Production and characterisation of chemically cross-linked collagen scaffolds

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Abstract

Chemical cross-linking of collagen-based devices is used as a means of increasing the mechanical stability and control the degradation rate upon implantation. Herein, we describe techniques to produce cross-linked with glutaraldehyde (GTA; amine terminal cross-linker), 4-arm polyethylene glycol succinimidyl glutarate (4SP; amine terminal cross-linker), diphenyl phosphoryl azide (DPPA; carboxyl terminal cross-linker) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; carboxyl terminal cross-linker) collagen films. In addition, we provide protocols to characterise the biophysical (swelling), biomechanical (tensile) and biological (metabolic activity, proliferation and viability using human dermal fibroblasts and THP-1 macrophages) properties of the cross-linked collagen scaffolds.

Keywords: Collagen cross-linking; Biophysical characterisation; Biomechanical Properties; Cytotoxicity; Macrophage response

1. Introduction

Collagen is the most abundant extracellular matrix (ECM) protein in mammals. Fibril-forming collagens (type I, II, III, V, XI) represent 90 % of the total collagen content in the body, whilst their fibrous structure empower containing tissues with their specific viscoelastic properties [1]. Collagen type I comprises of two α1(I) and one α2(I) polypeptide chains that tangle up in a triple-helix conformation [1,2]. It is stabilised via two cross-links in the helical region and one more in each telo-peptide region (*N*- and *C*-terminal), through the action of lysyl oxidase [3,4], sugar-mediated [5,6] and trans-glutaminase [7] cross-linking. These cross-links are responsible for the quarter-staggered arrangement of collagen molecules in lateral and head-to-tail fashion, resulting in the formation of cross-striated fibrils with exceptional mechanical properties [8,9].

Considering its abundance in native ECM supramolecular assemblies and its cell-recognition signals, collagen type I is favoured for tissue engineering and drug delivery applications [10]. However, collagen type I extraction from animal tissues involves breakage of the native cross-links and consequent loss of its mechanical integrity / stability [11,12]. Thus, reconstituted forms of collagen need to be exogenously cross-linked, through chemical, physical or biological approaches [12-14] to tune the mechanical properties and the degradation rate of the produced scaffolds upon implantation [15-17]. Although physical (e.g. dehydrothermal [18,19], ultra-violet irradiation [20,21]) and biological (e.g. microbial [22,23] or tissue-type II [24,25] trans-glutaminase) cross-linking methods have been assessed, the resultant cross-linking is weak. To this end, amine or carboxyl terminal chemical cross-linking methods are preferred. However, customarily employed chemical cross-linkers, such glutaraldehyde (GTA, amine terminal) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, carboxyl terminal), are often associated with cytotoxicity [26-29], foreign body response [30,31] and macrophage pro-inflammatory response [32-34]. Although alternative cross-linkers, such as 4-arm polyethylene glycol succinimidyl glutarate (4SP; amine terminal) [35-38] and diphenyl phosphoryl azide (DPPA; carboxyl terminal) [39,40], have been proposed and have shown promise, they have yet to be directly compared.

We have previously described assays to characterise collagen and to quantify thermal and biochemical properties of collagen-based preparations, biomaterials and tissue specimens [41-45]. Herein, we provide protocols to fabricate cross-linked with GTA, 4SP, EDC and DPPA collagen type I films and to characterise the effect of the cross-linking treatment on the biophysical (swelling), biomechanical (tensile) and biological (metabolic activity, proliferation and viability using human dermal fibroblasts and THP-1 macrophages) properties of the resultant scaffolds.

2. Materials

2.1 Collagen cross-linking and film preparation

All materials, unless otherwise is stated, were purchased from Sigma-Aldrich (Ireland).

1. Sodium hydroxide (NaOH): 1M in water.
2. Hydrochloric acid (HCl): 1M in water.
3. Phosphate buffered saline 1x and 10x (PBS, Fisher Scientific).
4. Collagen type I stock solution (6 mg/ml): Dissolve 180 mg of freeze-dried collagen type I in 30 ml of 0.05 M acetic acid.
5. GTA cross-linker solution (0.625 %): dilute 6.25 μl of 25 % GTA (Sigma-Aldrich) in 244 μl of PBS 1x (see **Note 1**).
6. 4SP cross-linker solution (20 mM): dissolve 50 mg of 4SP-SG (Jenkem Technology USA; MW 10,000) in 0.25 ml PBS 1x. Vortex thoroughly until the powder dissolves.
7. DPPA cross-linker solution (1 %): dilute 2.5 μl of DPPA (Sigma-Aldrich) in 0.25 ml of 2 % dimethylformamide (DMF) (see **Note 1**).
8. EDC cross-linker solution (1000 mM): dissolve 98 mg of 2-(N-moprholino)ethanesulfonic acid (MES) in 10 ml of distilled water and adjust the pH to 5.5. Dissolve 115 mg of N-Hydroxysuccinimide (NHS; Sigma-Aldrich) in 5 ml of MES buffer. Dissolve 48 mg of N(3-Dimethylaminopropopyl)-N'-ethylcarbodiimide hydrochloride (EDC; Sigma-Aldrich) in 0.25 ml of the MES+NHS solution (see **Note 1**).

9. Silicon or PDMS non-adherent mould (3x4 cm).
10. Screw-cap tubes (15 ml, Sigma-Aldrich).
11. Bench-top vortex.
12. Laminar flow hood.

2.2 Swelling assay

1. Phosphate buffered saline 1x (PBS; Fisher Scientific).
2. Tweezers.
3. Laboratory digital milligram scale.

2.3 Tensile test

1. Scalpel and blades.
2. Ruler.
3. Cutting board.
4. Phosphate buffered saline 1x (PBS; Fisher Scientific).
5. Tweezers.
6. Digital calliper.
7. Tensile test machine with 10N load cell (Zwick Roell).

2.4 Cell culture

1. Primary human dermal fibroblasts (DF; ATCC).
2. Human derived leukemic monocyte cells (THP-1; ATCC).
3. Culture media for DF: DMEM – high glucose supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin and streptomycin.
4. Culture media for THP-1: RPMI-1640 supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin and streptomycin.

5. Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich).
6. Lipopolysaccharides from Escherichia coli O55:B5 (LPS; Sigma-Aldrich).
7. Cell culture silicone rings to fix the films on the plates: 18.6 mm of diameter ace O-rings (Sigma-Aldrich).
8. Sterile 24-well plates (Sarstedt).

2.5 Cell proliferation and morphology assay

1. 4', 6-diamidino-2-phenylindole (DAPI; 1:3000 dilution in PBS 1x from 1mg/ml stock).
2. Optional: Rhodamine phalloidin (1:150 dilution in PBS 1x from 0.2 mg/ml stock, Thermo Fisher Scientific).
3. Paraformaldehyde (PFA; 4 %).
4. Phosphate buffered saline 1x (PBS; Fisher Scientific).
5. Triton-X 100 solution (Fisher Scientific, 0.2% in PBS)

2.6 Metabolic activity assay (alamarBlue®)

1. Sterile phosphate buffered saline 1x (PBS; Fisher Scientific).
2. alamarBlue® solution (Invitrogen; 10 % in PBS).

2.7 Cell viability assay

1. Dimethyl sulfoxide (DMSO; Sigma-Aldrich).
2. Phosphate buffered saline 1x (PBS; Fisher Scientific).
3. Calcein AM (Invitrogen; 4 mM) in DMSO.
4. Ethidium homodimer-1 (Sigma-Aldrich, 2 mM) in DMSO.

3. Methods

3.1 Collagen cross-linking and film preparation

All steps detailed in this paragraph need to be performed on ice to avoid denaturation of collagen and prevent its premature gelation. The temperature of the centrifuge, if possible, should be set to 4 °C before starting the procedure. The following instructions describe the production of a film of 3x4 cm dimensions, for other mould dimensions the volume of the reagents need to be scaled up.

1. Mix 0.42 ml of PBS 10x with 0.2 ml of 1 M NaOH in a 15 ml screw-cap tube using a bench-top vortex.
2. Add 4.2 ml of the collagen stock solution to the mixture and mix thoroughly with the vortex.
3. Use the pH meter and 1 M NaOH to neutralize the solution (pH 7.2-7.4).
4. Add the 0.25 ml of the chosen cross-linker and mix with the bench-top vortex (see **Note 2**).
5. Centrifuge the solution at 2,000 rpm for 10 seconds to remove the bubbles.
6. Pour 4 ml of the solution on the non-adherent mould (3x4 cm).
7. Incubate the sample (mould and collagen) at 37 °C for 1h.
8. Dry the sample in the flow hood at room temperature (RT) for at least 24 h.
9. Use tweezers to peel-off the collagen film from the non-adherent surface.
10. Store the films in a dark and dry environment until use.

3.2 Swelling assay

1. Use a blade and a scalpel to cut five pieces (n=5) from the collagen film and record their dry weight using a laboratory scale (see **Note 3**).
2. Incubate the samples in PBS 1x at RT overnight.
3. Blot the samples with filter paper to remove the excess surface water.
4. Weigh the samples in the laboratory scale and record their wet weight.
5. Calculate the swelling ratio (%) as follows: [(wet weight – dry weight) / dry weight] * 100%.

3.3 Tensile test

1. Use a scalpel with a sharp blade and a ruler to cut the collagen film in three stripes of 1x4 cm (see **Note 4**).
2. Hydrate the samples overnight in PBS 1x at 37 °C (see **Note 5**).
3. Blot the samples with filter paper to remove the excess surface water.
4. Using the callipers, take 5 measurements of the thickness of the sample in the middle region (see **Note 6**).
5. Insert the sample into the upper grip of the tensile tester and tighten (see **Note 7**).
6. Bring the crosshead into an appropriate range for the sample and affix the sample to the lower grip (see **Note 8**).
7. Set up the software from the mechanical tester to the following tensile test parameters, pre-load: 0.01 N, pre-load speed: 5 mm/min, test speed: 10 mm/min.
8. Initiate the test and export the results to excel.
9. Calculate the following parameters: stress at break (MPa), strain at break (%) and Young's modulus (MPa), using the width and the thickness of the samples. Stress at break = Force at break / original cross-sectional area of the sample, Young's modulus = slope of the linear region of the stress (MPa) / strain (no units) curve (see **Note 8**).

3.4 Cell culture

3.4.1 Scaffold preparation

1. Using a scalpel with a blade cut the collagen films into 1x1 cm squares.
2. In the laminar flow hood, place the films in 24-well plates (see **Note 9**).
3. Use the UV from the laminar flow hood for 1 h to partially sterilise the collagen scaffolds in the plates. Keep the lid of the plates open during the process.
4. Fix the collagen films to the bottom of the plates with the help of autoclaved tweezers and silicone rings.

5. Remove the excess of cross-linker by washing the films three times with sterile PBS 1x for 20 min.
6. Sterilise the films with 0.5 ml of 70 % ethanol for 30 min.
7. Wash the films three times in sterile PBS 1x.

3.4.2 DF seeding

1. Incubate the sterilised films at 37 °C and 5 % CO_2 overnight with specific media for the DF cells.
2. Discard the media and seed the DF onto the various samples at 20 x 10^3 cells/cm^2 with 0.5 ml of fresh DMEM supplemented media. Incubate the fibroblast for 3, 5 and 7 days at 37 °C and 5 % CO_2. Change media every 3-4 days.

3.4.3 THP-1 seeding

1. Incubate the sterilised films at 37 °C and 5 % CO_2 with 1 ml cell-specific media for THP-1 cells for 3 days.
2. Collect the RPMI supplemented media incubated with the scaffolds (pre-conditioned media) and filter it through 0.2 μm sterile filters. Keep it at 37 °C in the water bath until further use.
3. Seed THP-1 into new 24-well plates and onto the scaffolds at 26 x 10^3 cells/cm^2 with THP-1 complete media supplemented with 100 ng/ml of PMA to induce adherent mature macrophage-like state for 6 hours.
4. Wash off non-attached cells with sterile PBS 1x.
5. Add 0.5 ml of the filtered pre-conditioned media to the macrophages attached to the 24-well plates TCP and 0.5 ml of fresh RPMI supplemented media to the macrophages attached to the scaffolds. Create a positive control for macrophage inflammatory response by culturing TCP-seeded macrophages with 100 ng/ml of LPS in RPMI complete media and a negative control with only complete media. Incubate macrophages for 24 h and 48 h at 37 °C and 5 % CO_2.

3.5 Cell proliferation and morphology assay

1. At the appropriate time-point remove media from cells.
2. Gently wash scaffolds with PBS 1x.
3. Add 0.5 ml of 4 % PFA and allow the sample to fix for 30 min at RT (see **Note 10**).
4. Wash the sample with 1x PBS.
5. Incubate the samples with 0.2 ml of 0.2 % Triton-X solution for 5 min.
6. Wash the sample with PBS 1x .
7. Add rhodamine phalloidin solution enough to cover the bottom of the well and/or scaffolds (around 150 μl).
8. Incubate 1h at RT. Protect the samples from the light during this procedure and in subsequent manipulations.
9. Wash the sample with PBS 1x.
10. Add 150 μl of DAPI solution and incubate for 5 min at RT.
11. Wash the sample three times with PBS 1x (see **Note 11**).
12. Image the samples in a fluorescence or confocal microscope using a 10X objective and an excitation/emission wavelength of 358/461 nm for DAPI and 540/565 nm for rhodamine phalloidin.
13. Take 4-5 representative images of every sample.
14. Use ImageJ or other software for an automated counting of the nuclei per image (**Note 12)** and for morphology assessment (**Note 13**).
15. Graph the number of cells per area to evaluate cell proliferation.

3.6 Cell metabolic activity assay (alamarBlue®)

1. Cell metabolic activity can be assessed by the incubation of the cells with 10 % alamarBlue® for 2 h on the day of the time-point, as per manufacturer's protocol (**Note 14**).

2. Cell metabolic activity is expressed as % of reduced alamarBlue® and normalised to the activity of cells in TCP.

3.7 Cell viability assay

1. Prepare staining solution by diluting 1:1000 the calcein AM and the ethidium homodimer-1 stock solutions with PBS 1x.
2. Add 150 μl of DMSO to a well with cells alone (no scaffold) to create a negative control with dead cells. Place the plate in the incubator for 20 min.
3. Remove culture medium from the wells and wash cells with PBS.
4. Add 150 μl of staining solution to each scaffold.
5. Incubate at 37 ºC, 5% CO_2 for 30 min.
6. Wash the sample with PBS 1x (see **Note 11**).
7. Image the samples in a fluorescence or confocal microscope using a 10X objective and an excitation/emission wavelength of 495/515 nm and 528/617 nm for Calcein AM and ethidium homodimer-1, respectively.
8. Take 3-5 images of every sample.
9. Count viable (green) and dead (red) cells with ImageJ or another software.
10. Calculate cell viability (%) by dividing the number of alive cells by the total number of cells.

4. Notes

1. For safety reasons, prepare these cross-linking solutions in the chemical hood. GTA, DPPA and EDC are toxic if swallowed, inhaled or in contact with eyes or skin. Use personal protective equipment (PPE) when manipulating these samples outside the fume hood.
2. After the addition of the cross-linker, collagen gelation will take place within 1-5 min (depending on the cross-linker used). To avoid collagen gelling in the tube, the next two steps (centrifugation and pouring of the solution on the mould) need to be performed rapidly.

Alternatively, devices like dual syringes or 3-way stopcocks might be used to mix the collagen solution with the cross-linker, followed by direct pouring of the resulting mixture on the PDMS moulds (skip the centrifugation step).

3. For an accurate estimation of the swelling of the collagen samples, the minimum recommended weight per sample piece is 10 mg.
4. When cutting the collagen film in stripes for tensile testing, caution must be taken not to damage the middle region of the sample (test region). Any defects in that region should result in automatic rejection of the sample. If possible, samples cut in the dog bone-shape are preferred, as a narrower than the edges middle area promotes stress concentration in the middle of the sample (this may not be possible in very fragile samples). Tensile test assays are typically carried out in quintuplicate (n=5), meaning that in this case at least two collagen films (3x4 cm) per condition are needed to obtain an accurate measurement of the scaffolds' mechanical properties.
5. C-shaped sample holders made of water-resistant materials can be used to maintain the collagen films in a stretched position preventing the samples from folding over onto themselves when incubated in PBS 1x, which facilitates sample thickness measurement and loading in the tensile test machine. Bench protector sheets made of paper and a laminated waterproof polyethylene layer can be used to make these sample holders. Dry collagen stripes can be glued at their ends to two sample holders in a 'sandwich' conformation. After affixing the sample in the tensile test machine, sample holders need to be cut in their middle region to negate potential bias during testing.
6. Two glass panes can be used to give a consistent thickness measurement for the samples: use the callipers to measure the thickness of the glass panes alone, and then repeat the measurement with the sample inserted between the panes.
7. It is essential to ensure that the placement of the sample is at 90 ^{o} angle to the grip so as to negate potential bias upon test.

8. Young's modulus should be extracted from the linear region of the stress/strain curve in between the toe and the failure regions typical of collagen materials.
9. The number of technical replicates (wells per condition) typically ranges between 3 and 5. The number of plates to consider depends on the number of time-points and experiments planned.
10. To minimize the toxicity risks associated with PFA, the fixation step should be performed in the chemical hood.
11. GTA cross-linking can lead to the auto-fluorescence of the collagen scaffolds when imaged with 358, 495, 528, 540 nm wavelengths. Washing the scaffolds thoroughly with PBS 1x diminishes the effect of this phenomenon.
12. Automated counting with ImageJ in confluent cells can lead to inaccuracies. If cells are confluent or over-confluent (nuclei superposition), manual counting is recommended.
13. Elongation degree of THP-1cells can be employed to assess the immune reaction *in vitro*. To do so, using ImageJ manually describe the shape of 25-50 cells per image and measure the shape features aspect ratio, circumference and roundness. Consider as elongated cells those with an aspect ratio higher than 3.0 or with an aspect ratio superior to 2 and circumference and roundness lower than 0.5. Calculate the percentage of elongated cells in 4 - 5 images for each condition. The presence of a high number of clusters (aggregates of 5 or more cell) can also be assessed as an indication of inflammatory reaction.
14. When assessing the metabolic activity of cells on scaffolds, these must be moved to new wells with the help of sterile tweezers to prevent the action of cells attached to surrounding TCP.

5. Acknowledgements

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6. List of Tables

Table 1: Swelling ratio, thickness and mechanical data of the produced collagen films. GTA significantly decreased water absorption. GTA and DPPA significantly reduced thickness. GTA induced the highest stress at break. DPPA induced the highest strain at break. GTA induced the highest modulus. # indicates statistically significant difference ($p < 0.05$).

Cross-linking agent	Swelling ratio (%)	Thickness (μm)	Stress at break (MPa)	Strain at break (%)	Young's modulus (MPa)
NC	100 ± 16	178 ± 4	0.03 ± 0.01	8.33 ± 3.00	0.20 ± 0.04
GTA	21 ± 9 #	67 ± 6 #	0.19 ± 0.04 #	4.19 ± 2.82	1.95 ± 0.33 #
4SP	80 ± 9	232 ± 33	0.07 ± 0.03 #	13.28 ± 3.13	0.38 ± 0.04
EDC	125 ± 7	185 ± 39	0.03 ± 0.01	15.78 ± 3.08	0.10 ± 0.03
DPPA	91 ± 23	83 ± 12 #	0.05 ± 0.01	18.22 ± 1.89 #	0.22 ± 0.01

7. List of figures

Figure 1- Dermal fibroblasts (DF)

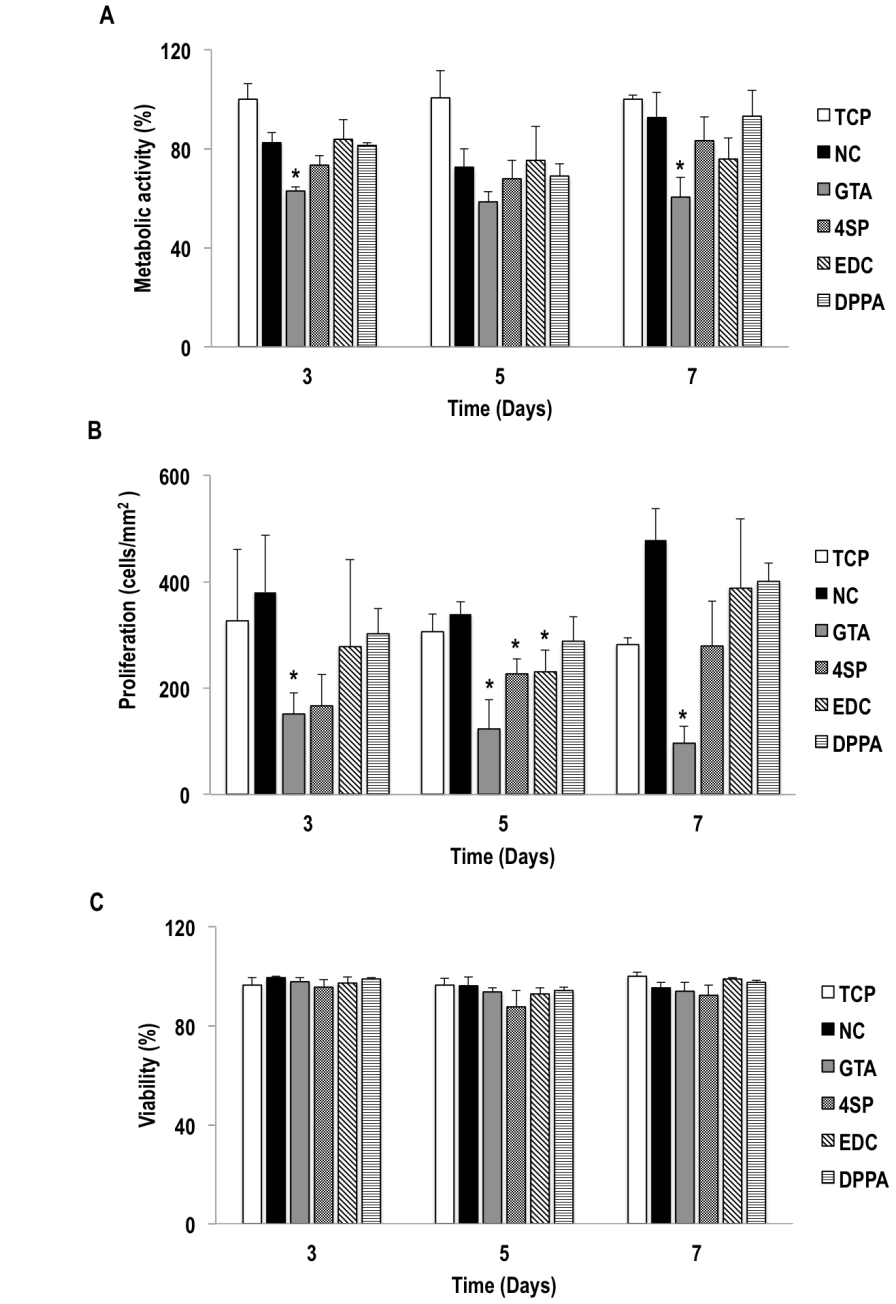

Figure 1: Metabolic activity (A), proliferation (B), viability (C) and rhodamine phalloidin (red) and DAPI (blue) images (D) of DF seeded onto the collagen scaffolds. DF on GTA cross-linked scaffolds presented reduced metabolic activity at day 3 and 7; and reduced proliferation at all three time-points in comparison to the NC samples. None of the cross-linking treatments significantly affected DF viability (C). DF adopted a spindle-shaped morphology in all the groups except for GTA, on which cells spread in all directions (D). Treatments: non-cross-linked collagen films (NC), collagen films cross-linked with glutaraldehyde (GTA), 4-arm PEG succinimidyl glutarate (4SP), carbodiimide (EDC), and diphenylphosphoryl azide (DPPA). Tissue culture plastic (TCP) was used as control. Significance compared to *NC group ($p < 0.05$).

Figure 2- THP1 on films

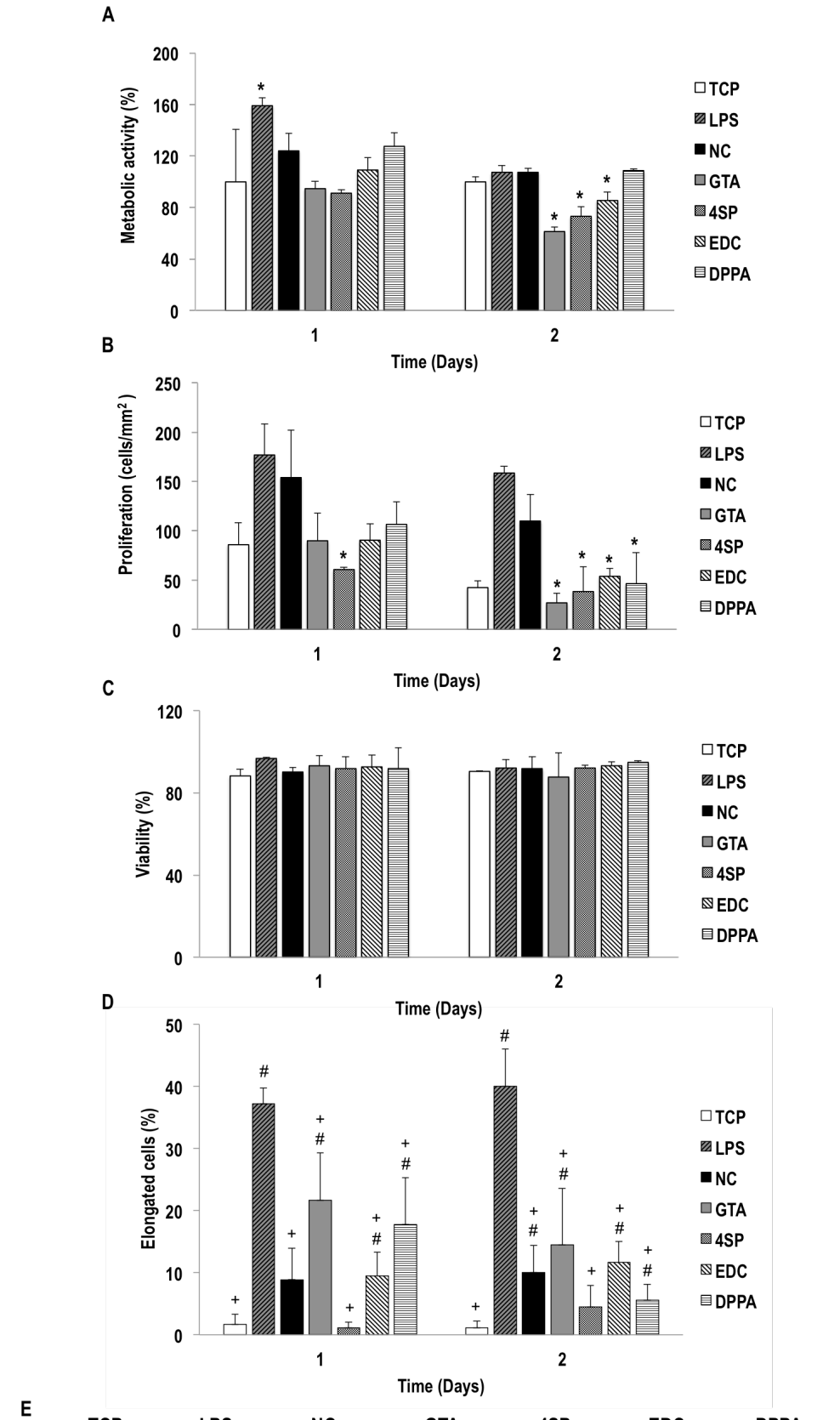

Figure 2:

Metabolic activity (A), proliferation (B), viability (C), elongated cells proportion (D) and rhodamine phalloidin (red) and DAPI (blue) images (E) of THP-1 seeded onto the collagen scaffolds. By day 1, LPS significantly increased THP-1 metabolic activity (A); and 4SP significantly reduced THP-1 proliferation (B) in comparison to the NC samples. By day 2, GTA, EDC and 4SP significantly reduced THP-1 metabolic activity (A); and all the cross-linking treatments significantly reduced THP-1 proliferation (B) in comparison to the NC. None of the cross-linking treatments significantly affected macrophage viability (C). All treatments elicited a significant lower proportion of elongated cells than LPS, whereas THP-1 in GTA, EDC and DPPA presented a significant higher number of elongated cells than TCP at both time points, likewise NC at day 2 (D). THP-1 adopted a rounded morphology, independently of the treatment and time in culture. Some elongated macrophages (white arrows) were observed predominantly in LPS and GTA treated samples, whereas cell clusters (black arrows) were mainly observed in LPS condition (E). Treatments: non-cross-linked collagen films (NC), collagen films cross-linked with glutaraldehyde (GTA), 4-arm PEG succinimidyl glutarate (4SP), carbodiimide (EDC), and diphenylphosphoryl azide (DPPA). Tissue culture plastic (TCP) and lipopolysaccharides in TCP (LPS) were used as controls. Significance compared to *NC group, compared to #TCP and compared to +LPS group ($p < 0.05$).

Figure 3- THP1 on pre-conditioned media

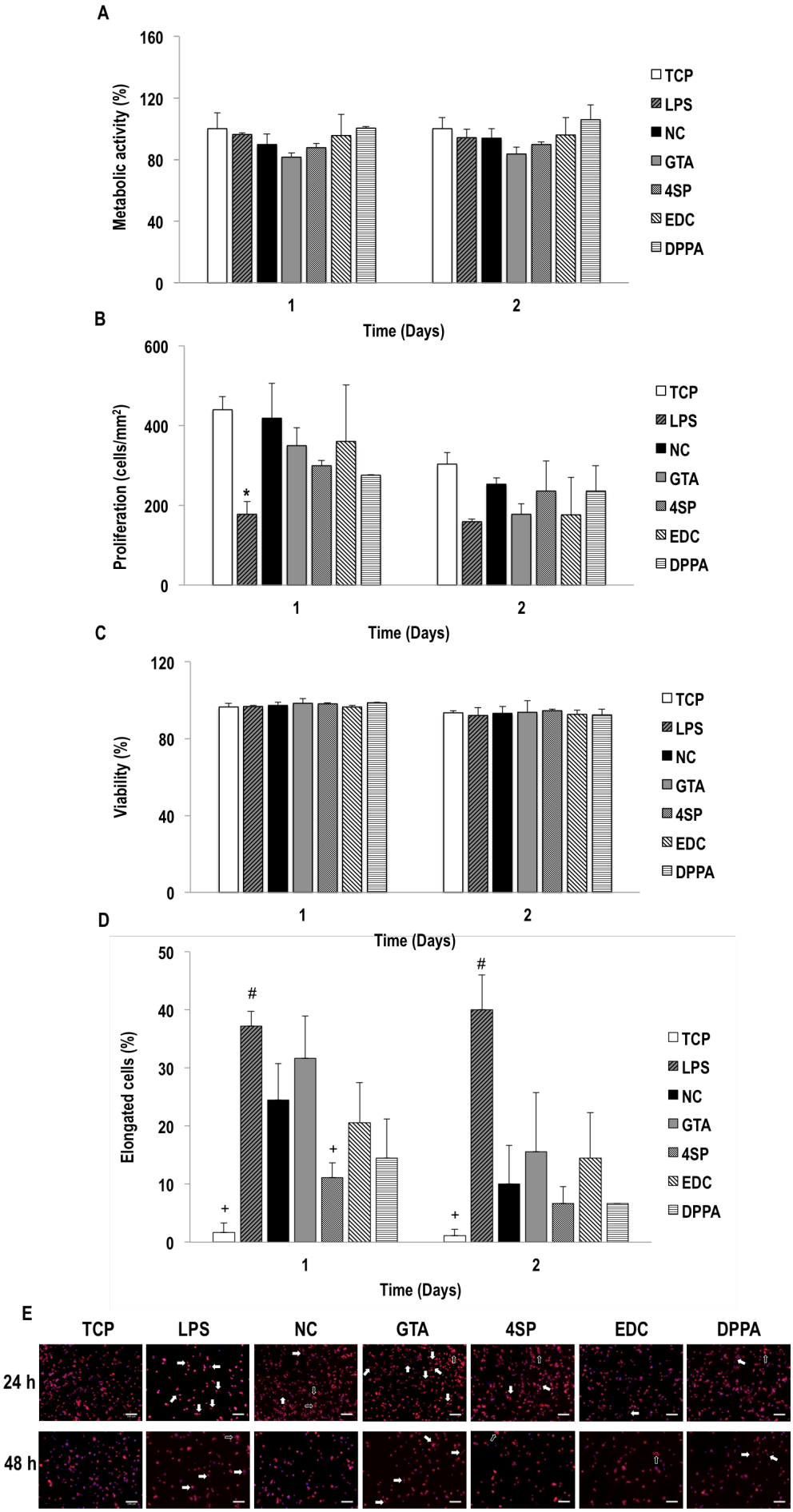

Figure 3: Metabolic activity (A), proliferation (B), viability (C), elongated cells proportion (D) and rhodamine phalloidin (red) and DAPI (blue) images (E) of THP-1 cultured with pre-conditioned media from the cross-linked collagen films. None of the cross-linking treatments significantly affected THP-1 metabolic activity (A). Only cells treated with LPS showed a decreased proliferation in comparison with the NC samples (B). None of the cross-linking treatments significantly affected macrophage viability (C). LPS showed a higher number of elongated cells than TCP at both times points. All treatments showed values higher than TCP and lower than LPS. Significant differences were found between LPS and TCP at both time points, whereas only 4SP showed a significant difference with LPS at day one (D). THP-1 adopted a rounded morphology, independently of the treatment and time in culture. Some elongated macrophages were observed (white arrows) predominantly in LPS, NC and GTA treated samples. Some cell clusters were observed (black arrows) in all the conditions except for TCP (E). Treatments: non-cross-linked collagen films (NC), collagen films cross-linked with glutaraldehyde (GTA), 4-arm PEG succinimidyl glutarate (4SP), carbodiimide (EDC), and diphenylphosphoryl azide (DPPA). Tissue culture plastic (TCP) and lipopolysaccharides in TCP (LPS) were used as controls. Significance compared to *NC group, compared to #TCP and compared to +LPS group ($p < 0.05$).

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