D4.1 – REPORT ON THE NANOPILLAR BIOCOMPATIBILITY

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Abstract:	This document summarizes the first test on living tissues and cells' nanopillar biocompatibility and tissue and cell survival. The deliverable summarizes the first work done in this area and it confirms that StretchBio does not face any critical restriction related to the use of biomaterials in the proposed devices' architecture.

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Disclosure Statement:

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Executive Summary

The overall goal of the StretchBio project is the design, development, fabrication and validation of an advanced label-free and compact nanosystem for the continuous monitoring and quantification of mechanical stresses in “ex vivo” fresh tissue biopsies. The first step to accomplish this objective is to test living tissues and cells’ nanopillar biocompatibility and tissue and cell survival. This deliverable summarizes the first work done in this area during the first year of the project.

Several cell lines (Caco-2, lung cancer cell lines and Schneider’s cells) and tissues from *Drosophila* (*Drosophila* imaginal discs) have been seeded on different nanodevice surfaces and their properties evaluated: tests have been performed to analyse cell viability, such as Alamar cell staining, LIVE/DEAD viability/cytotoxicity assay and trypan blue cell count. In addition, nanosurfaces were coated with different substances to compare cell viability in coated and non-coated surfaces.

All cell lines tested do not show mortality when seeded on surface devices, and only Drosophila imaginal discs, when seeded on 7’-RIE treated substrate, showed a stressful effect of this type of substrate on the disc.

The here-reported work shows promising result to attain the project objectives and it will be further developed during the next months of the project.

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1 Introduction

1.1 Purpose of the document

This deliverable summarises the effort to assess living tissue-nanopillar biocompatibility and tissue survival in nanopillars. Here, we analyse different cell lines and *Drosophila* tissues to test their biocompatibility with the surfaces of the future project devices.

1.2 Background

Different cell lines and primary cultures have been used in this work. All the cell lines were grown in a monolayer.

Caco-2 cells, also named human colonic adenocarcinoma cell line, are the most widely used models in intestinal drug studies in pharmaceutical research. Although limitations such as their tumour origin and the absence of the intestinal physiological context must be considered when extrapolating the data obtained “in vitro”, this cell line has the advantage of being easy to handle, reproducible, and adaptable to automatic “high-throughput screening” which make them relevant as a good toxicokinetic tool for predicting acute gastrointestinal toxicity (1–3).

In addition to Caco-2 cells, two cell lines derived from lung cancer were also used, NCI-H358 and A-549 cell lines. NCI-H358 cell line was isolated from a primary bronchioalveolar lung carcinoma, which is a non-small cell carcinoma of the lung which harbours a KRAS mutation (4). A-549 cells line are derived from adenocarcinomic human alveolar basal epithelial cells (5).

Drosophila larval epithelial sacs, called imaginal discs, can be dissected easily for further molecular or cellular analysis. While the bulk of the wing disc comprises epithelial cells, it also includes neurones and glia, and is associated with tracheal cells and muscle precursor cells. Studies in wing imaginal discs have made key contributions to many areas of biology, including tissue patterning signal transduction, growth control, regeneration, planar cell polarity, morphogenesis and tissue mechanistic (6).

1.3 Related documents

This deliverable builds upon the findings made in the first stages of WP4. No ethical concerns have been considered taking into account the assessment made in the deliverable D1.3. OEI – Requirement No.12.

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2 Material and Methods

2.1 Nanopillar surfaces

All the substrates assessed for biocompatibility assays were generated inside the consortium by the project partners: five surfaces composed of different materials were used to test cell viability and biocompatibility: Si n-type, Si p-type, processed Si Lito + RiE, SiO_2 968mm and SiO_2 303.5mm. Definite nanopillar structures (5‘and 7‘pillars) were also tested.

2.2 Cell lines culture

Caco-2 cells were cultured in D-MEM media supplemented with 10% FBS, 1mM glutamine and 1U/ml penicillin/streptomycin. NCI-H358 were cultured in RPMI1640 medium with 10% of FBS. A-549 cell line was cultured in D-MEM supplemented with 10% FBS.

Schneider 2 cells (S2 cells) were derived from a primary culture of late-stage *D. melanogaster* embryos: they were cultured with commercial Schneider medium.

2.3 Wing imaginal discs

Larvae from Drosophila (genotype w; $\frac{TREdsRED}{CyO}$; $\frac{MKRS}{TM6B}$,), were dissected to isolate wing imaginal disc. The wing discs were transferred to the preheat (25°C) culture medium (Schneider’s culture medium supplemented with 2% of heat-inactivated FBS, 1,25% Fly extract and 5µg/ml insulin). The medium was changed three times by negative pressure on a silicone tube before being transferred to the substrate.

2.4 Coating substrates

Different coating substrates were tested to evaluate the optimal conditions to different material surfaces (Table 1). In addition, we studied the viability properties of different cells to the different coated and non-coated surfaces. In Caco-2 cells we tested Collagen type I coating, in S2 poly-L-lysin, and in lung cancer cell lines we tested gelatine and Matrigel coatings.

Coating	Concentration
Collagen type I, rat tail	1,1µg/ml
Poly-L-Lysin	0,1mg/ml
Gelatine	1%
Matrigel	0,3 mg/ml (dil 1:60 of stock)

Table 1. Coating substrates tested to evaluate the optimal conditions to different material surfaces

2.5 Cell viability studies

To analyse cell viability, we performed Alamar Blue assay and LIVE/DEAD viability/cytotoxicity assay (Thermo-Fisher Scientific). The Alamar Blue cell viability reagent is a ready-to-use resazurin-based solution that functions as a health indicator by using the reducing power of living cells to quantitatively measure viability. LIVE/DEAD viability /citotoxicity assay allows two-colour discrimination of live from dead populations based on membrane integrity, esterase

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activity, metabolic activity, or structural segmentation. Plasma membrane integrity is determined by ethidium homodimer-1 which enters cells with a compromised plasma membranes to bind DANN and emit a red fluorescence; live cells are identified by Calcein AM, a fluorogenic cell-permeant dye that is converted to a green fluorescence after interaction with intracellular esterase's.

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3 Results

3.1 Cell viability assays on coated surfaces

The percentage of Caco-2 cells seeded in 24-well plates that remained viable after being exposed to the substrates for a 6-day period was ascertained by means of Alamar Blue, a colorimetric assay that detects metabolically active (living) cells. None of the nanodevice substrates showed a decreased cell viability regardless collagen coating (Figures 1 and 2).

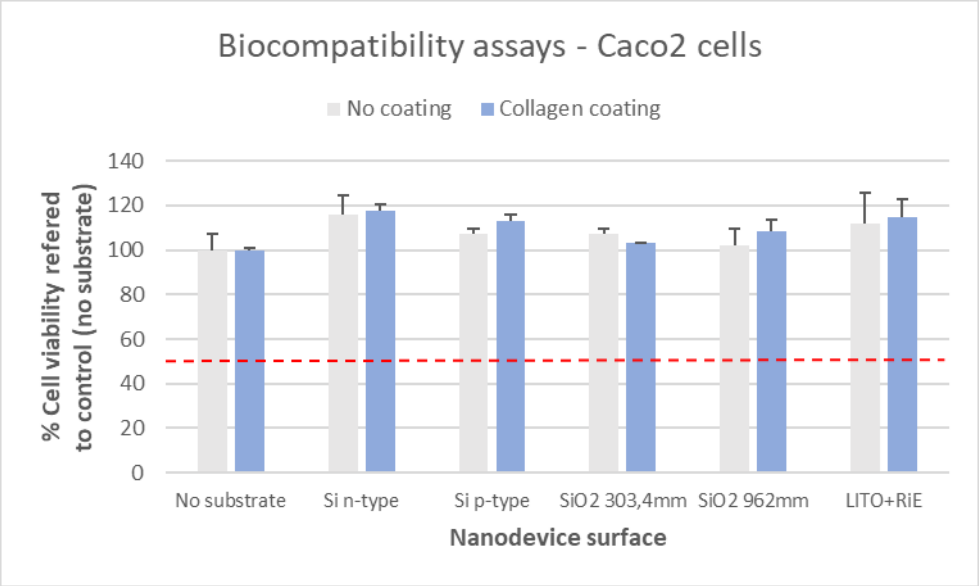

Figure 1. Representation of the biocompatibility values of both the assessed substrates and the no substrate case (control group) after 6 days of culture with Caco-2 cell line. Data are represented as mean ±SD. The red line indicates the median lethal dose (LD50) at which there is 50% cell mortality.

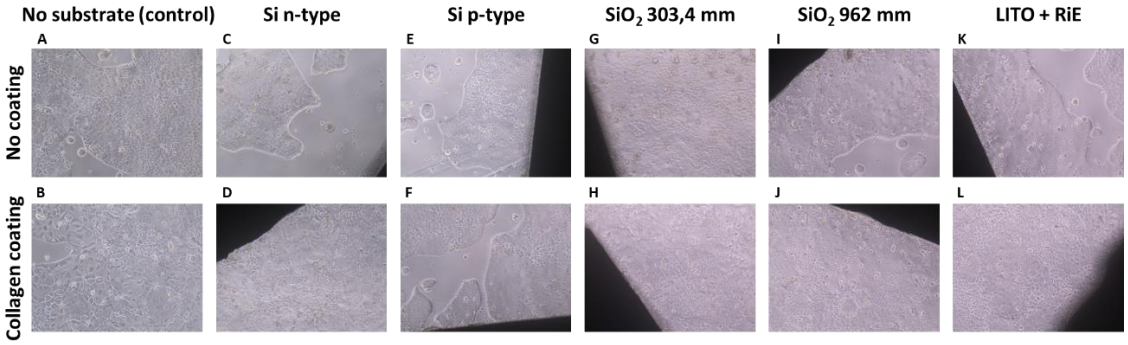

Figure 2. Phase contrast optical microscopy images (100x) of the Caco-2 cell line cultured with no substrate (A-B) and with potential nanodevice substrates (C-L) with or without collagen coating after 6 days of cell culture.

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To further explore if Caco-2 cells can be grown on the surface of the potential nanodevice substrates, the slides of substrates were placed on a 24-well plate. Caco-2 cells were seeded on the top of each slide with 20 µL of cell culture medium (DMEM supplemented with 10% FBS, 1 mM glutamine, and 1 U/mL penicillin/streptomycin), and leave them to be attached for 4 hours at 37ºC and 5% CO_2. After this period, additional 300 µL of cell culture medium was added to each well. After 48 hours in culture, cell medium was changed and incubated for additional 72h at 37ºC and 5% CO_2.

Similarly, at the end of this period (day 6 of culture), the number of viable cells were assayed by means of the Alamar Blue cell viability reagent according to the manufacturer's protocol. Data shown in Figure 1 indicate the percentage of Caco-2 cells seeded on the top of the slide substrates that remain viable after being cultured for a 6-day period. Compared to the control group (no substrate), none of the nanodevice substrates assessed showed a decreased cell viability.

When we performed the viability assay in lung cell lines, A-549 and NCI-H358 cells, after five days in culture, no differences were observed between coated and non-coated surfaces. The polyanionic dye calcein is well retained within live cells, and the emitted fluorescence can be measured (ex/em~495nm/~515 nm). Red fluorescence was produced when EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids (ex/em~495nm/~635 nm). No red fluorescence was detected in any condition tested. Green fluorescence was measured as relative fluorescence units (RFU) (Figure 3).

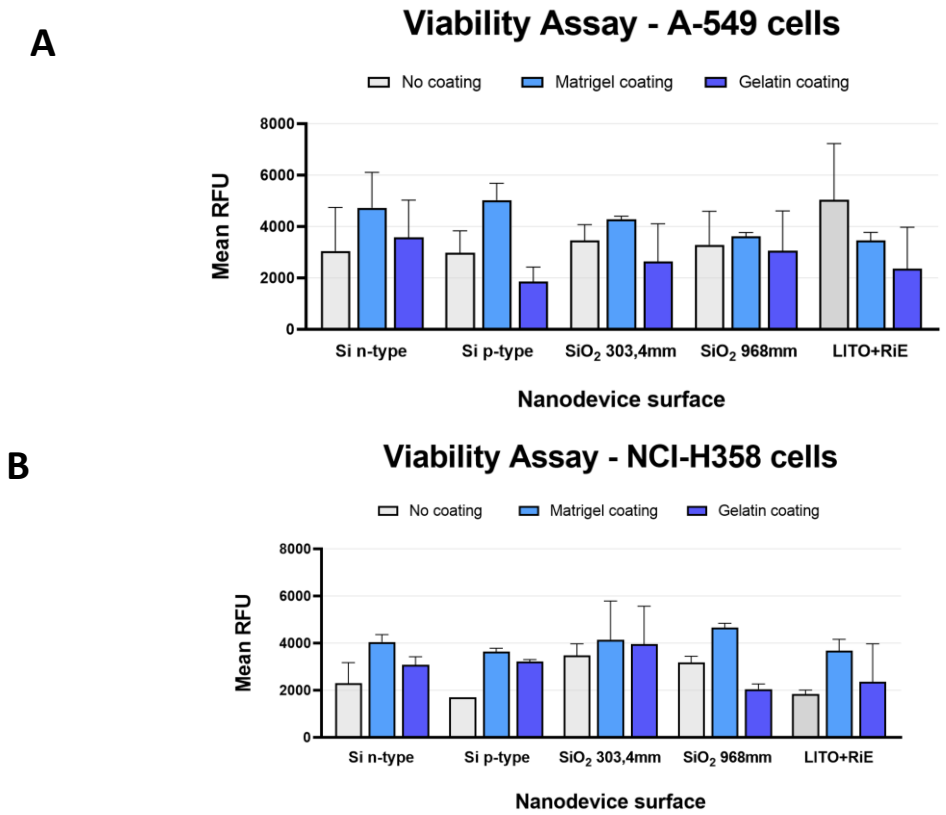

Figure 3. Viability assays (A-549 cells & NCI-H358 cells). a) Viability assay in A-549 cell line showed no differences between coated and non-coated conditions in any of nanopillar substrates tested. b) No significant differences were observed between the different surfaces analyzed both with coating and without.

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Both lung cancer cell lines proliferate in a uniform manner on 5' and 7'-RIE treated surfaces (Figure 4).

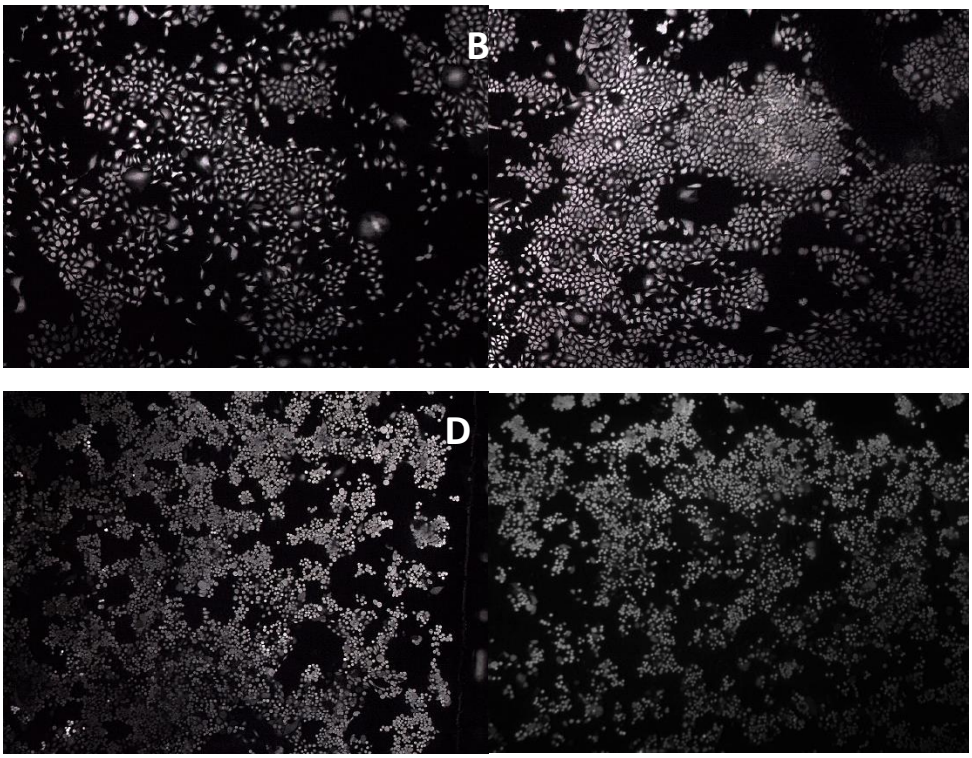
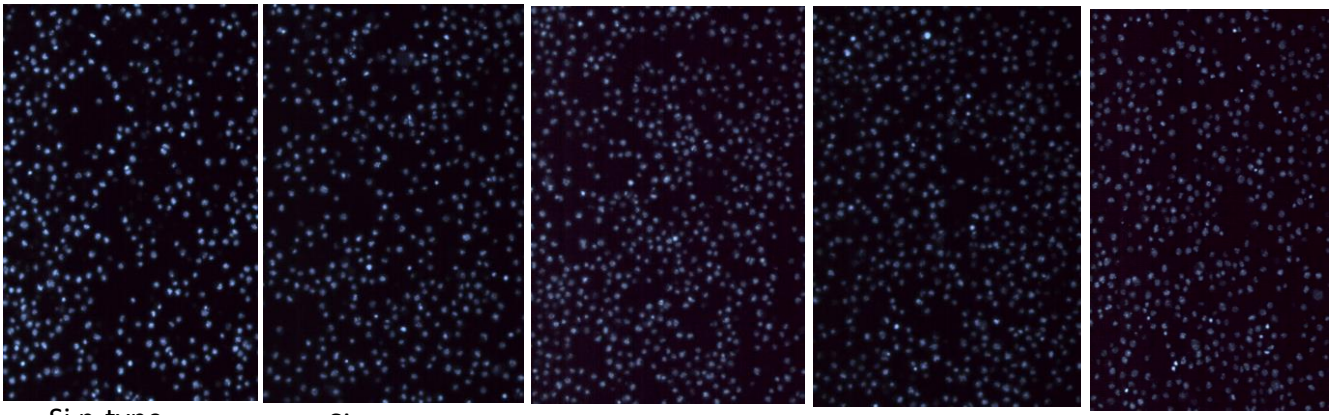


Figure 4. A-549 cell line grown on a 5-' (a) and 7'-RIE treated surfaces (b). NCI-H358 cells grown on a 5-' (c) and on a 7'-RIE treated surface (d). (Magnification x40).



	Mean cell nº
Si n type	4086,333
Si p type	3483
SiO2 303,4nm	3617
SiO2962	3753,667
Processed Si	3301,6667

Figure 5 Schneider's (S2) cells from Drosophila, showed no differences in cell viability when cultures on nanopillar surfaces when coated with poly-L-lysin. (Magnification x200).

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Results of *Drosophila* imaginal discs incubated on substrates showed the stress levels when placed on a glass slide (no substrate) or the two types of silicon substrates.

The fact that there are no significant differences, in any time point, between the "no substrate" condition and the "Processed Si 7'" condition could imply that is the direct contact of the wing imaginal disc to the material the reason for the stress.

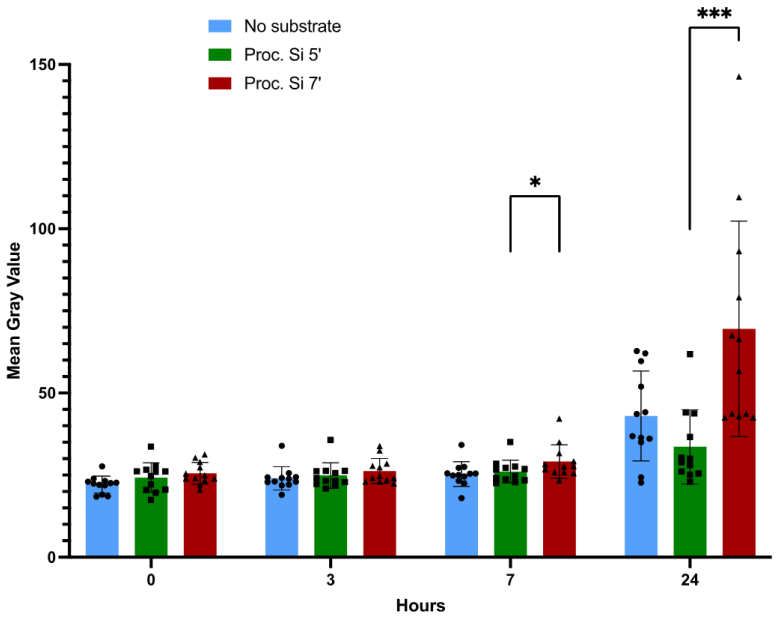

Figure 6. Stress effect of samples as function of time and substrate. The data highlight a stressful effect of the "Processed Si 7'" type of substrate on the disc, already statistically significant after 7 hours, but clearly visible after 24 hours of culture.

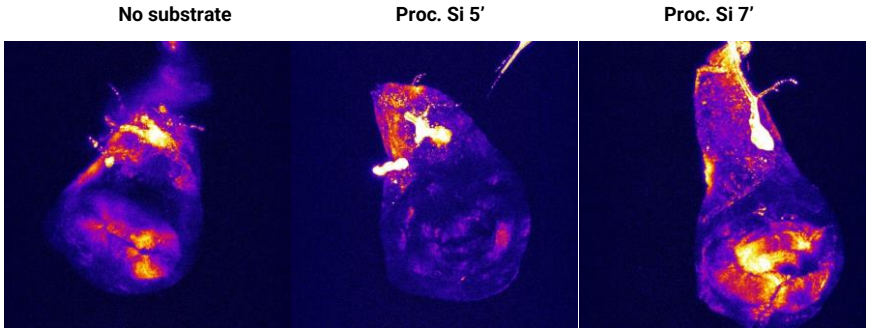

Figure 7. Maximum pixel value projection of discs after 24 h of culture.

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4 Conclusions

This deliverable builds upon the findings made in the first stages of WP4 of StretchBio project.

All cell lines analysed (Caco-2 cells, A-549, NCI-H358 and S2 cells) showed no differences in terms of viability, independently of the nanodevice tested, including coating and non-coating surfaces. In *Drosophila* imaginal discs, we observed a stressful effect of 7'-RIE treated substrate, that could be caused by the direct contact of the substrate with the tissue or to a stressful (or toxic) effect of the substrate on the culture medium.

The here-reported results are provisory and they will be further developed in the coming months. At the time being, any significant issue regarding the biocompatibility of tissues and cells which could jeopardize StretchBio concept has been identified.

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