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DELIVERABLES REPORT

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DESCRIPTION	METHODS AND PROCEDURES FOR BIOLOGICAL EVALUATION OF SCAFFOLDS
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During the first 12 months of the project, the partners defined and shared a set of general guidelines for in vitro evaluation of the biological performances for scaffolds designed and prepared during the project.
The specific set of biological tests and the experimental methods can vary depending on the material, the specific scaffolds and the intended final application. Therefore, the present document aims to address general matters such as selection of cell phenotype, optimization of cell seeding routine and evaluation of essential cell-biomaterial compatibility.

Design of the cell culture experiment and optimization of cell seeding procedures

Preliminary operations.
The following points should be regarded general guidelines for the experimental activities and should be followed, whenever possible. Some of the steps may not apply even in specific cases such as cell encapsulation in hydrogels or scratch test.

- Define field of application and the goal of the experiment
- Define a suitable sterilization method and verify the effects of sterilization of the scaffold composition, properties and performances (mechanical and dimensional stability, morphological and microstructural modifications, chemical stability of the scaffolds as a whole and also of the single components in case of multicomponent composite scaffolds)
- Selection of the cell line
- Immortalized cell lines (both cancer-derived cells or cells that have been artificially manipulated to proliferate indefinitely) can be used in the preliminary stages of scaffold biological characterisation. At this stage, the experimental work should aim to assess possible cytotoxic effects of the scaffold/biomaterial, to compare different cell seeding methods, to evaluate preliminary aspects of cell-biomaterial and cell-scaffold interaction, cells viability after un-common processing techniques (for example, cell encapsulation and storage in hydrogel matrices), to establish a proof-of-concept of cell infiltration ability.

The origin of cells could be either human or animal, depending on the availability on the different sites. A variety of cell lines are eligible to be used in this stage including, but not limited to NIH-3T3, MG63, MRC5. In a second stage of the work, stem cells such as adipose-derived mesenchymal stem cells, bone marrow mesenchymal stem cells should be considered.

- Define the composition of the cell culture medium to be used accordingly with cell type selected. This step is particularly important in case of MSCs; two options should be taken into consideration: A. Use Growth Factors (GFs) enriched medium to drive and direct the required differentiation or to preserve a specific phenotype. B. GFs-free medium to evaluate the potential of scaffold and biomaterial to direct and drive MSCs differentiation or cell plasticity
- Plan seeding method taking onto account shape, size, composition, wettability of the scaffold. It is also important to consider possible specific needs of specific cell lines and whether the scaffold is supposed to be seeded in wet or in dry conditions.
- Define time points for biological evaluations, considering a culture time up to 3 days as early stages of cell behaviour.
- Define a set of evaluation techniques accordingly with the goal of the experiment. In general the following factors should be considered: cell viability (Alive&Dead staining), evaluation of cell morphology, cell proliferation behavior and cell activity, visual (immunostaining) and quantitative evaluation of protein expression (representative protocols are reported in Attachment).
- Write an experimental protocol, addressing the following points: aim of the experiment, sterilization method, cell seeding method and seeding density, definition of experimental groups, timetable of the experiment, characterization techniques and number of technical replicates (calculation of the total number of samples to be seeded and the total number of cells needed for the experiment), define internal control groups (cells on TCP, un-seeded scaffolds, …). A scheme of the experimental protocol is reported in attachment.
- Submit the experimental protocol to the local advisor for approval. Approved protocols should be completed at the end of the experiment with results, comments, suggestions, and warnings.

<u>Cell seeding efficiency and optimization steps</u>

In principle, the individuation of a suitable and effective seeding methods is not trivial. At the same time, the correct number of cells that should be seeded on a scaffold cannot be determined *a priori*. In case of non-standard culture conditions and novel substrates with unknown behavior, a set of preliminary tests with limited number of samples and time points are strongly suggested.

In general, a well-designed and well-pondered cell culture experiment should proceed in this order:

- Cell expansion to obtain the number of cells required for the test.
- Preliminary experiments should be performed to evaluate the optimal cell seeding concentration. At least three different concentrations should be used, and efficiency evaluated in terms of cell adhesion, proliferation.
- Materials and Scaffolds should be prepared in an appropriate number depending on characterization methods scheduled and considering statistical significance; sterilization (to be performed the day of the seeding or in advance). Sterilization

method should be previously selected and the impact on materials evaluated (ex. Polymeric materials at least Mw should be controlled)
- Preconditioning of the scaffold with cell culture medium or serum solutions can e performed to speed up cell adhesion (from 1 hour to 12 hours of incubation depending on the size, porosity and wettability of the scaffold)
- Cell detachment, counting and preparation of cell suspension at the required concentration.
- Seeding of the scaffolds with the required number of cells (previously evaluated)
- Remember to prepare control samples
- Considering moving the samples in a new well after few days (to avoid the contribution of the cells on the bottom of the TCP)
- Check cell viability, distribution, scaffold colonization in the short and medium term (up to few days), in particular in case of non-standard culture conditions and scaffolds.

The above procedures and planning steps can be applied to the biological evaluation of biopolymeric scaffolds and with minor modifications to the evaluation of the activity and behavior of cells encapsulated in hydrogel matrices. On the other side, many of the steps above cannot be directly applied to the case of biological evaluation of biopolymers as signaling molecules directly added to cell culture medium or released during the cell culture test by the scaffold.

As an example, we report here two case studies: the effect of silk sericin fractions and keratin molecules on fibroblast activity, with potential application in wound healing.

Evaluation of the biological activity of biopolymer as signaling molecules[1]

A. The effect of sericin fractions on cell activity and behavior

The goal of the study is to characterize and biologically evaluate fractions of sericin extracted from different silk strains with different methods and different extraction times. Extracted sericin fractions should be investigated in terms of amino acid composition by high performance liquid chromatography (HPLC) or SDS-PAGE and molecular weights by gel filtration chromatography (GFC). The biological activity of sericin proteins is assessed by culturing 3T3 mouse embryonic fibroblast cells in the presence of sericin-enriched culture media. The effect of different sericin samples on cell adhesion, morphology, proliferation and collagen production are evaluated. The findings of this study can be useful for the selection of silk sources and sericin extraction conditions.

Cell expansion

Fibroblast 3T3 cells are cultured in Dulbecco's Modified Eagle Medium with high glucose Euroclone, Milan, Italy) containing 10% fetal bovine serum (FBS), supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Fungizone® amphotericin B (Gibco®, Life Technologies, Monza, Italy). The culture

[1] The cell culture procedures and evaluation tests can slighly vary in the different groups depending on reagents and instrumentation availability on site and country/university regulations. However, the proposed protocols shouldbe used as reference documents for the planning of the test, the design of characterization methods, the number of replicates requested.

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medium are changed every other day, until cells reached confluence. Then, cells are harvested with 0.25% trypsin in PBS, and re-suspended in fresh medium. The cells are seeded on polystyrene 48-well plates at a density of 2×10^4 cells per well with 500µL of culture medium and incubated under standard culture conditions (5% CO_2, 37°C). After 4 h, the medium should be removed and replaced with 450 µL of medium and 50 µL of sericin sample, obtaining a sericin-enriched culture medium with a sericin concentration of100 µg/mL. Culture medium is replaced with fresh sericin-enriched medium every other day.

Cell morphological characterization by CLSM

After 24 h of culture in presence of sericin in the medium, the cells are fixed and permeabilized with 4% paraformaldehyde solution in PBS with 0.2% Triton X-100. Cells are labeled with Oregon Green® 488 Phalloidin (Molecular Probes®, Life Technologies, Monza, Italy) for the visualization of filamentous actin (F-actin) and 4',6-Diamidino-2-Phenylindole, dilactate (DAPI, Molecular probes®) to stain cell nuclei. The morphology of adhered cells is observed confocal laser microscope.

Cell proliferation

Cells number and proliferation can be assessed using PicoGreen® dsDNA quantitation assay (Quant-iT PicoGreen® dsDNA Assay, Invitrogen™, Grand Island, NY, US), following manufacturer's instructions. At predetermined time points (day 1, day 3, and day 7), culture medium is removed and cells were washed with PBS. Cells are covered with 500 µL of 0.05% Triton-X in PBS, and stored at -20°C until analysis. Later, samples are thawed at room temperature and the supernatant is transferred to a 1.5 mL tube and sonicated for 10 s with a Hielscher ultrasonic homogenizer (UP400S, 400 watt - 24kHz, amplitude 50%, from Hielscher Ultrasonics, Teltow, Germany). Extracts of 100µL arethen placed in a black 96-well plate, and mixed with 100 µL of PicoGreen® working solution. Five independent samples are analyzed for each experimental condition. Fluorescence intensity iss measured with a 200 microplate reader (excitation wavelength: 485 nm, emission wavelength: 535 nm). Fluorescence measurements are taken in triplicate. DNA content of each sample was determined on the basis of a calibration curve generated with a double-stranded DNA standard provided with the kit. According to manufacturer's instructions, the approximate number of cells per sample was estimated from DNA content by the conversion factor of 7.7 pg DNA per cell.

Determination of soluble collagen production

Supernatants were collected from the cells cultured in the presence of sericin samples. The total amount of soluble type I collagen was assayed using the Sircol™ Collagen Assay kit (Biocolor, Carrickfergus, UK). The test was conducted following the manufacturer's instructions and the collagen content was determined using a Tecan Infinite 200 microplate reader by measuring absorbance values at a wavelength of 500 nm. Four independent samples were analyzed for each experimental condition. Fluorescence measurements were performed in triplicate. The specific value of collagen content per cell was calculated by normalizing collagen amount with respect to the cell number obtained by PicoGreen® assay.

B. The effect of keratin proteins on cell migration and proliferation

The term "keratin" defines a family of structural, filament-forming proteins found in epidermal and corneous tissues like hair, nails, horns, claws, hooves, turtle scute, whale baleen, beaks, and feathers. Being insoluble and heavily cross-linked via sulfur bridges, some keratin-based structures are between of the toughest biological materials. Keratins extracted from hair and wool represents a whole new family of biomaterials for applications like TE

scaffolds, drug delivery and wound healing. The presence of the primary amino acid sequence of cellular-binding motifs, i.e. RGD (Arg-Gly-Asp), in the keratin make it useful for tissue scaffolds, by promoting cell adhesion and activation by interaction with integrins.

Preparation of keratin/medium solution

1.0 mg/ml keratin/medium solution was prepared by dissolving keratin from camel hair (CA_K) and cashmere (CS_K) into standard NIH 3T3 medium. The solutions were filtered through a 0.22 μm filter (Euro Clone, Milan, Italy) for sterilization.

Cell culture

Murine embryo fibroblast (NIH 3T3, ATCC number: CRL-1658) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM l-glutamine and 1% antibiotic/antimycotic in a humidified atmosphere of 5% CO2 at 37 °C, changing the medium every third day. Cells (at passage number 8) were collected by trypsin and were seeded in a 96-well plate (3×103 cells/well in 0.2 ml medium) for lactate dehydrogenase activity assay (LDH assay) and a 6-well plate (1×105 cells/well in 2 ml medium) for scratch test, and cultured in standard NIH 3T3 medium.

Scratch test – to evaluate cell proliferation and migration ability in the contest of wound healing.

Once the cell confluence reached 70%, a linear scratch was made in the cell monolayer using a sterile 100 μl plastic pipette tip. 2 ml PBS per well was added to wash the cellular debris. The PBS was removed and 2 ml of keratin/medium solution (1.0 mg/ml) and standard NIH 3T3 medium (control group) were added. Photographs were taken by a microscope equipped with an automatic stage (Nikon Ti, Nikon, Japan) on day 0 and day 1. The images were post-processed by software (GIMP 2) to calculate the decreasing ratio of blank area for samples and controls. For each time point, samples and controls were in triplicate and three points were selected to image each scratch.

Appendix 1. Experimental protocol for biological evaluation of scaffolds (Template)

Experiment TITLE

Aim of the experiment

Describe the aim of the experiment in brief, and summarize keypoint in the table below…

cell lines	
performed assays	
estimated time point	
main reagents and materials	
starting time	
keywords	

Summary:

Experimental groups

List here experimental groups and code-name of the samples.

Group #	*Sample name*

Experimental design

- Sterilization procedure
- Cells and cell culture medium

Cell culture medium composition

Product	*Code*	*Quantity*

Cell seeding details

- cell concentration:
- cell suspension total volume
- total number of samples:
- total number of cells needed:
- total volume:
- cells/mL

Charcterization techniques

Experimental scheduling

Provide info about cell seeding date and experimental time points

Results and observations

Fill this section at the end of the experimental activities, summarize results and observations, take note of the problems encountered and lessons learned. Report the document to the local advisors.

Appendix 2. General protocol for cell fixation and nuclei/cytoskeleton staining

Cell fixation

1. Remove medium
2. Wash samples twice with PBS, 5' each
3. Fixation in 4% paraformaldehyde (PFA) [equivalent of Formalin 10% solution]→ incubate at RT (room temperature) from 20 min to 1 h according to the sample size
4. Wash three times with PBS at RT, 5' each
5. Samples can be stored at 4 °C in PBS for some days

Cell permeabilization

6. Remove PBS
7. Permeabilize cell membrane with Triton X-100 solution (0.2% in PBS) → incubate at RT from 20 min to 1 h according to the sample size
8. Wash three times with PBS at RT, 5' each

Nuclei (DAPI) and cytoskeleton (Alexa Fluor® Phalloidin/OregonGreen) staining

9. Remove PBS
10. Calculate the total needed amount of PBS according to the sample size (typically 200-400 µL for a 48-well TCP sample are required)
11. Add 5.4 µL of DAPI stock solution for 25 mL of PBS and 5 µL of cytoskeleton staining* stock solution for each sample → incubate at RT from 20 to 50 min (protect from light!!)
12. Wash three times with PBS at RT, 5'each
13. Leave samples in PBS and store them at 4°C, protected from light

Appendix 3. General protocol for immunostaining

Cell fixation

1. Remove medium
2. Wash samples twice with PBS, 5' each
3. Fixation in 4% paraformaldehyde (PFA) [equivalent of Formalin 10% solution]→ incubate at RT (room temperature) from 20 min to 1 h according to the sample size
4. Wash three times with PBS at RT, 5' each
5. Samples can be stored at 4 °C in PBS for some days

Cell permeabilization

6. Remove PBS
7. Permeabilize cell membrane with Triton X-100 solution (0.2% in PBS) → incubate at RT from 20 min to 1 h according to the sample size
8. Wash three times with PBS at RT, 5' each

Blocking and antibody staining

9. Remove PBS
10. Add blocking solution (BSA 1% in PBS) → incubate at RT for 1 h
11. Add 1:200 (or check the suggested dilution in the datasheet of the antibody in use) solution of primary antibody in BSA 1% overnight at 4°C (or 1-2 h at RT, shaking)
12. Wash three times with PBS at RT, 5' each
13. Add 1:1000 (or check the suggested dilution in the datasheet of the antibody in use) solution of secondary antibody in PBS at RT for 1 h on an orbital shaker (protected from light!!)
14. Wash three times with PBS at RT, 5' each
15. Leave samples in PBS and store them at 4°C, protected from light

Appendix 4. Quantification of DNA

Assay method (for Thermo Fisher Scientific product Picogreen)

0.250 µL of 0.05% Triton-X are poured in each well. After 20 min the content of each well is transferred in a 1 mL vial and moved to a −20 °C refrigerator until the test is carried out.

PicoGreen assay	
reagent composition	(samples 1:1 in TE buffer 20×) 1:1 in PicoGreen 200× reagent
assay support	48-well TCP (test); 96-well TCP (data record)
initial cell density	2k encapsulated cells/well – 0.25 ml medium/well
repetitions	standard curve in triplicate; each sample in duplicate; 4 acquisition for each repetition
data record	fluorescence excitation wavelength: 485 nm fluorescence emission wavelength: 538 nm
notes	keep samples on ice! protect samples from light after the addition of the reagent! calculate the requested amount of reagents in advance!

1. Sonicate each sample for 10 seconds using the tip of an ultrasonic processor (cycle: 1, amplitude: 40%). Wash the tip in distilled water for a few seconds after each sonication;
2. put immediately the samples on ice;
3. dilute the total needed amount of TE buffer 20× in DI water;
4. dilute the samples 1:1 in TE buffer to obtain 500 µL of final volume in a 48-well plate;
5. dilute DNA lambda adding 20 µl of the stock solution (100 µg/mL) in 980 µl of TE buffer (final concentration 2 µg/mL);
6. prepare the sample for the standard curve in a 24-well plate using different composition of TE buffer and DNA lambda (4 mL of TE buffer are needed);
7. transfer the sample for the standard curve (100 µL in triplicate) to a black 96-well plate. If the sample number requires the use of 2 or more plates, a calibration curve has to be prepared in each plate;
8. transfer the samples for the test (100 µL in duplicate) to the black 96-well plate;
9. prepare the PicoGreen working solution in order to get a 200-fold dilution of the stock solution in TE buffer **(protect from light!!!)**
10. add 100 µL of PicoGreen working solution to each well and incubate at room temperature for 2-3 minutes (* final concentration values are reduced to their half after adding the PicoGreen working solution).

Example of calculation for 24 samples, 1:1 dilution in TE buffer:

- *24*2 (test) + 8*3 (calibration) = 72 total samples*
- *TE buffer for sample dilution: 24*250 µL = 6 mL*
- *TE buffer for calibration (standard): 4 mL*
- *TE buffer for PicoGreen working solution dilution: 72*100 µL = 7.2 mL*
- *total TE needed: (6+4+7.2) mL*1.2 = 20 mL*
- ***TE buffer: 1 mL concentrated TE buffer (20×) in 19 mL DI water***
- ***PicoGreen working solution: 45 µL PicoGreen stock solution (200×) in 9 mL TE buffer (tot 7.2*1.2mL)***

Appendix 5. Alamar Blue Assay (Resazurin)

It is a colorimetric/fluorimetric assay based on the incorporation of an oxidation/reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth.

- Dissolve 0.025gr of Resazurin in 100mL of PBS w/o Mg^{2+}
- Prepare fresh cell medium (enough for all your samples) adding 10% of Resazurin previously dissolved
- Change the medium to your samples adding medium with Resazurin previously prepared (add the right volume according to your sample size)
- Incubate at 37°C for at least one hour protected from light
- Transfer 100uL of supernatant to 96 well-plate (duplicates for each samples)
- Measure fluorescent intensity with excitation wavelength at 535nm and emission at 590nm, or measure absorbance at a wavelength of 570nm and 600nm. Medium and Alamar reagent will be the negative control.

➔ It is better to have at least 4 replicates for each sample

➔ To determine the incubation time and to avoid the saturation of the system, according to the specific cell type and proliferation rate, it is better to perform a preliminary analysis: different cell concentrations (till the maximum of cells that you expect to have at the end of your experiment) are analyzed at different incubation time (1h, 2h, 3h, 4h, 5h, 6h). See AlamarBlue assay protocol (ThermoFisher Scientific – DAL 1025).

Appendix 6. ALP (Alkaline Phosphatase Activity - Abcam)

Standard curve preparation

Always prepare a fresh set of standards for every use.

Discard working standard dilutions after use as they do not store well.

Prepare a 1mM pNPP standard by diluting 40µL pNPP 5mM Standard in 160 µL of Assay Buffer.

Using 1mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	*p*NPP 1 mM Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount *p*NPP in well (nmol/well)
1	0	300	120	0
2	10	290	120	4
3	20	280	120	8
4	30	270	120	12
5	40	260	120	16
6	50	250	120	20

Each dilution has enough amount of standard to set up duplicate readings (2 x 120 µL).

Samples Preparation

Remove the medium from the well

Wash twice in PBS

Add 1mL of 0.1% Triton-X and wait 30 minutes at RT

Store at -20° until use

Thaw at RT and sonicate each sample 20 seconds on ice

Centrifuge 3 minutes at 13000g

Transfer 50µl of cell lysate into a 96 well-plate and add 60ul assay buffer (Triton-X 100 should use as a blank). For each sample use a triplicate.

Add 20µl of the 0.5 mM substrate solution (diluted 10 times from 5mM with assay buffer)

Incubate for 30 minutes at 25°C and protect from light

- Add 10µl of ALP enzyme solution to each standard well
- Add 20µl of stop solution for each sample and standard
- Measure fluorescence intensity at 360/440 nm (Ex/Em)