Supplementary Information (SI) for Biomaterials Science.
This journal is © The Royal Society of Chemistry 2026

# Bioinspired Synergistic Chitosan–Graphene–Tannin Hydrogel Orchestrates Inflammation Resolution and Accelerates Skin Tissue Repair

Isleidy Ruíz[a*], Luisbel González[b], Julia Eduarda Schneider Gregória[c,d], Cintia Sena Bueno[c,d], Maria Esméria Corezola do Amaral[c,f], Guilherme Ferreira Caetano[c,d,e*], Katherina Fernández[a*]

[*]Author corresponding: isruiz@udec.cl;caetanogf@fho.edu.br;kfernandeze@udec.cl

[a] Laboratorio de Biomateriales, Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Concepción, Concepción, Chile.

[b] Instituto de Ciencias Aplicadas, Facultad de Ingeniería, Universidad Autónoma de Chile, Santiago 8581151, Chile

[c] University Center of Hermínio Ometto Foundation, FHO, Araras 13607-339, Brazil.

[d] Graduate Program of Orthodontics, University Center of Hermínio Ometto Foundation, FHO, Araras 13607-339, Brazil.

[e] Division of Dermatology, Department of Internal Medicine, Ribeirao Preto Medical School, University of Sao Paulo, Sao Paulo 05508-060, Brazil.

[f] Núcleo de Ciência da Saúde -Centro Universitário da Fundação Hermínio Ometto, Araras, SP-Brasil

**Synthesis of Graphene Oxide (GO) and Reduced Graphene Oxide (rGO)**

GO synthesis was carried out by the oxidation of natural graphite powder using the modified Hummers method.[1] In a typical example, 2.25 g of graphite were added to a mixture of 30 mL of $H_3PO_4$ and 270 mL of $H_2SO_4$. Once dissolved, 13.5 g of $KMnO_4$ were slowly added and heated for 1 h at 40 °C. The mixture was then cooled to 25 °C, and the oxidation was stopped by adding $H_2O_2$ (60% *v/v*) until a color change from black to dark green was observed, without the presence of foam. Subsequently, it was centrifuged at 5000 rpm for 20 min and the precipitate was washed with HCl (10% *v/v*) until a light brown color was achieved. After that, three Milli-Q water washes were carried out, and it was centrifuged at 9000 rpm for 20 min. In this stage, the absence of chlorides was verified using $AgNO_3$. Then, a wash with ethanol and several washes with Milli-Q water were carried out to eliminate traces of alcohol. The solution was then dialyzed for 72 h, in 12 kDa dialysis bags and in 5 L of Milli-Q water, with water changes every 24 h. The dialyzed solution was placed in a cryogenic bath for 2 h at −40 °C and lyophilized for 72 h.[2]

GO reduction was performed by the method proposed by Xu et al. (2010). A solution of 0.24 g of tris(hydroxymethyl)aminomethane in 200 mL of Milli-Q water was prepared, to which 0.05 g of DA and 0.1 g of GO were added. Once the GO dispersion was achieved, the pH was adjusted to 8.5 with HCl (5%) and sonicated for 20 min in an ice bath. Then, it was heated at 60 °C for 24 h with constant and gentle stirring to induce the reduction process. Subsequently, the sample was filtered through Whatman® No.1 filter paper and the retained solid was dialyzed in Milli-Q water for 72 h, with water changes every 24 h. When the dialysis was finished, the sample was filtered and dried at room temperature.[3]

**Pinus Radiata Bark Extract Production (TA)**

Pinus radiata bark extracts were produced through a pilot-scale extraction process, as described by Bocalandro et al. (2012).[4] For this purpose, a reactor volume of 4 $m^3$ and a vapor heating system composed of a shell and a tube heat exchanger with 6 m2 heat transference area were used. In addition, a recirculation circuit for the extracted solution was implemented. Briefly, the Pinus radiata bark was ground with a double-knife mill to an average size lower than 20 mm. Then, the bark was dried at room temperature to a humidity of 24.5% (dry weight), and 100 kg (dry weight) of bark was soaked in an ethanol/water solution at a 1:20 ratio (w/v) for 120 min at 120 °C. Subsequently, the ethanol was evaporated in a vacuum (absolute pressure 0.05 bar) at room temperature. Thus, the water-insoluble particulate material after decanting and the water-soluble polyphenol fraction were obtained. Finally, the water-soluble polyphenols were lyophilized at room temperature and the obtained extracts were stored in sealed amber glass containers for further analysis.

**In vitro characterization**

**Hydrogels cytotoxicity.** These experiments were conducted using a cell density of $10^4$ cells/ mL. First, 1 mL of DMEM medium was added to 10 mg of material and to individual materials (rGO, PF and CS) to promote full contact. After 24 h of incubation at 37 ° C, the supernatant was recovered and mixed with 5% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotics (100 units/mL of penicillin and 100 units/ mL of streptomycin). The supernatant from each sample was added to the cells and incubated for 48 h at 37 °C under humidified air with 5% (v/v) $CO_2$. At the end of the incubations, the supernatants were removed, and the cells were washed with PBS, pH 7.4. Then, 100 μL of fresh DMEM

medium was added to the cells and 5 mg/mL MTT solution was added for the determination of cell viability. The plates were incubated for 4 h at 37° C with $CO_2$, then 25 µ L of the medium was removed and 50 µL of dimethyl sulfoxide (DMSO) was added to the wells. After 10 min, the supernatant was removed by aspiration, and the formazan crystals were dissolved in DMSO (100 µ L per well), followed by shaking for 5 min. The absorbance was determined using a microplate reader (Biotek Synergy 2, Agilent, CA, USA) at a wavelength of 540 nm. The cell viability (%), relative to control cells, was calculated from Equation (1):

$$Kill\ (\%) = \frac{A_{test}}{A_{control}} * 100 \quad (1)$$

where $A_{\text{is}}$ and $A_{\text{control}}$ are the absorbance values of the wells (with the material) and control wells (without the material), respectively. DMEM medium was used as a positive control.

**In Vitro Wound Healing Assay (Scratch Test).** An *in vitro* wound healing assay was performed according to previously described experimental procedures [5], with slight modifications. Briefly, human dermal fibroblast cells (50,000 cells/well) were seeded into a 24-well plate and incubated at 37 °C for 48 h in a humidified atmosphere with 5% $CO_2$. Subsequently, a vertical scratch was manually created in the middle of the human dermal fibroblast monolayer, using a 200 µL sterile pipet tip. Then, each material was fixed on CellCrown 24 inserts (Corning Incorporated, PA, USA) and placed on the 24-well plate without touching the surface. The wound closure rate and the cell migration were monitored over time (48 h) using an IncuCyte light microscope (Sartorius S3, USA). Finally, the images were analyzed using ImageJ software. The wound closure rates were calculated according to Equation (2):

$$Rate\ of\ wound\ closure(\%) = \frac{(A_0 - A_t)}{A_0} * 100 \quad (2)$$

where $A_0$ is the initial wound area and $A_t$ is the wound area after each time interval.

**In vivo characterization**

**In Vivo Wound Healing Assay.** The wound healing assay was performed on 36 adult male Wister rats (mean weight 190–210 g) from the Animal Hospital of the Faculty of Medicine, Hermínio Ometto Foundation University Center, SP, Brazil, Department of Clinical Medicine. These rats were housed in collective cages for 7 days before the surgical procedure. During the study, they were transferred to individual cages maintained at room temperature (22°C) under controlled light conditions (12–12 h light/dark cycles) and received a solid diet and water supplemented with Vitagold® (Tortuga, São Paulo, Brazil) ad libitum.

**Surgical Procedure and Groups.** The animals were anesthetized by intraperitoneal injection of a 2.5% tribromoethanol solution (100 μL/10 g; Sigma, USA). The animals' backs were shaved, and after asepsis with 70% ethanol, two full-thickness circular incisions were made in the dorsocervical region with a sterile histological punch (1.5 cm in diameter), the thickness of which encompassed all skin layers. The excised skin from each animal was preserved for subsequent histological and biochemical analysis, representing samples from the initial day (day 0) without treatment.

The groups, consisting of 9 animals each, were separated according to wound treatment: in group 1, both wounds of each animal were treated with chitosan hydrogels (circular, 2.0 cm in diameter) pre-moistened with 0.9% saline; in group 2, both wounds of each animal were treated with chitosan-reduced graphene oxide hydrogels (circular, 2.0 cm in diameter) pre-

moistened with 0.9% saline; In group 3, both wounds of each animal were treated with chitosan-reduced graphene oxide-tannin hydrogels (circular, 2.0 cm in diameter) pre-moistened with 0.9% saline; in group 4, the wounds were treated only with 0.9% saline (150 µL each). The wounds of all animals in both groups were covered with gas, and the dressing was changed daily after hydration with 0.9% saline (100 µL) until the follow-up day. The hydrogels were removed only after euthanasia of the animals at 2, 7, and 14 days.

Wound area was calculated using ImageJ software to analyze re-epithelialization using the wound healing rate (WHR) according to equation 3.

$$WHR(\%) = \frac{(A_0 - A_t)}{A_0} * 100 \quad (3)$$

Where $A_0$ corresponds to the day of surgery (day 0) y $A_t$ corresponds to the day of euthanasia (days 2, 7 o 14).

Histological features were assessed by the same specialist (blinded for treatment). Wound histological assessment scoring included: reepithelialization (0 = none; 1 = partial; 2 = complete but immature/thin; 3 = complete and mature), neovascularization (0 = none; 1 = up to 5 vessels/high-power field [HMF]; 2 = 6–10 vessels/HMF; 3 = >10 vessels/HMF), amount of granulation tissue (0 = none; 1 = scant; 2 = moderate; 3 = abundant), and inflammatory cells (0 = none; 1 = scant; 2 = moderate; 3 = abundant).

**Western blotting.** Tissue samples were placed in liquid nitrogen and macerated with a hammer in a stainless steel support. Subsequently, the lyophilized samples were homogenized in a buffer containing 1% Triton-X-100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium

orthovanadate, 2 mM PMSF and 0.01 mg/mL of antiprotease cocktail using the Politron® equipment. The homogenate was centrifuged at 12,000 rpm at 4 °C for 20 minutes to separate the sample according to its density. Subsequently, the supernatant was aspirated and its proteins were quantified with the biuret reagent. Subsequently, the samples received Laemmli buffer added with 200 mM dithiothreitol in a 5:1 ratio. Finally, they were subjected to a temperature of 100°C for 5 minutes. After protein quantification, 50 µg of total protein was subjected to 12% polyacrylamide gel electrophoresis (SDS-PAGE) in a BioRad Minigel apparatus with electrophoresis buffer. Initially, they were subjected to 80 V up to the gel stacking line and subsequently to 120 V up to the gel resolution line. A molecular weight marker with established molecular weight values was used in each gel. The proteins separated in the gel were transferred electrically to a nitrocellulose membrane using a BioRad® apparatus for approximately 1.5 hours at 120 V, kept on ice. To reduce nonspecific binding, the PVDF membrane was incubated in 5% BSA diluted in basal solution at room temperature for 1 hour. After blocking, the membrane was washed three times with basal solution and incubated with specific antibodies diluted in 3% BSA blocking solution, and kept at 4°C overnight with gentle shaking. The primary antibodies used are described in Table S2 (1:5000). The following day, they were washed with basal solution and incubated with secondary antibody for approximately one hour. Approximately 2 ml of chemiluminescence solution in a 1:1 dilution of the reagents from the Thermo Fisher® commercial kit was then added for approximately two minutes. Light emission was detected and captured using the SysGen® photodocumentation system. Optical density was read using ImageJ® software. The results were normalized by comparing the expression with GAPDH.

**Table S1.** Skin irritation assessment: scoring scale for erythema, scaling, and edema

| Reaction | Irritation Score |
|---|---|
| Formation of erythema and eschar | |
| Without erythema | 0 |
| Mild erythema (barely noticeable) | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema (red) tending to eschar formation | 4 |
| Edema formation | |
| No edema | 0 |
| Mild edema (barely noticeable) | 1 |
| Well-defined edema (edges of the area well defined by magnification) | 2 |
| Moderate erythema (increase approx. 1 mm) | 3 |
| Severe edema (increase of more than 1 mm and extension beyond the area of exposure. | 4 |
| Maximum possible score for irritation | 8 |

**Table S2.** TaqMan assays used for Western blot and RT-qPCR

| TaqMan Assays | Product Codes |
|---|---|
| *Gapdh*<br>*Glyceraldehyde-3-Phosphate Dehydrogenase* | *Rn01775763_g1* |
| *Vegf*<br>*Vascular endothelial growth factor* | *Rn01511602_m1* |
| *COL I*<br>*Collagen type I* | *Rn01463848_m1* |
| *COL II*<br>*Collagen type III* | *Rn01437681_m1* |
| *MPO*<br>*Myeloperoxidase* | *Rn01460205_m1* |
| *CD68*<br>*Glycoprotein CD68* | *Rn01495634_m1* |
| *Arg1*<br>*Arginase 1* | *Rn00691090_m1* |
| *IL-6*<br>*Interleukin 6* | *Rn01410330_m1* |
| *IL-10* | *Rn01495634_g1* |

| | |
|---|---|
| *Interleukin 10* | |
| *IL-1β* | *Rn00580432_m1* |
| *Interleukin 1β* | |
| *IL-1rn* | *Rn02586400_m1* |
| *Interleukin 1 receptor antagonist* | |

**Table S3:** Quantitative results of *in vivo* assay

| Samples | Re-epithelialization | Neovascularization | Granulation tissue | Inflammatory cells |
|---|---|---|---|---|
| | | **Untreated** | | |
| **2 days** | 0 | 2 | 1 | 2 |
| **14 days** | Marked epidermal acanthosis. Loose connective tissue is seen in the papillary and reticular dermis. | | | |
| | | **CS** | | |
| **2 days** | 1 | 2 | 2 | 3 |
| **14 days** | There is mild acanthosis, no hyperkeratosis. There are no traces of skin appendages in the wound area yet. | | | |
| | | **CS-rGO** | | |
| **2 days** | 1 | 2 | 1 | 3 |
| **14 days** | Complete maturation of the epithelium, but epidermal ridges or marked dermal papillae are not yet observed. Loose connective tissue is observed in the papillary and reticular dermis. There is a greater presence of inflammatory cells than fibroblasts. | | | |
| | | **CS-rGO-TA$_3$** | | |
| **2 days** | 2 | 3 | 3 | 2 |
| **14 days** | Good differentiation of the epithelium. Dermal papillae and epidermal ridges are clearly visible, with many blood vessels close to the epithelial basement membrane. Differences are seen between the loose connective tissue of the papillary dermis versus a denser and more organized/molded tissue in the reticular dermis. In addition, the generation of developing hair follicles is seen. No hyperkeratosis or acanthosis is observed. | | | |

Re-epithelialization (0 = none; 1 = partial; 2 = complete but immature/thin; 3 = complete and mature). Neovascularization (0 = none; 1 = up to 5 vessels/high power field [HMF]; 2 = 6-10 vessels/HCF; 3 = >10 vessels/HCF). At this point, 3 visual fields were analyzed, and an average was obtained. Amount of granulation tissue (0 = none; 1 = scant; 2 = moderate; 3 = abundant) Inflammatory cells (0 = none; 1 = scant; 2 = moderate; 3 = abundant).

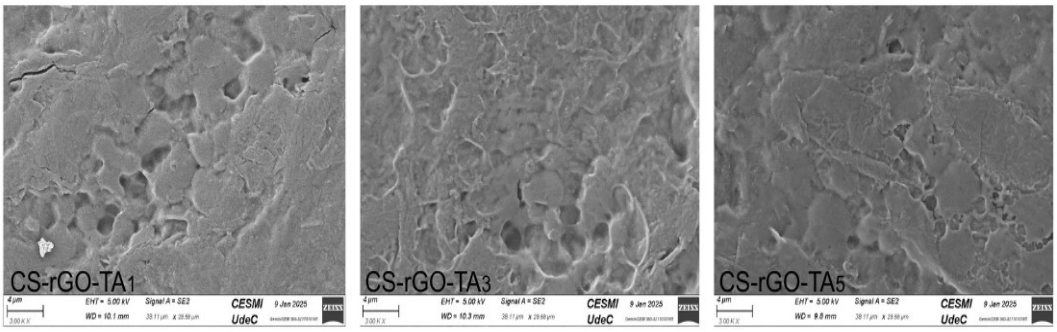

Fig. S1 SEM images of the synthesized hydrogels

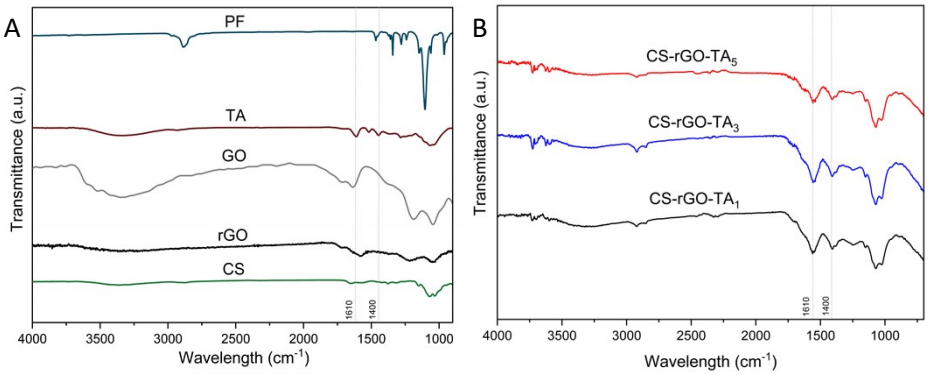


Fig. S2 FTIR spectra of the raw materials (A) and the synthesized hydrogels (B)

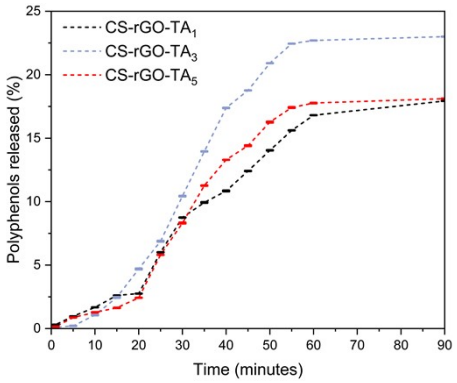


Fig. S3 Release profiles of polyphenols over time

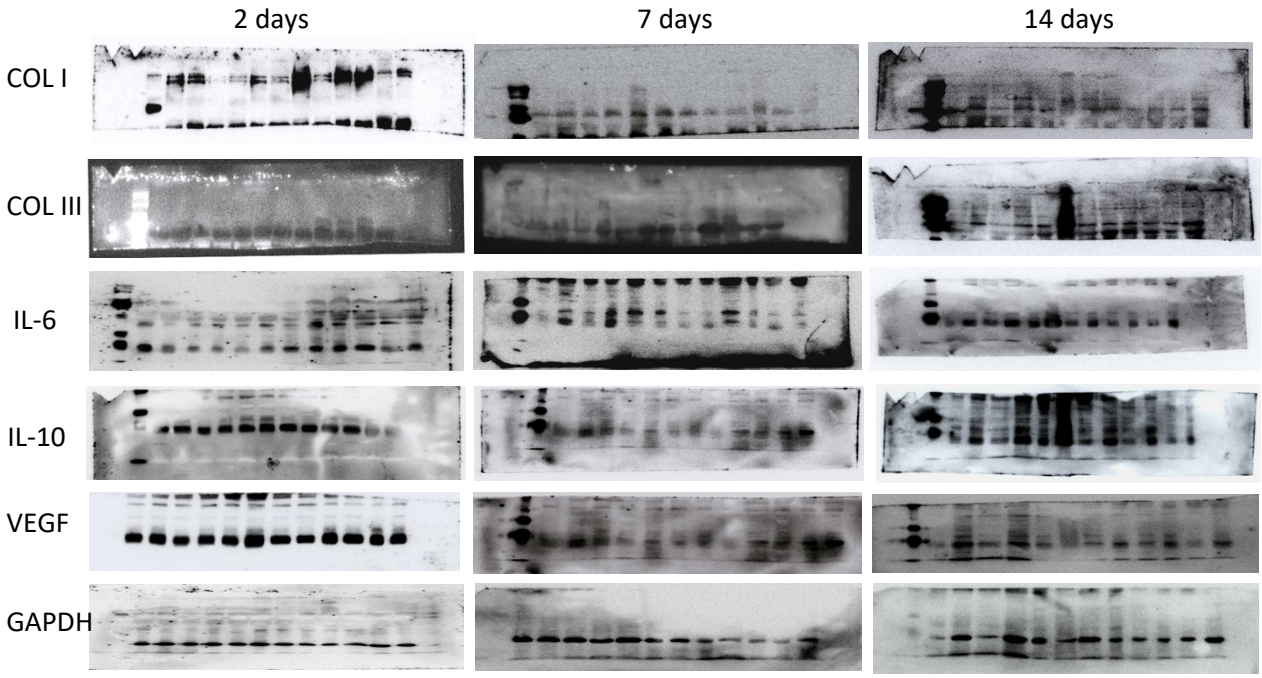


Fig. S4 Images of the gel from the Western blotting assay

**Reference**

(1) Hummers, W. S. Jr.; Offeman, R. E. Preparation of Graphitic Oxide. *J Am Chem Soc* **1958**, *80* (6), 1339. https://doi.org/10.1021/ja01539a017.

(2) Chen, J.; Yao, B.; Li, C.; Shi, G. An Improved Hummers Method for Eco-Friendly Synthesis of Graphene Oxide. *Carbon N Y* **2013**, *64*, 225–229. https://doi.org/10.1016/J.CARBON.2013.07.055.

(3) Qun Xu, L.; Jing Yang, W.; Neoh, K.-G.; Kang, E.-T.; Dong Fu, G. Dopamine-Induced Reduction and Functionalization of Graphene Oxide Nanosheets. *Macromolecules* **2010**, *43* (20), 8336–8339. https://doi.org/10.1021/ma101526k.

(4) Bocalandro, C.; Sanhueza, V.; Gómez-Caravaca, A. M.; González-Álvarez, J.; Fernández, K.; Roeckel, M.; Rodríguez-Estrada, M. T. Comparison of the Composition of Pinus Radiata Bark Extracts Obtained at Bench- and Pilot-Scales. *Ind Crops Prod* **2012**, *38*, 21–26. https://doi.org/https://doi.org/10.1016/j.indcrop.2012.01.001.

(5) Schuhladen, K.; Raghu, S. N. V.; Liverani, L.; Neščáková, Z.; Boccaccini, A. R. Production of a Novel Poly(ε-Caprolactone)-Methylcellulose Electrospun Wound Dressing by Incorporating Bioactive Glass and Manuka Honey. *J Biomed Mater Res B Appl Biomater* **2021**, *109* (2), 180–192. https://doi.org/10.1002/jbm.b.34690.