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Development of photosynthetic sutures for the local delivery of oxygen and recombinant growth factors in wounds

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ABSTRACT

Surgical sutures represent the gold standard for wound closure, however, their main purpose is still limited to a mechanical function rather than playing a bioactive role. Since oxygen and pro-regenerative growth factors have been broadly described as key players for the healing process, in this study we evaluated the feasibility of generating photosynthetic sutures that, in addition to mechanical fixation, could locally and stably release oxygen and recombinant human growth factors (VEGF, PDGF-BB, or SDF-1α) at the wound site. Here, photosynthetic genetically modified microalgae were seeded in commercially available sutures and their distribution and proliferation capacity was evaluated. Additionally, the mechanical properties of seeded sutures were compared to unseeded controls that showed no significant differences. Oxygen production, as well as recombinant growth factor release was quantified *in vitro* over time, and confirmed that photosynthetic sutures are indeed a feasible approach for the local delivery of bioactive molecules. Finally, photosynthetic sutures were tested in order to evaluate their resistance to mechanical stress and freezing. Significant stability was observed in both conditions, and the feasibility of their use in the clinical practice was therefore confirmed. Our results suggest that photosynthetic gene therapy could be used to produce a new generation of bioactive sutures with improved healing capacities.

Statement of significance

Disruption of the vascular network is intrinsic to trauma and surgery, and consequently, wound healing is characterized by diminished levels of blood perfusion. Among all the blood components, oxygen and pro-regenerative growth factors have been broadly described as key players for the healing process. Therefore, in this study we evaluated the feasibility of generating photosynthetic sutures that, in addition to mechanical fixation, could locally and stably release oxygen and recombinant human growth factors at the wound site. This novel concept has never been explored before for this type of material and represents the first attempt to create a new generation of bioactive sutures with improved regenerative capabilities.

1. Introduction

Adult human wound healing is characterized by tissue repair rather than regeneration. In this regard, surgical procedures have evolved to become less invasive, and biomaterials more biocompatible, however, scarring still represents a major problem in modern surgery. This has deleterious consequences for the life-quality of patients, including mechanical [1–3], cosmetic [4,5], and economical aspects [6]. Moreover, in most cases scar tissue does not represent a functional tissue by itself, as it is mainly composed of extracellular matrix.

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Disruption of the vascular network is intrinsic to trauma and surgery, and consequently, wound healing is characterized by diminished levels of oxygen, nutrients, immune cells and growth factors in the regenerating tissue. Among all those factors, oxygen is a crucial molecule for wound healing due to its pivotal role in cell metabolism and signaling [7]. Moreover, it is well described that oxygen is a key molecule for the most critical steps in wound healing, such as cell proliferation, collagen synthesis, re-epithelialization and anti-bacterial immune response [8]. Above all, the importance of oxygen for wound healing becomes clear when it comes to non-healing wounds which tend to be hypoxic [9–11].

Furthermore, pro-regenerative growth factors have been described as key players for the healing process, and as such, they have been considered pro-regenerative therapeutic targets in numerous biotechnological approaches [12–17]. Among those factors, the critical role of the vascular endothelial growth factor (VEGF) has been broadly discussed because of its established proangiogenic potential [18–23]. Furthermore, platelet-derived growth factor (PDGF) is implicated in blood vessel formation and early hematopoiesis [9,18,24]. Chemokines have also been shown to be critical factors for wound healing and, particularly, the stromal derived factor 1 (SDF-1α) is mainly responsible for stem cell homing to wounded tissues [17].

Evidence of the use of surgical sutures has been documented for more than 32,000 years [25,26] and to date they still represent the most common approach to fix damaged tissues. Although materials and procedures have been modified over the years, the primary function of surgical sutures remains the same, in primarily holding tissues together, therefore relying on the patient́s own regenerative capacity to properly close the wound. This issue becomes even more relevant when the patient́s healing capacity is compromised, as seen in different physiological conditions such as aging or inadequate nutrition [27–30], and is also a hallmark for several chronic diseases including diabetes and peripheral arterial disease [10,31].

Recently, our group has introduced photosynthetic biomaterials as a novel approach to deliver oxygen to tissues independently of blood vessel perfusion. This approach is based on the incorporation of photosynthetic cells into different materials and has shown to be effective *in vitro* [32] and *in vivo* [33]. In this setting, seeded photosynthetic cells use the energy of light to trigger water photolysis, thus the material itself is able to produce oxygen upon light stimulation. Moreover, we further demonstrated that the use of molecular biology tools allows to genetically engineer such photosynthetic cells to additionally deliver recombinant growth factors *in situ* [34].

In this work, we propose that standard commercially available surgical threads can be bio-activated with genetically modified microalgae to release both oxygen and recombinant growth factors directly into the wound area.

2. Methods

2.1. Cell culture of C. reinhardtii

Cell-wall deficient *C. reinhardtii* strains UVM4-GFP and UVM4-VEGF [34] were grown photomixotrophically on either semisolid Tris Acetate Phosphate (TAP) medium or in liquid TAP medium supplemented with 1% (w/v) sorbitol and kept under continuous white light exposure (2000 lx, ~30 μE), using a lamp with the full spectrum of light (Nano Light, 11 Watt, Dennerle, Vinningen, Germany). Cell concentration in the cultures was determined using a Casy Counter TT (Roche Diagnostics, Mannheim, Germany).

2.2. Cell seeding in the threads

Polyglactin 910 undyed, braided, gauge size 0, 3–0 and 5–0 Vicryl suture threads (Ethicon, NJ, USA) were used (3.5, 2, and 1 according to European Pharmacopoeia, equivalent to 0.35, 0.2 and 0.1 mm respectively). Unless stated otherwise, the strings were cut into 3 cm pieces under sterile conditions. The pieces were winded and submerged into a $5 \cdot 10^7$ cells/mL microalgae solution and incubated at room temperature under constant illumination (2000 lx, ~30 μE) with no agitation. After 24 h, *C. reinhardtii*-seeded thread pieces were transferred to a new petri dish with fresh TAPS medium and kept in culture for 1, 4, 7, 10 and 14 days adding fresh TAPS every 3 days.

2.3. Light and confocal imaging

Pictures of the threads at different time points were taken with a Keyence vhx-900F microscope and with an inverted phase-contrast microscope (Axiovert 25, Carl Zeiss AG, Oberkochen, Germany). Obtained images were analyzed with the AxioVision SE64 Rel. 4.9.1 software (Carl Zeiss AG, Oberkochen, Germany).

2.4. Chlorophyll measurement

Three centimeter pieces of each gauge threads were seeded with *C. reinhardtii* cells as described before and incubated for 1, 4, 7, 10 and 14 days at room temperature under continuous light exposition. Then, sutures were placed in 250 μL of dimethyl sulfoxide (DMSO) and put under agitation for 10 min. After that, the optical density was measured at 435 nm (Nanodrop, Thermo scientific, Waltham, MA, USA). Additionally, the procedure was repeated using six centimeter pieces of the 0 gauge threads. Unseeded threads kept under the same conditions were used as blank. Results were obtained from three independent experiments, with three experimental replicates for each condition.

2.5. Scanning electron microscopy (SEM)

Suture threads were seeded with *C. reinhardtii* and incubated for 7 days as described before. After fixation in 3% glutaraldehyde and dehydration with graded ethanol, samples were air-dried and sputtered with gold to 20 nm thickness (Baltec, SCD 005; Leica Microsystems, Wetzlar, Germany). A voltage of 3.0 kV was used for the scanning electron microscopy analysis (JSM 6390, Jeol, Tokyo, Japan).

2.6. Oxygen release quantification

Oxygen concentration *in vitro* was measured by placing the thread pieces in Oxodishes® and using the Sensor Dish® Reader (SDR; PreSens GmbH, Regensburg, Germany) according to the manufacturer's instructions. Twelve centimeter thread pieces were seeded with *C. reinhardtii* as described above, kept in culture for 1, 4, 7, 10 days and then placed in the Oxodishes. The Sensor Dish with the Oxodish and samples were kept inside an incubator at 1% oxygen, 35 °C and 100% humidity during the length of the experiment. For light stimulation, a lamp with the full spectrum of white light (Nano Light, 11 W, Dennerle, Vinningen, Germany) was placed 40 cm away from the sample. Oxygen concentration was recorded, and measurements stopped, when saturation of the system was reached and maintained for at least 1 h. The results show the amount of time (hours) that it takes for each condition to reach and maintain the maximum oxygen concentration that is possible to measure with the PreSens-system. This maximum amount of oxygen corresponds to 52.3% pO_2, which is equivalent to 250% of the amount of oxygen present in air saturated water (250% air sat.). Three repetitions of three replicates each were performed for this assay; unseeded threads were used as negative control.

2.7. Co-culture with fibroblasts/HIF-1α quantification

Seeded sutured pieces were kept in culture for 10–14 days and then coated with a fibrin glue solution to prevent microalgae growth outside the thread segment. For the fibrin coating, seeded threads were dipped in fibrinogen and thrombin (Tisseel, Baxter) diluted 1:2 with TAPS, and allowed to polymerize before addition of TAPS medium. 3T3 fibroblasts ($1 \cdot 10^6$ cells per well) were seeded in six-well plates and after 24 h, 36 cm photosynthetic coated threads were placed in each well and kept under light exposition and hypoxic conditions (1% oxygen) at 35 °C for 16 h with 2 mL culture media (1:2 TAPS and DMEM without phenol red, with glutamine and 10% FCS). Control consisted of 3T3 cells kept under same conditions but using unseeded threads. Afterwards, supernatant was removed quickly by inverting the plates, and then placed in liquid nitrogen. Cell extracts were obtained by lysing the cells with 200 μL lysis buffer specified by the manufacturer in the ELISA kit. Total protein extracts were quantified by BCA assay (Thermo Scientific, Waltham, MA, USA). Then, Hypoxia-inducible factor 1-alpha (HIF-1α) was quantified by ELISA according to the manufacturer's instructions (DYC1935-2, R&D Systems, Minneapolis, MN, USA).

2.8. Gene modification of microalgae

The coding sequences for the human stromal cell-derived factor 1 (accession number P48061) and human full-platelet-derived growth factor subunit B (accession number P01127) were adapted to the codon bias of *C. reinhardtii* and inserted into the transformation vector as described before [34]. Briefly, the synthetic genes pBC1-PDGF-B and pBC1-SDF-1 were assembled from synthetic oligonucleotides and polymerase-chain-reaction (PCR)-products (Gene Art AG, Life Technologies, Regensburg, Germany). In order to target the transgenic proteins for secretion into the culture medium, the respective sequences were inserted into the expression cassette derived from the endogenous *PsaD* gene 5′ and 3′ UTRs regulatory elements, and behind the export sequence encoding the leader peptide of the *C. reinhardtii* extracellular enzyme arylsulfatase (ARS2) (Fig. 4). The pBC1-CrGFP_131 basis vector [35] contained the APHVIII resistance gene for selection on paromomycin, whose expression is controlled by the HSP70/RBCS2 promotor [36]. The transgene fragments were cloned into the vector backbone pBC1-CrGFP_131 using NdeI and EcoRI cloning sites. The final constructs were verified by sequencing. The plasmid DNA was replicated and purified from transformed *E. coli* K12 (dam + dcm + tonA rec−) bacteria, and the plasmid concentration determined by UV spectroscopy. $1 \cdot 10^7$ UVM4 *C. reinhardtii* cells were suspended to a volume of 300 μL and vortexed with Ø 0.5 mm glass beads for 20 s in the presence of 5 μg of the respective plasmid DNA. After transformation, cells were seeded in TAPS liquid medium and incubated overnight in the dark with continuous shaking. Then, the algae were seeded on TAP-agar plates containing 10 μg/mL paromomycin and incubated for the first three days under dim light. The plates were then moved to standard light exposition (2000 lx, ~30 μE) until the paromomycin resistant colonies were large enough to be transferred to a fresh plate. Clones were subsequently maintained on solid TAP medium under selective conditions.

2.9. DNA extraction

Genomic DNA from 50 mL of *C. reinhardtii* cells was extracted using phenol/chloroform/isoamyl alcohol (25:24:1) (Carl Roth GmbH, Mannheim, Germany) and chloroform/isoamyl alcohol (24:1). DNA was then precipitated with ice-cold isopropanol and washed twice with ethanol 70%. Pellets were air dried for 5 min and re-suspended in 40 μL H_2O.

2.10. Polymerase chain reaction

The integration of the recombinant gene was confirmed by PCR using gene specific primers pairs (Metabion GmbH, Planegg, Germany; and Sigma-Aldrich, Taufkirchen Germany). Primer sequences used were hVEGF-165-Fwd 5′-GAAGTTCATGGACGTG TACC-3′ and hVEGF-165-Rev5′-TTGTTGTGCTGCAGGAAG-3′ (258 bp), hPDGF-B-Fwd 5′-AACGCCAACTTCCTGGTG-3′and hPDGF-B-Rev 5′-GTGGCCTTCTTGAAGATGGG-3′ (164 bp), hSDF-1α-Fwd 5′- CGTGAAGCACCTGAAGATCC-3′ and hSDF-1α-Rev 5′-CTTCAGCTTGGGGTCGATG-3′ (103 bp), and psaD-Fwd 5′-CCGTCAC CGTCTTCGAATAAT-3′ and psaD-Rev 5′-GCTAACAGTATGGCT CACTCTC-3′ (440 bp).

2.11. Cytokine release from photosynthetic sutures

Genetically modified algae were used to seed thread pieces and were kept in culture for 1, 4, 7, 10, and 14 days. At each time-point, threads (9 cm) were transferred to new wells with fresh TAPs liquid medium, and 24 h later, supernatants were collected, centrifuged and stored in −80 °C. Concentration of the recombinant growth factors in the supernatants was measured using the human VEGF, PDGF and SDF-1α Enzyme-linked Immune Sorbent Assays (ELISAs) respectively, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA: VEGF Quantikine® ELISA kit (DVE00), Human PDGF-BB Quantikine® ELISA kit (DBB00), and Human CXCL12/SDF-1 DuoSet ELISA kit (DY350)).

2.12. Human cells culture

Human Umbilical Vein Endothelial cells (HUVECs, PromoCell GmbH, Heidelberg, Germany) were seeded on a 12 well-plate ($1 \cdot 10^5$ per well), cultured for 24 h and then starved for 16 h before activation. Similarly, human Adipose-derived Stem Cells (hu ASC) (PT-5006, Lonza, Basel, Switzerland) were seeded in 12 well-plates ($6.5 \cdot 10^4$ per well) or in 96 well-plates ($0.3 \cdot 10^4$ per well), cultured for 24 h and starved for 16 h using RPMI 1640, with stable glutamine and 2.0 g/L $NaHCO_3$ (Biochrom, Berlin, Germany) supplemented with 1% fetal calf serum (heat inactivated FCS, Biochrom GmbH, Berlin, Germany) and 1% antibiotic/antimycotic (100x ab/am; Capricorn Scientific, Ebsdorfergrund, Germany).

2.13. Preparation of conditioned media using the released cytokines

In order to recover the recombinant proteins, 30 mL of the supernatants from confluent *C. reinhardtii* cultures (ca. $2 \cdot 10^7$ cells/mL) were passed through a 0.22 μm filter. Then, the human growth factors were recovered by centrifugation (3000*g*, 47 min) using filtration tubes capable of retaining peptides above 30 kDa (Vivaspin 15R Hydrosart, Sarstedt, Goettingen, Germany). In another filtration step under the same conditions, the diluent medium was changed to RPMI 1640. Afterwards, the media were supplemented with 1% fetal calf serum (heat inactivated FCS, Biochrom GmbH, Berlin, Germany).

2.14. In vitro activity of released cytokines

In order to evaluate the biological activity of the released cytokines (rhVEGF, PDGF and SDF-1α), a receptor phosphorylation assay was performed. For this purpose, $1 \cdot 10^5$ HUVECs (PromoCell GmbH, Heidelberg, Germany) per well were cultured for 24 h in 12 well plates and then starved for 16 h before activation. Cells were then stimulated for 5 min, with either 50 ng/mL recombinant

VEGF-165 (Preprotech, NJ, USA), or the concentrated protein supernatants of confluent cultures of the genetically modified or wild-type strain. Cells were then snap-frozen by submerging the plate in liquid nitrogen and lysed in RIPA-buffer with phosphatase inhibitors (Phosphatase Inhibitor Mini Tablets; Pierce-Thermo Fisher Scientific Inc, IL, USA) and proteinase inhibitors (PIC; BD Pharmingen, NJ, USA), Pefabloc SC-Protease Inhibitor (Carl- Roth, Karlsruhe, Germany), cOmplete (Roche, Basel, Switzerland), and phenylmethylsulfonyl fluoride (PMSF; SigmaAldrich, MO, USA). Cells were scratched from the well-floor, and lysates were homogenized by pipetting up and down and stored at −80 °C for further analysis. Detection of receptor and phospho-receptor was performed in a semi-quantitative way by using a Human Phospho-VEGF R2/KDR DuoSet IC ELISA kit according to manufacturer's instructions (cat. DYC1766-2, R&D Systems, Minneapolis, MN, USA). For the evaluation of PDGF, the same procedure was performed but using huASCs (PT-5006, Lonza, Basel, Switzerland), 10 ng/ml recombinant PDGF-BB (Preprotech, NJ, USA), and Human Phospho-PDGF R beta DuoSet IC ELISA kit (cat. DYC1767-2, R&D Systems, Minneapolis, MN, USA). For SDF-1α, huASCs (PT-5006, Lonza, Basel, Switzerland), 30 ng/mL SDF-1α/CXCL12 (Preprotech, NJ, USA) and CXCR4 Colorimetric Cell-Based ELISA Kit (CBP1352, CytoGlow™, Sunnyvale, CA, USA) were used according to manufacturer's instructions.

2.15. Biomechanical characterization

Seeded threads measuring 7–8 cm were kept in culture for 1, 7 and 14 days as previously specified. Non-seeded sutures were incubated under the same conditions as controls. Sutures were subjected to a tensile strength test using a uniaxial digital Electronic Dynamometer D500 (Industria HP, Argentina). Sutures were clamped at both ends and the total length of the suture was recorded for future calculations. To avoid shear forces, sutures were mechanically allowed to move freely in the perpendicular plane. Sutures were loaded at a constant rate of 30 mm/min until failure, and the applied force and displacement were recorded throughout the whole process. Elongation was calculated by correcting displacement by the total length of each sample, which was then used in conjunction with the applied force to calculate the maximum force and elasticity. Samples were tested one after the other at a controlled temperature of 20 °C, and were kept hydrated at all times.

2.16. Ex vivo evaluation of photosynthetic threads

C. reinhardtii were seeded as before in whole polyglactin 910 undyed, braided, gauge size 0 Ethicon Vicryl suture threads (70 cm long), and kept in culture for 14 days. Threads were then used to evaluate their integrity, algae content and algae survival, after being used to suture human skin *ex vivo*. For this, photosynthetic threads were passed through full thickness human skin to resemble 45 stitches. Before the first stitch and after each following stitch, 1 cm of the thread was cut and placed in 250 μL DMSO for chlorophyll content quantification. Additionally, before the first and after the last stitch, another 1 cm piece was cut and placed in TAPS agar plates containing paromomycin to show algae viability after two weeks (Fig. 6A). The skin used for these experiments was abdominal full thickness human skin, harvested during abdominoplasty procedures. The skin has been transferred directly from the operating room to the laboratory to perform the experiments. The local ethical committee approved the study (204/17S), and informed consent was obtained from each participant.

2.17. Cryopreservation

Thread fragments (3 cm length) seeded with *C. reinhardtii* were incubated for 10 days as described before. Four fragments were placed individually in 0.5 mL of erythrosine solution (0.04 $g \cdot L^{-1}$) for 10 min at room temperature and then centrifuged (5 min, 14,100*g*). Four other fragments were placed in cryotubes containing TAP medium + 10% methanol as freezing medium for the algae as previously described [37], and then transferred to a Nalgene container and incubated at −80 °C for 24 h. Afterwards, sutures were thawed and transferred to new tubes containing 0.5 mL of erythrosine solution for 10 min at room temperature and centrifuged. Another four pieces were placed in cryotubes with TAP medium + 5% Sekusept, and incubated at 37 °C for 24 h as a control for death algae. One fragment from each group was placed in a TAPs agar plate and kept at room temperature under continuous light stimulation for 2 weeks. Viability of the algae in the threads was determined by a colorimetric assay, which relies on the selective adsorption or uptake of erythrosine by non-viable cells, hence, supernatants of the erythrosine solution were measured at 526 nm (Nanodrop, Thermo scientific, Waltham, MA, USA) as described by Béchet et al. [38]. Unseeded threads were also used as a blank-control, showing no adsorption of the reagent and the highest absorbance values. Results represent three independent experiments.

2.18. Statistical analysis

All assays were performed in at least three independent experiments. Data were expressed as mean ± SD. The GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. One-way ANOVA, or Two-tailed Student's t-tests were performed to evaluate the differences among groups. Differences among means were considered significant at $p \leq 0.05$.

3. Results

3.1. Photosynthetic activation of the surgical sutures.

C. reinhardtii were seeded in polyglactin sutures of different sizes and cultured for 10 days as described in the material and methods section. After seeding, all sutures showed an overall green color which was homogeneously distributed along the entire material (Fig. 1A). Moreover, we observed an increase in the intensity of the green color over time, which was confirmed by quantifying the total chlorophyll content at each time point for up to 10 days in culture (Fig. 1B). Furthermore, the results showed a direct correlation between the suture size and its chlorophyll content. Based on that, all further experiments were performed using 0.35 mm threads in order to maximize the number of algae within a photosynthetic suture. After showing that photosynthetic cells were capable of proliferating in the thread, the distribution and interaction of the microalgae with the material was studied in further detail by laser scanning confocal microscopy (LSCM) and scanning electron microscopy (SEM). LSCM results showed that the photosynthetic cells were located all over and in between the threads, but although its macroscopic distribution seemed to be homogenous, a further microscopic observation revealed areas with higher algae density than others (Fig. 2A). Additionally, SEM analysis confirmed the presence of algae in all filaments, in most cases forming micro-colonies (Fig. 2B). Next, growth dynamics and oxygen production was evaluated. To do this, sutures were seeded with algae and imaged at different time points up to 14 days, observing that cells grew in the suture surface as well as in the inner cavities of the material (Fig. 3A). This data was quantified by measuring the

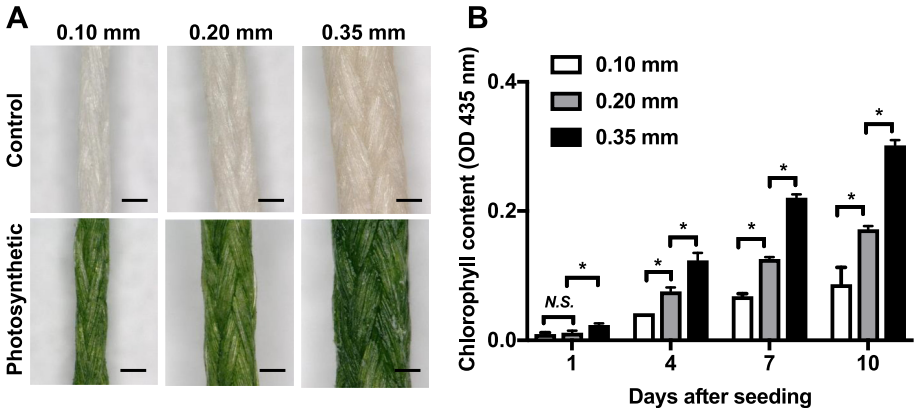

Fig. 1. Seeding of the microalgae in the threads. *C. reinhardtii* were seeded in polyglactin sutures of different gauges and cultured for 10 days. Compared to control threads, seeded threads showed an intense green color which is characteristic of the algae (A). An increase in the overall chlorophyll content was observed when increasing the gauge of the thread in all time points after seeding (B). The scale bar represents 200 μm in A, *p ≤ 0.001 in B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

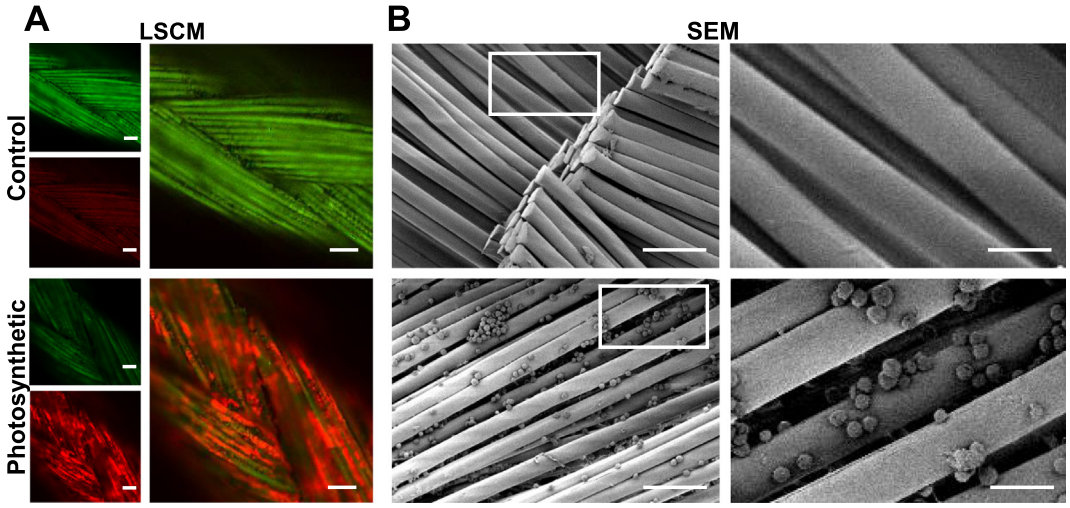

Fig. 2. Distribution of the microalgae in the threads. 10 days after seeding, Laser Scanning Confocal Microscopy (LSCM) shows the general distribution of algae (autofluorescence, red) along the entire thread (autofluorescence, green) (A). At the same time point, Scanning Electron Microscopy (SEM) analysis shows that *C. reinhardtii* were distributed in between the fibers of the threads (B). The scale bar represents 50 μm in A, 50 and 17 μm in B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chlorophyll content of the suture, which significantly increased during the first 10 days, and then stayed on a plateau from day 10 to 14 (Fig. 3C). As shown in Fig. 3B, photosynthetic oxygen production was observed by the formation of gas bubbles directly on the threads. This was further quantified and a significant increase in the oxygen content was observed between days 1–7 and days 10–14 (Fig. 3D). In order to determine if this increase in oxygen tension would be sufficient to induce a physiologically relevant effect in biological systems, photosynthetic sutures were placed in contact with hypoxic fibroblasts, in which a significant decrease in the expression of the hypoxic marker HIF-1a was observed (Fig. 3E).

3.2. Release of recombinant bioactive molecules

C. reinhardtii were genetically modified to synthetize and release rhPDGF-BB, or rhSDF-1α. For this, the coding sequence for the respective recombinant proteins was cloned into an endogenous expression cassette in the algae (Fig. 4A) and the correct insertion of the sequence was verified by PCR analysis of genomic DNA obtained from the genetically modified clones (Fig. 4B). In case of VEGF, a strain previously generated by our group was used [34]. Furthermore, the correct expression and release of the respective recombinant proteins over time were quantified by ELISA. Our results showed that threads seeded with genetically modified algae sustained a constant release of the selected recombinant human growth factors for at least 14 days *in vitro* (Fig. 4C). The concentration measured for each factor increased accordingly with the cultivation time after seeding, and reached the maxima of 9.836 ng/mL; 1.253 ng/mL and; 0.326 ng/mL, for rh-VEGF, rh-PDFG and rh-SDF1-α respectively. To test the bioactivity of the secreted recombinant factors we evaluated their ability to induce the phosphorylation of their respective receptors in cell culture (Fig. 4D). The results showed an increase in phosphorylation after stimulation with the commercially available recombinant factor (positive control) as well as with the recombinant molecule secreted by the algae. No phosphorylation was observed when cells were stimulated with the starvation medium (negative control) or the supernatant obtained from wild type algae. Due to technical issues, phosphorylation of CXCR-4 receptor could not be detected even for the positive controls (data not shown).

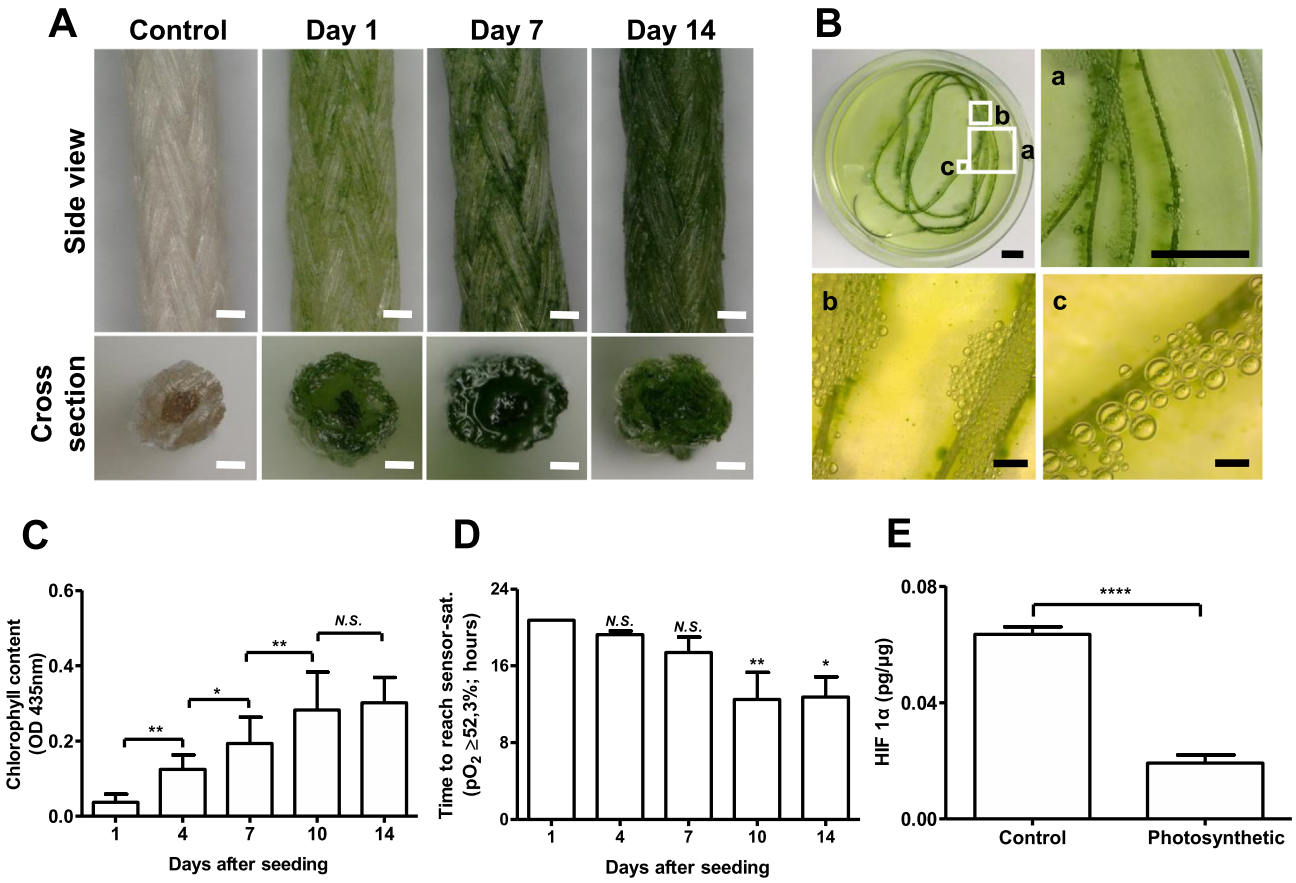

Fig. 3. Biocompatibility of the microalgae seeded into the threads. *C. reinhardtii* algae were seeded in 0.35 mm gauge threads. A significant increase in the green color intensity (A) and chlorophyll content (C) was observed within days, showing the survival and proliferation of the seeded microalgae in the threads. The algae in the threads remained photosynthetically active, as confirmed by visual observation of bubbles formation (B) and oxygen release measurement *in vitro* for at least 14 days (results are shown as time (hours) to reach sensor saturation) (D). When co-cultured with 3T3 fibroblasts ($1 \cdot 10^6$ cells per well in six-well plates) under light exposition and hypoxic conditions at 35 °C, oxygen released by seeded threads induced a significant reduction in the levels of the hypoxic marker HIF-1α (E) (units refer to pg of HIF-1α per μg of total protein in the analyzed samples). n = 5, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Scale bars represent 100 μm in A, and 1 cm (upper) and 500 μm (lower) in B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Biomechanical characterization

After confirming the feasibility to generate oxygen producing sutures, we decided to evaluate the effect of photosynthetic activation on the biomechanical properties of the material. For this, sutures were seeded with the algae, and then evaluated in stress-strain curves at different times after seeding, where almost identical deformation patterns were observed among the studied groups (Fig. 5A). Results showed that neither the algae nor the seeding protocol significantly modify the elastic limit (Fig. 5B), maximum force (Fig. 5C) and the Youngs module of the suture (Fig. 5D). The only exception was observed for the maximum force at day 14, where a significant decrease was observed for the photosynthetic material.

Once the mechanical stability of the suture was tested *in vitro*, we evaluated whether the mechanical stress induced by the process of suturing could affect the presence and viability of the algae in the thread. For that, we performed an *ex-vivo* simulation using photosynthetic treads to suture excised full-thickness human skin. As shown in the scheme (Fig. 6A), samples of 1 cm each were taken to verify the viability of the algae and chlorophyll content before suturing, and then after 15, 30 and 45 stitches. Interestingly, the chlorophyll content did not vary along the suturing process (Fig. 6B). Additionally, it was possible to re-grow the algae from the thread after stitching with it 45 times through the human skin, confirming that the seeded microalgae survived the friction induced by the suturing process (Fig. 6C).

3.4. Cryopreservation

In order to evaluate the feasibility of cryopreserving photosynthetic sutures, seeded sutures were frozen and cell viability was evaluated after thawing. Additionally, cell death was measured by a colorimetric assay where non-seeded sutures were used as control to evaluate the direct interaction between the material and the reagent. The results showed no significant differences in erythrosine adsorbance of photosynthetic sutures that were kept at room temperature or frozen. However, we did find statistical differences among the previous groups compared to a positive dead control, were seeded cells where previously stressed to death (Fig. 7A). We further evaluated the capability of algae to grow from the frozen photosynthetic sutures into agar plates, confirming their viable status (Fig. 7B).

4. Discussion

High cell proliferation and migration rates, as well as an increase in production and deposition of extracellular matrix are inherent processes in wound healing that require high energy consumption, therefore demanding appropriate levels of oxygen in mitochondria. Moreover, in wound healing oxygen is also required for the antibacterial defense that occurs through the generation of reactive oxygen species [39]. These high oxygen requirements are in contrast to the poor blood perfusion observed in injured tissues,

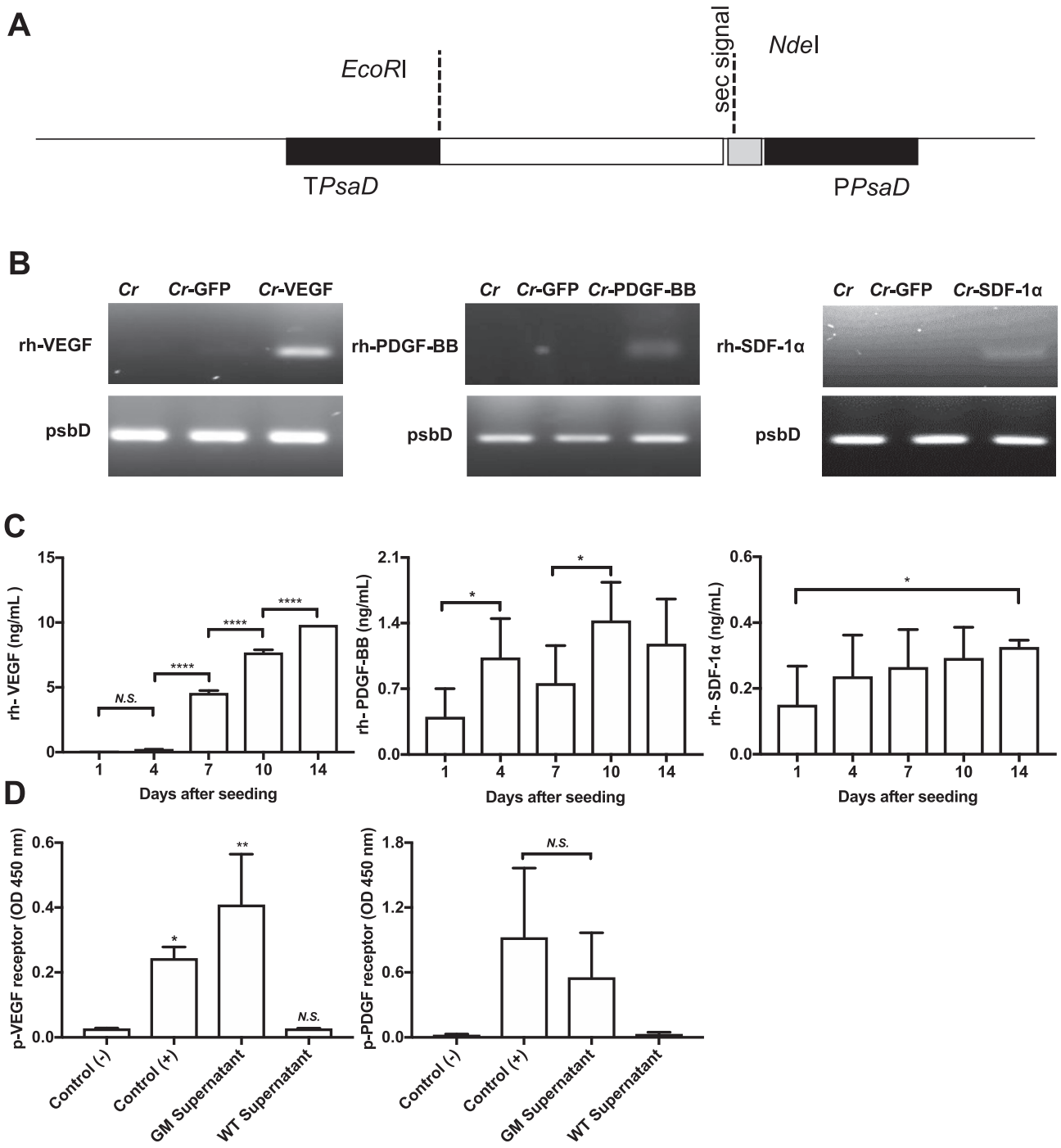

Fig. 4. Photosynthetic threads as source of recombinant bioactive molecules. Schematic representation of vectors used to express VEGF gene (A). The correct insertion of the codon adapted human VEGF-165 (263 pb), PDGF-β (165 pb) and SDF-1α (103 pb) into *C. reinhardtii* was verified by PCR using specific primers for the transgenes; untransformed wild type algae and GFP transformed algae were amplified as negative controls, and algae psbD (440 pb) was amplified as a positive control of the reaction (B). Gene modified algae were seeded in the threads and the secreted rhVEGF, rhPDGF and rhSDF-1α were quantified. Results showed a release of recombinant growth factors for up to 14 days *in vitro* (C). Supernatants of the seeded threads were used to stimulate cells in culture, and the bioactivity of the released growth factors was confirmed by quantifying the phosphorylation of its respective receptor in HUVEC (rhVEGF) or mesenchymal stem cells (rhPDGF and rhSDF-1) (D). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

where the native vascular network is disrupted. Therefore oxygen is broadly described as a fundamental molecule for tissue repair and regeneration, and its key role has been extensively described [8,40,41].

Surgical sutures are the gold standard for primary wound closure [25,42] and, over the last years, have significantly evolved to play a more active role in tissue repair and regeneration [6]. For instance, recent studies have shown that biologically active substances can be delivered using coated sutures [6,43–45] where, our group and others have described that cell-seeded sutures are a feasible way to deliver growth factors *in situ* [46–49]. Taking this into consideration, we decided to seed photosynthetic cells and use the suture itself as a local and permanent source of oxygen during wound healing.

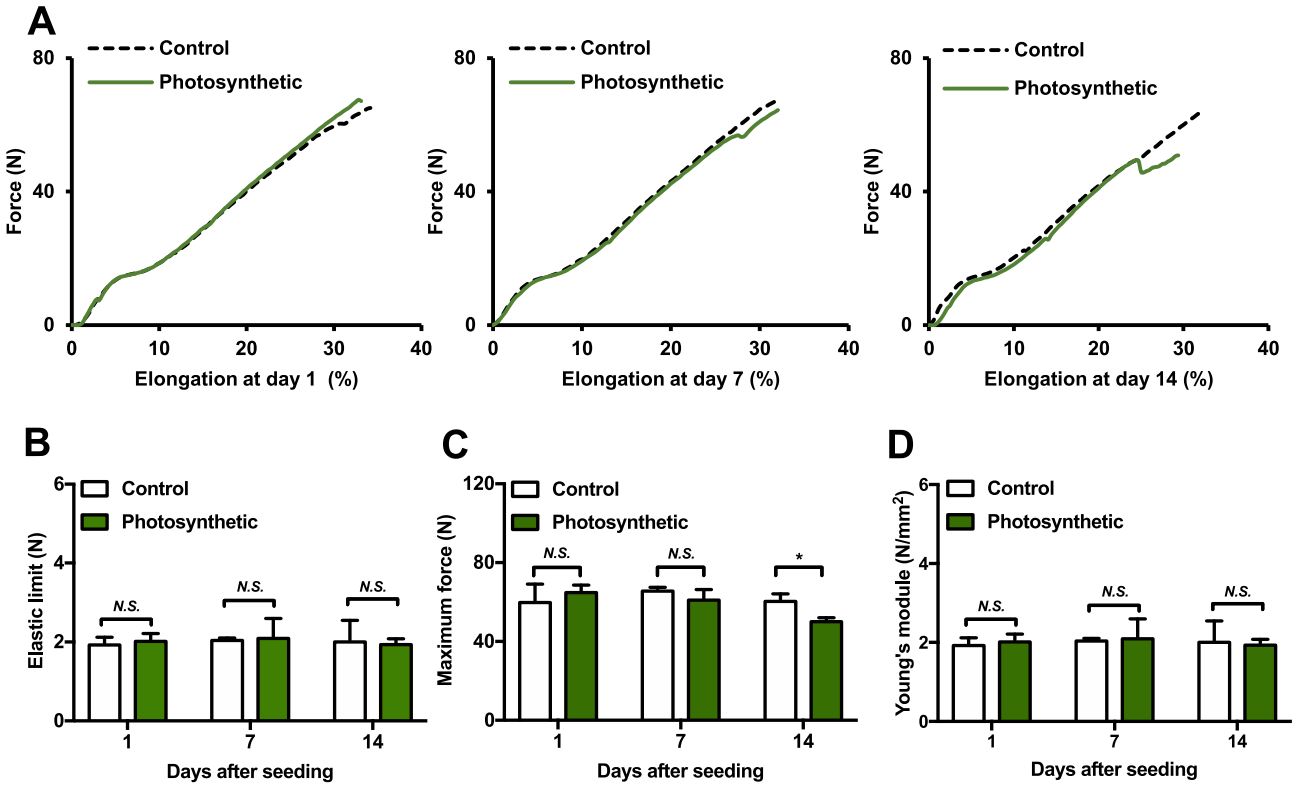

Fig. 5. Biomechanical properties of photosynthetic threads. A force-expansion profile at several time points (A) was performed to evaluate the elasticity (B), maximum force (C), and calculate Young's module (D) of the photosynthetic threads. $^{*}p \leq 0.05$.

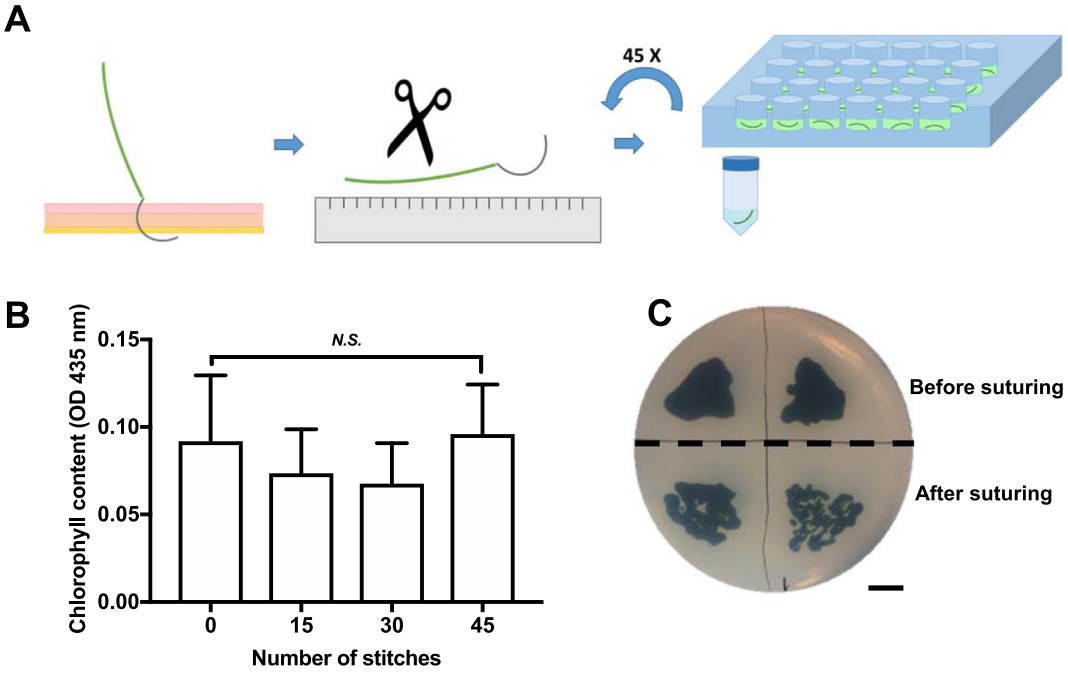

Fig. 6. *Ex vivo* evaluation of photosynthetic threads. After suturing a sample of full thickness human skin several times (A), the microalgae remained inside the thread and the chlorophyll content was not significantly diminished by up to 45 stitches (B). Viability of the remaining algae was confirmed by evaluating their capability to be regrown in agar plates (C). The scale bar represents 1 cm in C.

Here we show that sutures can be seeded with *C. reinhardtii*, a photosynthetic unicellular microalga, that was chosen because of being a well characterized model organism, categorized by the U. S. Food and Drug Administration (FDA) as "Generally Regarding As Safe" (GRAS) [50]. Additionally, we have previously shown that they do not elicit a significant immune response *in vivo* [33,34]. As shown in Fig. 1A, *C. reinhardtii* was homogenously distributed after seeding and, as expected, its loading and growing capacity was highly dependent on the increased surface provided by the inner filaments of bigger sutures. The role of the inner filaments as

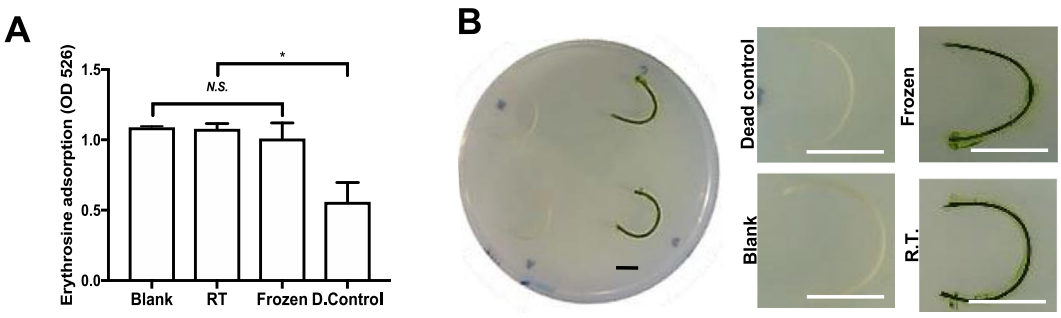

Fig. 7. Cryopreservation potential of photosynthetic threads. Seeded threads were frozen and cell viability was compared to unseeded threads (Blank), seeded but not frozen (kept at RT), or submitted to stressful conditions (Dead control). A quantitative colorimetric viability assay showed nonsignificant variations in cell mortality after freezing (A), which was confirmed by evaluating the capability of the algae to be regrown in agar plates (B). Scale bars represents 1 cm in B. $^{*}p \leq 0.001$.

scaffolding becomes clear in Figs. 2B and 3A, where the microalgae were observed to grow even in the deep cavities of the material. The ability of the algae to proliferate in polyglactin-910 is in accordance with our previous results using collagen-GAG scaffolds [32], and also with independent experiments describing the growth of these microalgae in 3D printed alginate scaffolds [51]. Therefore, photosynthetic cells have shown to be compatible with at least three commonly used biomaterials, supporting the notion that this photosynthetic strategy could be used in a broad spectrum of material-based approaches in regenerative medicine.

LSCM and SEM analysis show that microalgae grew all along the filaments, where they seem to be physically trapped rather than actively attached to the suture (Fig. 2B). This is expected for this kind of microorganisms which, in contrast to mammalian cells, do not actively attach through the classical cell-matrix or cell-cell interaction pathways. As a consequence, the algae density in the material could be underrepresented by microscopic techniques, where an important portion of cells are lost during the required washing steps. Interestingly, such cell loss was not observed after suturing *ex vivo*, where the stitching process itself did not diminish the total chlorophyll content of the threads (Fig. 5B), and has no apparent major effect in their further growing capability in agar plates (Fig. 5C). The fact that seeded cells remain in the suture after stitching, but not after washing, could be partially explained due to the majority of the algae not being located on the surface of the suture, but rather within the core of the threads, which protects them from the mechanical friction induced by the suturing process. Although microalgae remain after friction induced by the suturing process, their proliferation capacity or viability seems to be partially affected as observed in the agar plates, where the algae grew, but in lower densities than the control group (Fig. 6C). Nevertheless, the ability of the cells to stay in the material after suturing is a key aspect for this technology, and differs significantly from other approaches where sutures are coated [47,48,52] but not filled with cells. In this context, seeded cells are not only resistant to suturing, but also to cryopreservation, which could significantly facilitate the translation of this approach into clinical practice (Fig. 7). At this point, the issue of how to eliminate microalgae from the patient after treatment may arise. This is an important aspect to be considered for translational studies, however, our previous results show that this microalga survived for less than 14 days *in vivo* [34]. In any case, for safety issues, future approaches may consider the use of photosynthetic cells modified with suicide genes or the incorporation of light deprivation protocols after treatment.

After seeding, the microalgae actively release oxygen, as observed by the formation of bubbles over the threads (Fig. 3C), and by direct oxygen measurement in the culture dishes (Fig. 3E). Interestingly, oxygen production seems to follow the growth curve of the algae, releasing significantly more oxygen from day 1 to day 10, but stabilizing from day 10 to 14, where no significant increase in the chlorophyll content was observed (Fig. 3B). This correlation is not linear because at a certain time point algae density might interfere with oxygen production, most probably due to changes in the light penetration capacity of the material. To our knowledge, this is the first report on oxygen releasing sutures. However, other attempts to generate oxygen-releasing biomaterials have been previously described. Most of those approaches are based on chemical degradation of the biomaterial, generating concerns about the production of toxic metabolites [53]. Oxygen carriers have also been used as a sort of oxygen reservoir for implanted biomaterials [7,53], but this approach is highly limited to the fixed loading capacity of the carrier. In contrast, photosynthetic activation of materials provides a potentially unlimited source of oxygen, which is generated in a natural process that is biocompatible by itself. In this regard, our results showed that sutures remained photosynthetic for at least 14 days *in vitro*, as they did in the scaffolds when implanted *in vivo* in previous studies [33,34]. Here, it is important to notice that the development of photosynthetic biomaterials, including sutures, will necessarily require the establishment of new technologies to supply proper amount of light into tissues. Thus, for this particular application, a light emitting dressing would be required. This dressing will have to be designed considering the light-penetration properties of each particular tissue.

In addition to oxygen, several key molecules have been reported to be involved in the complex orchestration of the wound healing process. VEGF is the master regulator of angiogenesis and promotes proliferation and migration of endothelial cells, which makes it one of the main molecular targets to promote vascularization in tissue engineering and regenerative medicine approaches [20,54]. PDGF is another key factor involved along the whole process of blood vessel regeneration, recruiting cells and prompting vessel remodeling, by means of collagen turnover and cross-linking. Additionally, PDGF induces vascular smooth muscle cells and pericytes to release other angiogenic factors like VEGF [18,55]. Finally, stromal cell-derived factor 1 alpha (SDF-1α), also known as C-X-C motif chemokine 12 (CXCL12), is recognized for its capacity to recruit endogenous progenitor stem cells, particularly hematopoietic ones, and promote regeneration *in situ* [17]. Here, microalgae were genetically engineered to secrete recombinant VEGF, PDFG or SDF-1α which, in addition to oxygen, could contribute to create a pro-regenerative microenvironment directly at the wound site. Interestingly, the secretion curves of the algae seeded in the suture were highly dependent on the growth factor, and significant differences were observed in terms of amount and

reproducibility. For instance, the secretion of SDF-1α slightly increase over time, showing big standard deviations among replicates. In contrast, the secretion of VEGF was characterized by an exponential increase overtime and small standard deviations. Additionally, under the same conditions, the amount of secreted factors showed differences off up to 30 times among them (Fig. 4C). However, these quantities are comparable to physiological concentrations and would still be relevant to induce a response *in vivo* [56,57]. Our results indicate that the release of recombinant molecules from photosynthetic materials is a feasible approach to activate key molecular pathways (Fig. 4D), as shown by the bioactivation of the VEGF and PDGF receptors by the respective recombinant growth factors.

Because the therapeutic potential of surgical sutures relies on their mechanical properties, strain-stress curves were performed to determine variations in the elastic limit, maximum force and Young's module. Our results showed that the suturing material retains most of its original properties at different times after seeding; hence, showing that neither the seeding process itself nor the presence of the algae compromise their main function as tissue holders. Even though the maximum force decreased in the photosynthetic sutures after 14 days in culture, the clinical relevance of this observation may be overpassed considering that in many cases sutures are typically removed from patients after 7 to 10 days [25]; additionally, after 14 days the mechanical properties of the suture are expected to be affected by the normal biodegradation processes that occurs during the wound healing process *in vivo* [58]. Finally, the cryopreservation of the photosynthetic threads was proved to be possible. We find this to be relevant as it makes the photosynthetic threads amenable to be stored as a "ready-to-use" or "off-the-shelf" biomaterial, thus facilitating its clinical translation. However, sterilization and storage of such seeded sutures is still complex compared to standard sutures. Thus, further research should be performed to address these limitations.

In this work we showed that photosynthetic bioactivation could be used to generate sutures capable of constantly releasing oxygen and therapeutic recombinant growth factors directly at the wound site. Although further experiments are required to evaluate the safety and efficacy of this technology *in vivo*, this represents the first step to create a new generation of surgical sutures with improved regenerative capabilities.

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Competing interest

JTE is co-founder and VP of Technology at SymbiOx Inc. This startup did not provide any financial support to this work, but is closely related to some topics of this manuscript.

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