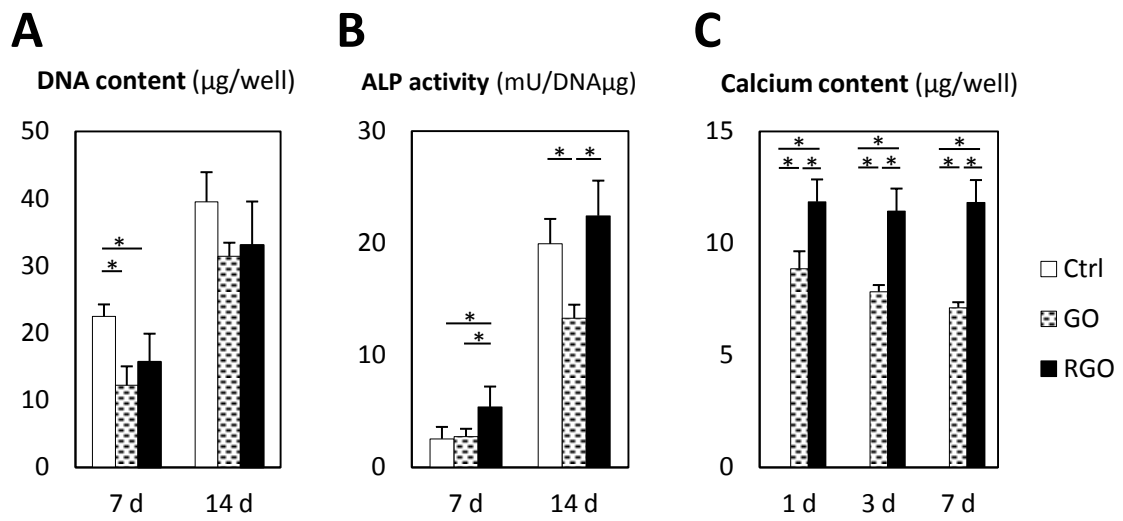


Fig.6

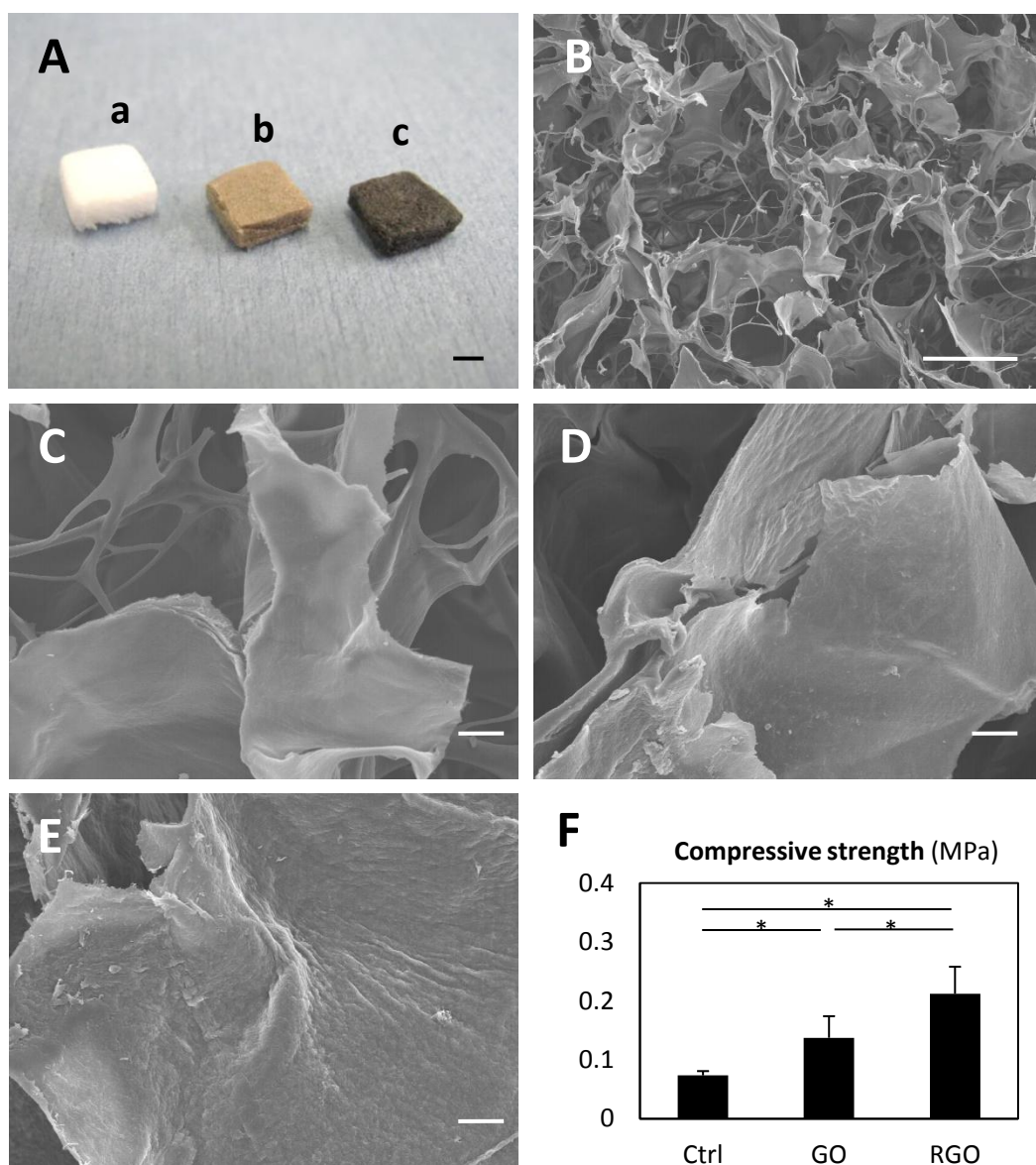


Fig.7

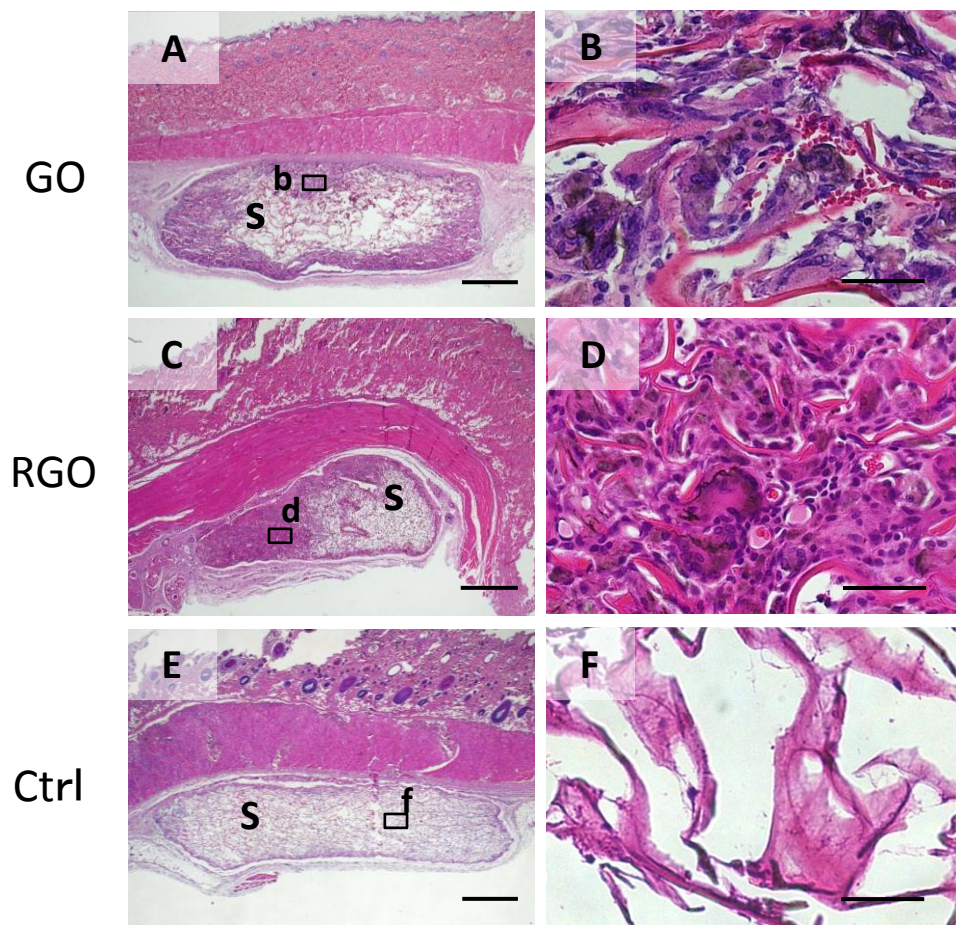


Fig.8

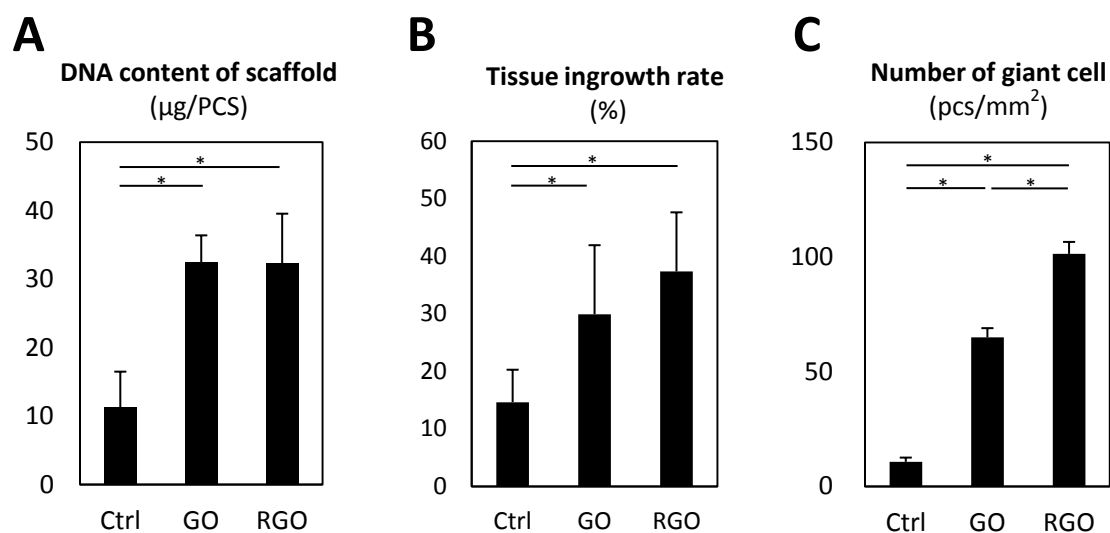


Fig.9