Universitat de Girona

Facultat de Ciències

Memòria del Treball Final de Grau

Títol del treball:

Focal adhesion regulation through microcontact printing of protein nanoparticles on self-assembled monolayers for cell guidance

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Data de dipòsit de la memòria a secretaria de coordinació: 27/05/2016

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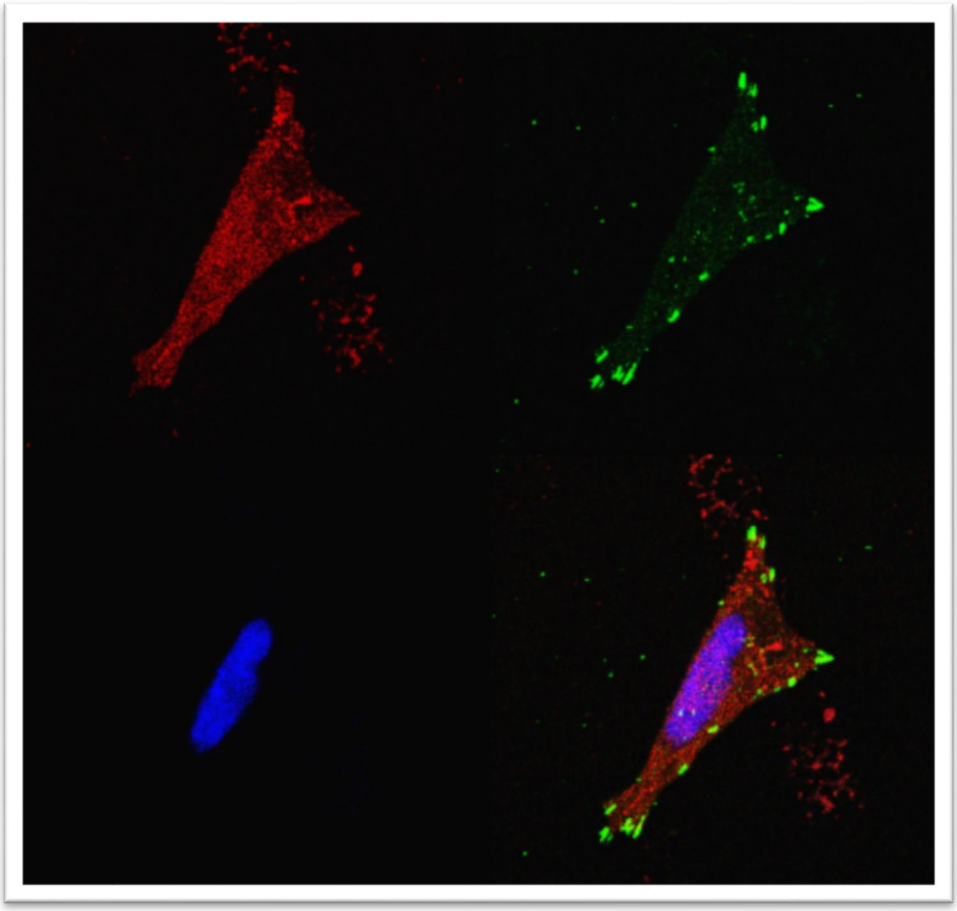

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Acknowledgements

Vull donar les gràcies a la Dra. Imma Ratera per haver-me donat l'oportunitat d'endinsar-me al món de la nanobiotecnologia i haver-me guiat dedicadament en la realització de tot aquest projecte. D'igual manera a l'Adriana Kyvik, per haver compartit la meva tasca i per haver-me donat un cop de mà sempre que ho he necessitat. A la Olivia Cano per haver-me convidat al grup de Nanobiotecnologia de l'IBB i per haver-me assistit durant la meva estada. També a tot el personal de l'ICMAB, de l'IBB i del SCAC que han format part de la realització d'aquest treball.

Ara que he arribo al punt culminant del Grau en Biotecnologia, vull donar les gràcies a aquells que m'han donat suport durant tot aquest temps: a la Muriel i en Brian per la seva ajuda. A en Miquel, i en general, a totes les amistats que m'han acompanyat durant aquest trajecte. A la meva família pel seu suport incondicional i la confiança que han dipositat en mi. I finalment, a l'Ana, perquè sense ella no hauria arribat fins aquí.

List of abbreviations

AFM: Atomic Force Microscopy
BSA: Bovine serum albumin
CV: Cyclic voltammetry
DLS: Dynamic Light Scattering
DMEM: Dulbecco's Modified Eagle Medium
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
FA: Focal adhesion
GFP: Green fluorescent protein
GRAS: Generally recognized as safe
HPLC: High performance liquid chromatography
IBB: Institut de Biotecnologia i Biomedicina
IBs: Inclusion bodies
ICMAB: Institut de Ciència de Materials de Barcelona
LB: Lysogeny broth
MAL: Maleimide
PBS: Phosphate-buffered saline
PdI: Polydispersity index
PDMS: polydimethylsiloxane
PEG: Polyethylene glycol
RT: Room temperature
SAM: Self-assembled monolayer
SCAC: Servei de Cultius Cel·lulars, Producció d'Anticossos i Citometria
SDS: Sodium dodecyl sulfate
SEM: Scanning Electron Microscope
THF: Tetrahydrofuran
UAB: Universitat Autònoma de Barcelona
µCP: Microcontact printing

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1. ABSTRACT

1.1 *English*

Bacterial inclusion bodies (IBs) are proteinaceous aggregates that are used as nanoparticulate materials to engineer the nanoscale topography. They assist cell culture, proving a positive impact not only on colonization and proliferation, but also on cell morphology. Self-assembled monolayers (SAMs) are formed as a result of a spontaneous self-organization of functionalized organic molecules onto appropriate substrates into stable, well-defined structures.

The interaction between different types of SAMs and IBs was studied: different self-assembled molecules give place to different interactions with IBs. Maleimide and OH terminated SAMs were produced, and this platform, surface functionalization using SAMs combined with IBs, was used to perform preliminary studies of cell guidance.

Escherichia coli and *Lactococcus lactis* IBs were produced, purified and characterized. Microcontact printing (μCP) using a polydimethylsiloxane (PDMS) stamp was carried out to provide a stripe pattern of IBs on the SAMs, thus providing IB substrate scaffolds in a pattern, which assisted growth and guidance of cultured neuroblastoma cells. Actin, nuclei and paxillin staining of the cells was performed and observation with confocal microscopy revealed that focal adhesions (FA) were formed specifically on the IB patterns. Cell alignment on the pattern and cellular bridges were also observed and it was determined that *L. lactis* IBs promoted more FA formation per cell than *E. coli* IBs.

This new approach opens new horizons in the field of tissue engineering and regenerative medicine towards the development of a new generation of innovative biotechnologically engineered biomaterials.

1.2 *Català*

Els cossos d'inclusió bacterians (IBs) són agregats proteics que s'utilitzen com a nanomaterials per dur a terme modificacions topogràfiques a escala nanomètrica. Afavoreixen el cultiu cel·lular demostrant un impacte positiu no només en la colonització i proliferació, també en la morfologia de les cèl·lules cultivades. Les self-assembled monolayers (SAMs) són auto-organitzacions espontànies de molècules orgàniques formant estructures estables.

S'ha estudiat la interacció entre els diferents tipus de SAMs i IBs: diferents molècules donen lloc a diferents interaccions amb IBs. S'han produït SAMs acabades en maleimide i OH, i posteriorment s'han funcionalitzat amb IBs per tal de dur a terme estudis preliminars d'orientaició cel·lular.

S'han produït, purificat i caracteritzat IBs de *Escherichia coli* i *Lactococcus lactis*. S'han imprès patrons de línies de IBs sobre les SAMs mitjançant la tècnica de microcontact printing (μCP) utilitzant segells de polydimethylsiloxane (PDMS), aconseguint així un substrat de IBs en un patró que afavoreix el creixement i orientació de cèl·lules de neuroblastoma cultivades. S'ha tenyit l'actina, el nucli i la paxilina de les cèl·lules per tal d'observar-les amb microscopi confocal, que ha revelat que les adhesions focals (FA) s'han format específicament sobre els patrons de IB impresos. També s'ha observat l'alineament cel·lular i la formació de ponts cel·lulars entre línies de IBs. A més, s'ha determinat que els IBs produïts per *L. lactis* promouen més formació de FA per cèl·lula que els IBs de *E. coli.*

Aquesta nova aproximació obre nous horitzons en el camp de l'enginyeria de teixits i de la medicina regenerativa cap al desenvolupament d'una nova generació de biomaterials innovadors biotecnològicament modificats.

1.3 *Español*

Los cuerpos de inclusión bacterianos (IBs) son agregados proteicos que se utilizan como nanomaterials para modificar topografías a escala nanométrica. Favorecen el cultivo celular demostrando un impacto positivo no solo en la colonización y proliferación, sino que también en la morfología de las células cultivadas. Las self-assembled monolayers (SAMs) son auto-organizaciones espontáneas de moléculas orgánicas formando estructuras estables.

Se ha estudiado la interacción entre los diferentes tipos de SAMs y IBs: Diferentes moléculas dan lugar a diferentes interacciones con IBs. Se han producido SAMs acabadas en maleimide y OH, y posteriormente se han funcionalizado con IBs para realizar estudios preliminares de orientación celular.

Se han producido, purificado y caracterizado IBs de *Escherichia coli* y *Lactococcus lactis*. Se han imprimido patrones de líneas de IBs sobre las SAMs mediante la técnica de microcontact printing (μCP) usando sellos de polydimethylsiloxane (PDMS), consiguiendo así un sustrato de IBs en un patrón que favorece el crecimiento y orientación de células de neuroblastoma cultivadas. Se ha teñido la actina, el núcleo y la paxilina de las células para observarlas con microscopio confocal, que ha revelado que las adhesiones focales (FA) se han formado específicamente sobre los patrones de IB imprimidos. También se ha observado la alineación celular y la formación de puentes celulares entre líneas de IBs. Además, se ha determinado que los IBs producidos por *L. lactis* promueven más formación de FA por célula que los IBs de *E. coli*.

Esta nueva aproximación abre nuevos horizontes en el campo de la ingeniería de tejidos y de la medicina regenerativa hacia el desarrollo de una nueva generación de biomateriales innovadores biotecnológicamente modificados.

2. INTRODUCTION

2.1 Nanobiotechnology and Nanomedicine

Nanobiotechnology is a recent scientific discipline that can be explained as the convergence between biotechnology and nanotechnology. The term nanobiotecnology is used to describe the applications of nanotechnology techniques for the development and improvement of biotechnological process and products [1]. For instance, the self assembly of molecules and the use of matrixes with nano-scale order for novel tissue engineering and regenerative medicine. Nanobiotechnology is defined as the application of nanotechnology and materials science to achieve breakthroughs in healthcare by the exploitation of the improved and often novel physical, chemical and biological properties of materials at the nanometer scale.

Among the different branches of Nanomedicine, regenerative medicine and cell therapies have been considered as one of the most promising tools for the development of the future medicine. As a consequence, the unraveling of the nature and mechanism of the biological processes behind these cellular and molecular interactions will constitute a breakpoint for the developing of the new generation of innovative biotechnologically engineered biomaterials capable of overcoming many important clinical and patient needs. Therefore, focusing on the mimicking of the extracellular matrix is essential in order to achieve such goals, since no cell tissue can be engineered nor grown without a bio-compatible substrate.

2.2 Surface engineering to mimic the extracellular matrix

Extracellular matrix (ECM) mimicking is an advantageous application of Nanobiotecnology. Many cells are adherent and must attach to and spread on a surface in order to survive, proliferate and function. In tissue, this surface is the ECM, an insoluble scaffold formed by the assembly of several large proteins that provide a wide range of biochemical and mechanical cues to cells [2]. These cues include several motifs that primarily interact with integrins to mediate adhesion among others. Materials that are modified with ECM ligands are important biomaterials, where coatings presenting a single biomolecular ligand give control over cell adhesion and subsequent cellular behavior [3]. Therefore, surface functionalization to replicate the ECM is useful in the study of artificial cell attachment, growth and proliferation and allows devising ways of creating artificial biological tissue.

2.2.1 Self-assembled monolayers

Self-assembled monolayers (SAMs) have been used in studies of cell adhesion, migration, proliferation, and differentiation, and are well-suited for a broad range of studies involving the interactions of cells with ECM [2]. SAMs are formed as a result of spontaneous, self-organization of functionalized organic molecules onto appropriate substrates into stable, well-defined structures. The final structure is close to or at thermodynamic equilibrium, and as a result, it tends to form spontaneously and rejects defects [6]. The SAMs are usually named based on the surface terminal group (constitutes the outer surface of the film and determines the properties of the surface) followed by spacer chain (connects the head and surface terminal groups) and the head group (binds strongly to a substrate) [6]. For instance, LA-PEG-MAL (used in this study) is Lipoic Acid – PolyEthylene Glycol – MALeimide (Figure 1, 2).

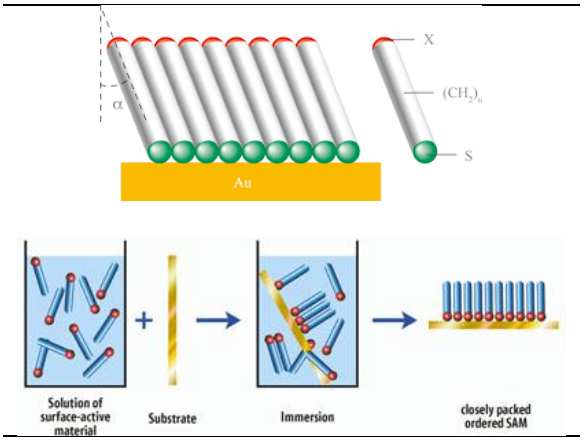


Figure 1. Top image shows a SAM disposed on a gold substrate [4]. Red color represents the terminal group, white is the spacer chain and green shows the head group. Second scheme shows the SAM spontaneous self-organization [5].

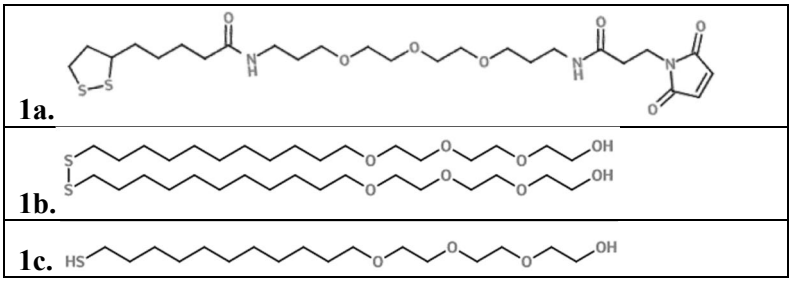


Figure 2: 1a. LA-PEG-MAL (Lipoic Acid – Polyethylene glycol – Maleimide) formed by maleimide as the surface terminal group for ligand immobilization. **1b**. HOEG3C11-S-S-C11EG3OH (OH - Triethylene glycol – S – S – Triethylene glycol – OH), non-reactive to maleimide since termination is not a thiol but a disulfide and **1c**. SH-PEG-OH (thiol - Polyethylene glycol – OH). Polyethylene glycol spacer chains act as surface coatings that prevent nonspecific adhesion of biomolecules to surfaces [7]. Sulfur atoms coordinate to the gold surface to give a densely packed and ordered hexagonal array of long chain molecules that are in an extended conformation [2].

In the field of ECM mimicking, SAMs have opened a wide range of possibilities as they are capable of imitate a bio-compatible substrate where cells can anchor, grow and proliferate [6].

2.3 Focal adhesions to regulate cell behavior

Cell anchoring to ECM is mediated by focal adhesions (Figure 3) among others. Integrins are cell membrane receptors that are activated by extracellular matrix (ECM) ligands [9]. The ligated receptors cluster and regulate chemical signaling by controlling the spatio-temporal assembly of enzymes and adaptors [10]. Studies on planar surfaces have demonstrated that such initial clustering results in focal complexes, which are small protein plaques of < 1 micron that consist of the integrin, phosphotyrosine and talin [11]. Using ECM proteins such as fibronectin is a strategy to stimulate focal adhesions creation as it plays an essential role in the adhesion of many cell types to extracellular matrices and artificial substrata. Substrate-dependent changes in the conformation of adsorbed fibronectin module integrin binding and control switching between proliferation and differentiation [12].

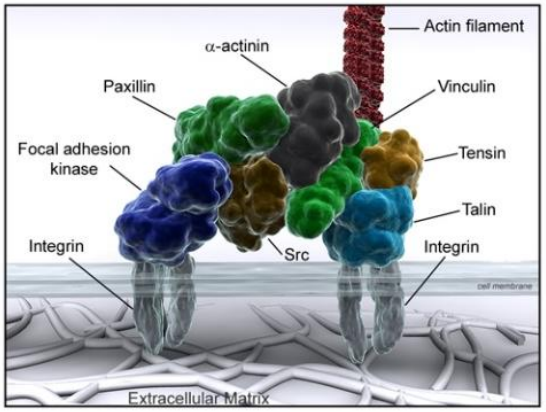


Figure 3. Composition of a focal adhesion [8].

2.4 Inclusion bodies as substrate for cell adhesion

In the framework of a collaboration of the NANOMOL Group with the group of Nanobiotechnology (IBB, UAB) it has been recently shown that a good strategy to accomplish cell growth and guidance is based in the use of SAM functionalized substrates decorated with biocompatible particles that integrate the mechanical (environmental topography) and biological control of cell proliferation.

Such particles are based on bacterial inclusion bodies (IBs) which are quite pure proteinaceous and mechanically stable bioadhesive nanoparticulate entities [13] ranging from around 50-500nm [14] to 1μm. It has been proven that when IBs are used as particulate materials to engineer the nanoscale topography, cell culture is assisted, proving a positive impact not only on colonization and proliferation but also on their morphology [13].

Up to now, IBs have been immobilized on surfaces through electrostatic interactions [13]. For IBs to become a proper substrate for nanomedicine applications, it would be preferable to attach them covalently to the surface in order to become a solid, robust and immovable support for cell incubation and posterior proliferation and guidance.

2.4.1 Escherichia coli and Lactococcus lactis inclusion bodies

Green fluorescent protein (GFP) IBs are useful nanobiomaterials that stimulate cell attachment, proliferation and differentiation and permit an easy characterization under UV microscope. IB production using *E. coli* is efficient and economically viable [15] but, on the other hand, safety concerns related to toxic compounds remaining on the product after purification are an obstacle to overcome. Bacterial endotoxins such as lipopolysaccharides (LPS) in *E. coli* limit this microorganism products applications in regenerative medicine as they are not safe to scale at human medicine.

For this reason, a novel IB production procedure [15] has been developed using *Lactococcus lactis* as production strain as lactic acid bacteria (LAB) have been classified as a GRAS (Generally Recognized As Safe) group of microorganisms by regulatory agencies. Therefore, *L. lactis* are a safer alternative for protein-based biomaterials production (Figure 4) for their broader applications in pharmaceutical industries.

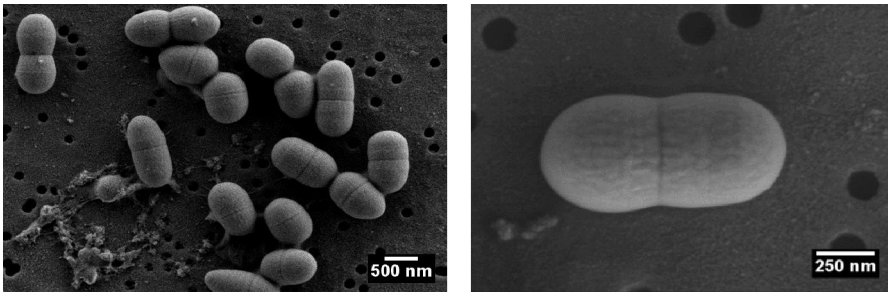


Figure 4. SEM images of *Lactococcus lactis* obtained by the research group of Prof. Antoni Villaverde from the Institute of Biotechnology and Biomedicine (IBB).

2.5 Maleimide as biofunctionalization and immobilization strategy

Tatkiewicz, W. *et al* successfully engineered protein-based nanoparticles (IBs) for cell guidance attaching them on surfaces through electrostatic interactions using amino-terminated SAMs [13]. The same group has also prepared amino-terminated silicon substrates and created IB-grafted surfaces that stimulate mammalian cell proliferation, proving the potential of IBs in tissue engineering and regenerative medicine [16]. Up to now, researchers have only focused on the utilization of amino-terminated SAMs to obtain protein nanoparticles adhesion on surfaces through weak electrostatic interactions.

On the other hand, several studies [17], [18] have demonstrated that maleimide permits a broad class of biologically active ligands to be covalently immobilized onto a monolayer. One of those is the RGD sequence (Arg-Gly-Asp) that serves as a ligand for the receptor-mediated adhesion of cells [18] and is a known ligand for cell integrin receptors. Since maleimide can be synthesized on a structure of polyethylene glycol

molecule with a sulfur-containing molecule head group (Figure 2) it can be arranged as a self-assembly monolayer and be used for studies of covalently attached ligands. Therefore, since IBs are protein-based nanoparticles presenting HS-terminated cysteine aminoacids, maleimide-terminated monolayers have been proposed to bind inclusion bodies to surfaces through maleimide reaction (Figure 5).

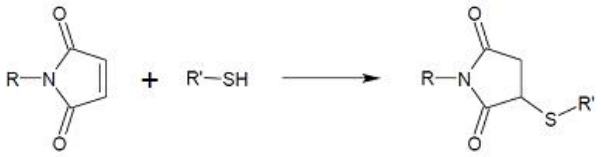


Figure 5. Reaction between a maleimide and a sulfhydryl group (IBs' cysteine residues contain a sulfhydryl group).

However, for regenerative medicine applications, IB and SAM patterns need to be stablished on surfaces in order to obtain appropiate substrates for proper cell adhesion and guidance.

2.6 Spatial control of biofunctionalization

In this context, microcontact printing (μCP) is one of the most versatile techniques for printing molecules and particles on surfaces. μCP extends conventional pattern printing to the μm-scale dimension where only the raised portions of the stamp come into contact with the stamped surface; the raised pattern of the stamp is therefore replicated on the stamp surface. The microcontact printing procedure consists of two principal steps: fabrication of polydimethylsiloxane (PDMS) stamps and printing (see section 4.7 and 4.8). PDMS is a commonly used polymer to prepare stamps as its elastomeric properties allow stamps to achieve conformal contact with the substrate to be stamped with little or no applied pressure. Stamps in μCP are cast from photolithographically generated resist patterns on master wafers as these provide excellent resolution. Once the master has been prepared and PDMS mixture poured over it, a stamp may be cast from it as PDMS is cured and then peeled off the master (Figure 6) [19].

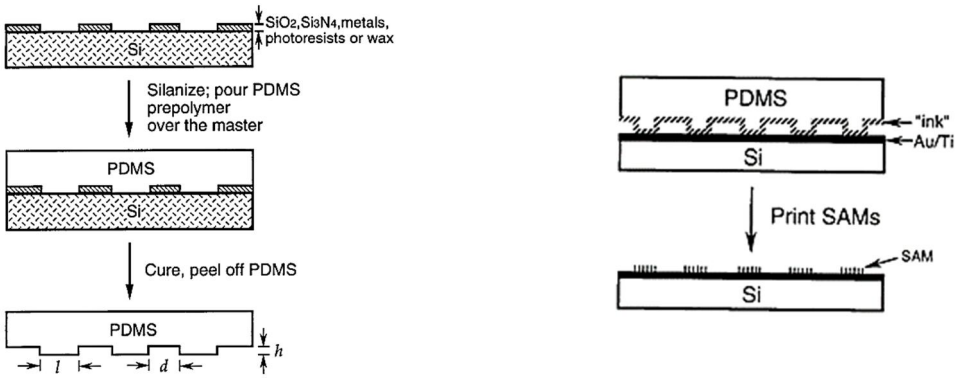


Figure 6. Left : preparation of PDMS stamp pouring PDMS to a patterned master, curing and peeling. Right : microcontact printing of SAMs using PDMS stamp [19].

However, μCP can also be used not only to generate SAM patterns, but also to decorate substrates printing IBs. Tatkiewicz *et all.* proved the potential of surface patterning with functional IBs as protein based nanomaterials for tissue engineering as they successfully achieved 5, 20 and 50nm IB stripe patterns using μCP. As stated in section 2.4.1, cells preferentially adhered to the IB areas and aligned and elongated according to specific patterns and chose the shortest way to reach new IB adhesion spots, as these nanoparticles stimulate cell attachment, proliferation and differentiation (Figure 7) [13].

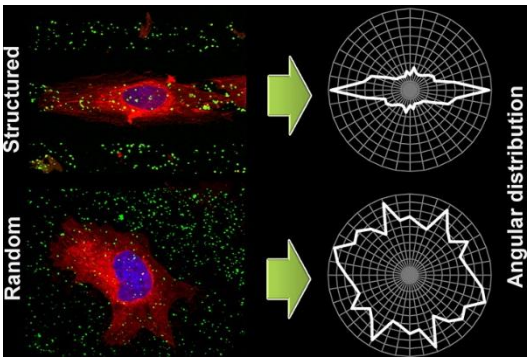


Figure 7. Impact of the IB patterning on the orientation of fibroblasts. Left: Confocal microscopy images of fibroblasts cultured on substrates functionalized with striped and random IBs patterns. GFP-derived IBs (green), cellular membrane (red) and nuclei (blue). Right: Radial distribution plots of cell membrane orientation. The spindle-like distribution for the pattern with stripes indicates a strong guidance of cell orientation [13].

2.7 Cell lines

Tatkiewicz *et all.* cultured human skin fibroblasts (1BR3.G) in order to analysis the influence of IBs on cell orientation [13]. As their precedents are a reference to our work, a similar cell strain had to be selected considering its morphology and growth rate. Human neuroblastoma SH-SY5Y INc cells were chosen to be used in this project as it has epithelial morphology with resembling characteristics to 1BR3.G cells (Figure 8).

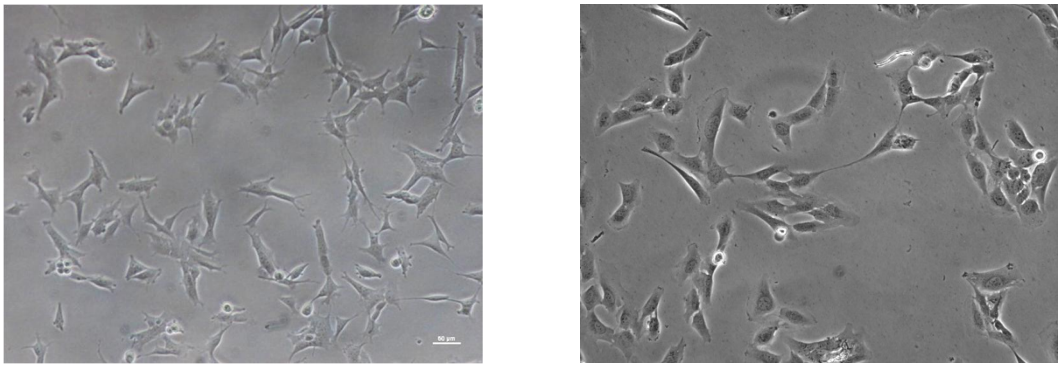


Figure 8. Optical microscopy images of SH-SY5Y cells on our culture (left) and 1BR3.G cells (right) photographed in Cell Culture Service (UAB).

3. OBJECTIVES

The main objectives of the present work are the production of protein nanoparticles to selectively functionalized surfaces for cell growth and study of focal adhesion regulation of cell behavior. More specifically, the objectives are:

i. *E. coli* and *L. lactis* IBs production

L. lactis and *E. coli* IBs need to be correctly produced and purified using recombinant strains in order to obtain a large amount of protein nanoparticle samples and therefore reproducible and comparable results.

ii. Selective interaction between IBs and SAMs

One of the targets of this study was to characterize the covalent interaction between IBs and the maleimide PEGylated SAM surface through thiol-maleimide reaction. For this purpose, we prepared separated SAMs, one containing maleimide molecules (LA-PEG-MAL) to covalently attach IBS on them and others incorporating non IB attaching SAM molecules (HO-EG3-C11-S-S-C11-EG3-OH and SH-PEG-OH). Observing noticeable differences on IBs adhesion between the different surfaces after strong rinsing was the main objective to prove the selective attachment of IBs only on the maleimide SAM (figure 9).

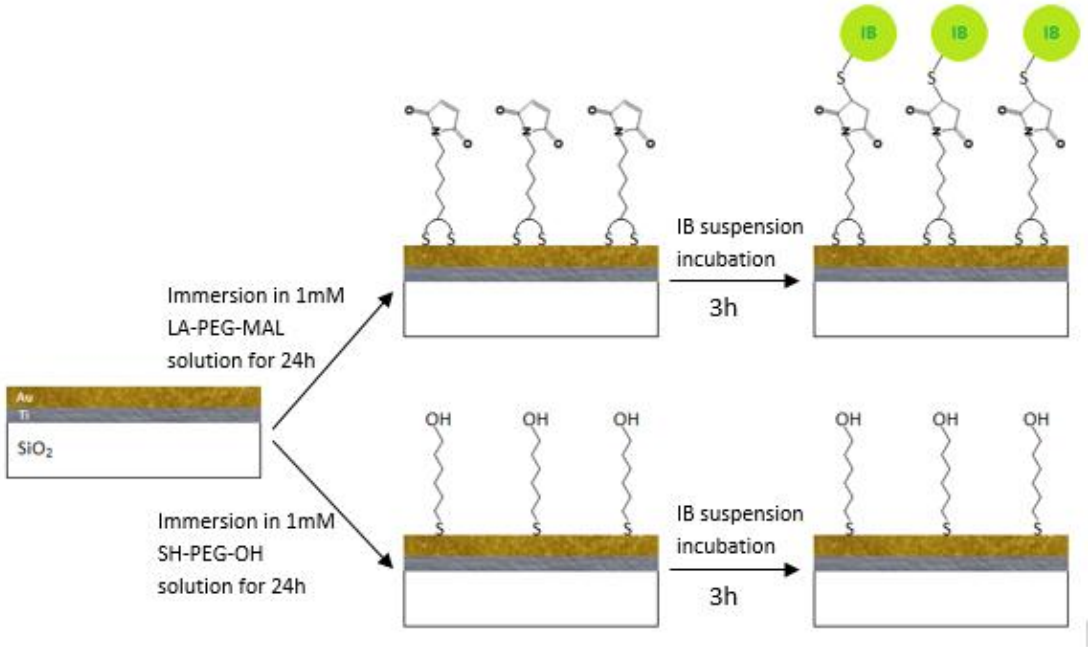


Figure 9. Scheme of the study of IB interaction with maleimide. At first, titanium and gold will be deposited by evaporation on SiO_2 wafers. Next step will be the functionalization with LA-PEG-MAL and SH-PEG-OH and finally IBs will be incubated on the surfaces. No IB adhesion is expected on SH-PEG-OH SAMs and covalent IB adhesion to maleimide SAMs is predicted.

The interaction between GFP IBs produced in *Escherichia coli* and *Lactococcus lactis* with maleimide-terminated self-assembled monolayer substrate will be studied using different characterization techniques such as AFM (Atomic Force Microscopy), optical UV microscopy, contact angle and cyclic voltammetry. Other techniques will be also useful to prove that the self-assembly protocol has been carried out correctly.

iii. Selective IB functionalization through μCP

Selective IB surface functionalization using μCP is proposed in order to generate IB patterns to investigate how IBs influence cell growth and guidance.

iv. Cell growth and study of focal adhesions on IB-patterned substrates

Cell culture will be performed in order to study the influence of IBs on cell growth and guidance. In order to accomplish this goal we will study if cells growing on IB patterned surfaces can form mature focal adhesions suggesting that such surfaces facilitate integrin clustering and activate other important signaling cascades. Focal adhesions were selected as main indicator of mechanism of cell attachment to the most favorable substrate for their growth. For the study of focal adhesion formation, a staining focused on cellular anchor proteins will be performed.

4. MATERIALS AND METHODS

4.1 Escherichia coli IB production and purification

IB production and purification was performed in the Nanobiotechnology group at the (IBB-UAB) leaded by Prof. Antonio Villaverde group under Dr. Olivia Cano supervision. All the material must be sterile and the procedure must be carried out in a laminar flow hood under sterility conditions.

E. coli Nc400 pTV1GFP strain was grown in LB rich medium supplemented with 300μl of ampiciline and 300μl of streptomicine in 300ml shake flasks at 37ºC and 250rpm until it reached the optical density of 0,715 units. Then the culture was induced to GFP IBs production using 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) during 3 hours. Purification started when the induced culture optical density reached 4,38 units. Then one small complete EDTA free pill was added to the broth. 3ml Lisozime 0,5 mg/ml was also added to the medium, and then the culture was incubated 2h at 37ºC and 250rpm. After this step, the culture was frozen overnight at -80ºC.

1,2ml Triton X-100 was added to the media when the culture defrosted. Culture was then incubated with agitation at room temperature for one hour. A first sterility control was then performed spreading 100µl of culture broth in an antibiotic-free LB Petri dish and incubated overnight at 37ºC. The rest of the culture was frozen overnight at -80ºC. Freezing/defrosting process must be repeated spreading 100µl of culture in an antibiotic-free LB plaque after each cycle, until no organism grows in the dish after 24 hours. After 3 cycles of freezing/defrosting, our broth was sterile.

75µl of NP-40 was then added to the mixture and kept under agitation for 1h at 37ºC and 250rpm. Culture was then centrifuged at 3900rpm during 30 minutes and the pellet was resuspended with 15ml of lysis buffer + Triton X-100. The mixture was then frozen overnight.

The culture was defrosted and centrifuged at 3900rpm during 15 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended with 15ml of PBS buffer. The mixture was then transferred to 1ml Eppendorffs (each one containing 10ml of the original broth). Samples were then centrifuged and the supernatant was discarded. GFP IBs were then purified and ready to be used. They were kept frozen at -80ºC until immediate use.

4.2 Lactococcus lactis IB production and purification

All the material must be sterile and the procedure were carried out in laminar flow hoods always under sterility conditions.

L. lactis strain was cultured in M17 medium enriched with 0.5% glucose and supplemented with 150µl of erythromycin and 75µl of chloramphenicol in 300ml shake flasks at 30ºC and 250rpm until it reached the optical density of 0,37. Recombinant gene expression was induced to GFP IBs production using 12.5ng/ml nisin during 3 hours.

Purification was started when optical density reached 3,8 units. Culture was distributed in 50ml sterile falcons and centrifuged 3900rpm during 30 minutes. The supernatant was then discarded and the pellet was resuspended in 30ml PBS/falcon which was then frozen overnight at -80ºC.

The sample was then defrosted and disrupted for three rounds using French Press at 15000 PSI. The resulting solution was frozen overnight at -80ºC and 600µl lisozime/falcon was added to the sample and kept under agitation during 2h at 37ºC and 250rpm when defrosted. Then the mixture was frozen overnight at -80ºC. 120µl Triton X-100/falcon was added after defrosting. Falcons were agitated for one hour at room temperature.

After Triton X-100, a first sterility control was performed spreading 100µl of culture broth in an antibiotic-free 0.5% glucose M17 broth Petri dish and left overnight at 30ºC.

The rest of the culture was frozen at -80ºC overnight. Freezing/defrosting process must be repeated spreading 100µl of culture in an antibiotic-free M17 broth plaque after each congelation cycle, until no organism grows in the media. After 3 cycles of freezing/defrosting, our broth was sterile.

75µl of NP-40 was added to the mixture and kept under agitation for 1h at 4ºC. 18µl 1M $MgSO_4$ and 18µl of 1mg/ml DNAse were added to each falcon and kept under agitation for 1 hour at 37ºC and 250rpm. The mixture was then centrifuged at 3900rpm during 30 minutes and the supernatant was discarded. The pellet was resuspended with 5ml lysis buffer + 0.5% Triton X-100. Falcons were then frozen overnight at -80ºC.

Tubes were centrifuged at 3900rpm during 30 minutes after defrosting and bacterial pellet was resuspended with 5ml of sterile PBS. Solution was then transferred to 1ml Eppendorfs (each one containing 10ml of the original broth). Aliquots were centrifuged at 1300rpm during 15 minutes and the supernatant was discarded. GFP IBs were then purified and ready to be used. They were kept frozen at -80ºC and defrosted for immediate use.

Table 1. *E. coli* and *L. lactis* culture parameters and supplements.

Microorganism strain	Strain	Agitation [rpm]	Initial broth filling [ml]	Temperature [ºC]	Medium	Supplements	Induction agent
E. coli	Nc400 pTV1GFP	250	300	37	LB	300µl ampiciline 300µl streptomicine	1mM IPTG
L. lactis	clP- htrA- pNZ8148-	250	300	30	M17	0.5% glucose 150µl erythromycin 75µl chloramphenicol	12.5ng/ml Nisin

4.3 Titanium and gold evaporation on silica substrates

For the preparation of SAMs, titanium (50nm) and then gold (100nm) were deposited on SiO_2 wafers using physical vapor deposition equipment inside the ICMAB Nanoquim service cleanroom. The titanium layer is used for a better gold adhesion to the substrate. The gold layer was deposited in order to provide an anchor layer to the thiol and disulfide-terminated molecules, since sulfur compounds display a great adhesion affinity to gold forming self-assembled monolayers.

Before the SAM deposition the substrates were treated with a cleaning procedure in order to remove all impurities and non-desired molecules: they were submerged first with dicloromethane, then ketone and finally into ethanol. They were then all dried under a stream of nitrogen and kept into a clean container. For the next cleaning step, the substrates were treated in the ultraviolet ozone cleaner for 20 minutes and they were immediately submerged into ethanol for 30 minutes. The substrates were then ready for the preparation of self-assembled monolayers.

4.4 Preparation of self-assembled monolayers

In this study SAMs are prepared using various molecules with different terminations and therefore functions: one molecule that can bind protein derivatives covalently (LA-PEG-MAL) and other molecules that do not react (HO-EG3-C11-S-S-C11-EG3-OH and SH-PEG-OH) in order to control protein adhesion (Figure 2).

Mixed SAMs were prepared by immersing the substrates in two 10mL methanolic 1mM solutions containing different LA-PEG-MAL (purchased from QUANTA BIODESIGN) and HO-EG3-C11-S-S-C11-EG3-OH (purchased from Prochimia) ratios under a controlled nitrogen atmosphere (Figure 1). Maleimide-containing mixed SAMs were prepared as 99:1 and 99:2 based on various studies [2], [3], [20], [21]. Substrates were immersed into solution overnight and they were then rinsed with HPLC ethanol to remove physisorbed molecule multilayers and dried under a nitrogen stream.

SAMs containing only one single kind of molecules were also prepared as 10ml 1mM solutions of all described PEGylated products. Substrates were submerged into 1mM ethanolic solution in a controlled nitrogen atmosphere for 24 hours to ensure thiol adhesion to the substrate. They were then rinsed using HPLC ethanol and dried under a nitrogen stream.

4.5 Deposition of IBs on mixed SAMs

E. coli and *L. lactis* GFP inclusion bodies were resuspended using first 1ml of PBS and then transferred into 9mL of PBS. Suspension was then sonicated during 10 minutes to disaggregate IBs. Previously prepared mixed SAM substrates were then immersed into the IB suspension and incubated in a plate shaker for three hours at room temperature. The resulting substrates were rinsed in ultrapure water before being observed under the characterization instrument. This ensures that weakly or non-bound IBs are washed out of the surface, proving strong IB adhesion to the mixed maleimide SAM.

4.6 Cyclic voltammetry

Cyclic voltammetry was used to prove the Michael-thiol addition reaction on a surface between maleimide SAMs and thiolated ferrocene. The electrochemical measurements using cyclic voltammetry (CV) were performed with SAMs immersed in a solution of THF:PBS, for which the pH was adjusted to 7.7 with 0.1M HCl and 0.1M NaOH. The gold substrate with the SAM was used as the working electrode, a Pt wire as a counter electrode and Ag/AgCl as the reference electrode. All solutions were purged with N_2 before their use.

4.7 Fabrication of microcontact printing PDMS stamp

For the fabrication of microcontact printing PDMS stamps, a specified 10:1 (w/w) ratio mixture of PDMS and curing agent (SYLGARD® 184 silicone elastomer kit) were mixed together and then exposed to the vacuum until air was completely removed. As the master we want to replicate contains µm-sized features and the polymer is highly viscous, removal of air bubbles with vacuum before curing of the polymer is necessary to ensure a complete filling. Mixture was then put in contact with the photolithographic patterned silicon master and it was cured for two days at 60ºC. Cured PDMS was finally peeled off the master.

4.8 Microcontact printing of IBs

IBs were stored at -80ºC until immediate use. Pellets were then brought to room temperature and they were resuspended using 1ml of PBS. The resulting dilution was transferred into 9ml PBS vial to obtain approximately 10ml of suspension, as described in Dr. Tatkiewicz thesis [22]. The suspension was sonicated for 10 minutes before PDMS stamp soaking.

Meanwhile 20µm stripe patterned PDMS stamps (Figure 10) were conditioned by sonication for 5 minutes in acetone, exposed to vacuum for 10 minutes, sonicated in a 10% solution sodium dodecyl sulfate (SDS) for 5 minutes conditioned in the same media for 5 minutes (detergent studies have shown that detergent-protein complexes detach from surfaces to a greater extent than proteins alone [22]), dried with a N_2 flow, dipped in MilliQ water to remove the excess of SDS and dried again with a N_2 stream.

Figure 10. Microscope picture of the PDMS microcontact printing stamp pattern used in this study.

The procedure was optimized for our SH-PEG-OH SAM after this point, using Dr. Tatkiewicz work as a reference. After conditioning, stamps were soaked in the IB PBS suspension for one hour. Stamps were then gently rinsed with Milli-Q water, dried under N_2 stream and placed on the SAM (section 4.4). A 10g weight was placed on the top of the stamp to provide repeatability and homogenous pressure. After one hour of contact, the stamp was carefully peeled off. Our printed wafer was then ready to be checked under fluorescence microscope for results. An overall flow chart of µCP sample preparation is represented in Figure 11.

Figure 11. Overall flow chart of the two-dimensional microscale structuration of IBs on surfaces. Each block represents an operation and arrows visualize the flow direction. Operations regarding PDMS stamp, IBs suspension, silicon substrate and μCP sample are marked in light blue, green, blue and purple, respectively [22].

4.9 Cell culture

Cell culture was performed using SH-SY5Y INc neuroblastoma cells in Servei de Cultius Cel·lulars, Producció d'Anticossos i Citometria (SCAC) facilities of the Institut de Biotecnologia i Biomedicina (UAB).

Subculture protocol was performed when culture reached 70% confluence. Media was aspired, 5ml DPBS was added and then aspired and 3ml of Trypsin were added (protease to detach cells from the flask). Flask was then incubated for 2 minutes at 37ºC and observed under microscope to check cell detachment. Trypsin was then neutralized using 6ml of Dulbecco's Modified Eagle Medium (DMEM) and centrifuged 5 minutes at 1400rpm. Supernatant was aspired and pellet in the tube was repeatedly hit to avoid cell aggregates.

Prior to the cell seeding substrates were gently rinsed with DPBS with BSA (3% w/2 in PBS) rinsed again with DPBS and introduced into the 24-wells plate, one substrate per well. SH-SY5Y INc were seeded at a density of 30.000 cells per well and incubated in 2ml DMEM supplemented with 2mM of L-glutamine and gentamicine (50μg/ml) at 37ºC in a 10% CO_2 humidified incubator for 22 hours. Additionally, medium was enriched with 10% of fetal bovine serum (FBS) for SH-SY5Y INc cell line.

4.10 Focal adhesion, nuclei and actin staining for confocal microscope analysis

After cell spreading the substrates were rinsed with warm DPBS, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and they were finally washed 2-3 times with DPBS. To achieve cell permeabilization, DPBS was removed and 3ml 0,1% Triton X were added on each sample and they were then washed three times with DPBS. It was then removed and 3ml of 1% BSA in PBS was added for 30 minutes in order to block cells.

100µl of primary antibody solution were prepared diluting paxillin antibody 1:400 in 1% BSA in PBS. Substrates were then incubated in a wet chamber for one hour at RT and they were later put in a 6 well plate with fresh PBS after incubation with the primary antibody. Samples were then washed with PBS on a shaker at 50rpm for 10 minutes. 100µl per sample of secondary antibodies were prepared diluting the antibody "mouse Alexa 488" 1:100, Hoechst 1:1000 and phallaoidin TRITC 1:50 in 1% BSA in PBS. Samples were then incubated in a wet chamber for 45 minutes at RT while covering with aluminum foil. After this time samples were washed in a 6 well plate previously filled with fresh PBS on shaker. Substrates were finally put in a new small petri dish and they were kept overnight in the fridge.

4.11 Instrumentation

AFM analysis was performed using 5500LS SPM AFM unit from Agilent Technologies. For determination of particle size distribution of different samples, we used a Zetasizer Nanoseries Nano-ZS (Malvern Instruments, U.K.). Substrates were observed under an Olympus BX51 microscope equipped with a CCD camera Olympus DP20. Fluorescence images were obtained with the help of Olympus U-LH100HG UV lamp and adequate filters. In order to obtain confocal images, samples were analyzed at spectral confocal microscope Leica TCS SP5 AOBS (Leica Microsystems, Mannheim, Germany). Contact angle measurements were carried out by Contact Angle Measuring System DSA 100 from KRÜSS.

5. RESULTS

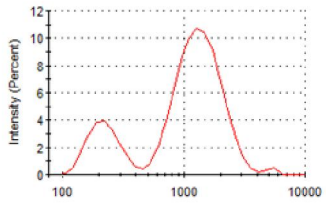
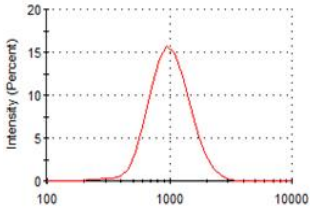
Substrates functionalized with SAMs, as well as IB incubated surfaces were characterized using multiple instruments in order to extract information about SAM composition and IB bonding. Each technique gave different data that could be used not only by itself but complementing other microscopy results to determine the IB characteristics, surface topography, composition and adherence to inclusion bodies.

5.1 Characterization of IBs produced in E. coli and L. lactis

5.1.1 Characterization of IBs using Dynamic light scattering analysis

E. coli and *L. lactis* IB size distribution and Z-Potential characterization of each kind of protein nanoparticles was carried out through Dynamic light scattering (DLS) as the quality of the inclusion body production can be estimated using this method. Samples were resuspended and sonicated for 10 minutes immediately before analysis at 25ºC and 188,3kcps count rate and 0,85mm measurement position.

Table 2. Dynamic light scattering results on size and Z-Potential, and particle size distribution graphs.

	Size average (nm)	Polydispersity index (PdI)	Z-Potential (mV)	Size distribution by intensity (nm)
E. coli	684,9	0,525	-10,86	
L. lactis	911,2	0,127	-11,36	

L. lactis IB suspension analysis results (Table 2) show that particle size average of the freshly prepared IBs is 911,2µm. One single peak in the size distribution by intensity chart demonstrates our suspension contains homogeously-sized IBs ranging around 1µm. Polydispersity index (PdI) is a dimensionless parameter that indicates the degree of homogeneity of the suspension and if the sample has a suitable size distribution. PdI=0,127 shows that size distribution is suitable for the analysis and it demonstrates that a proper purification process has been carried out.

On the other hand, *E. coli* IBs size average is wider due to the different-sized nanoparticles in the suspension as size distribution by intensity chart displays (228 and 1428 peaks with 20.9% and 78.1% of intensity respectively). PdI=0,525 indicates that size distribution is heterogeneous.

The higher the solubility of a protein is, the less prone to form protein nanostructures it is [15]. Z-potential measurements show that nanoparticles present negatively charged surfaces with negative values ranging from -10.86 to -11.36 mV (table 2), being an indication of unstable suspensions (unstable suspension Z values range from -30 to +30, all other positive or negative values indicate sample stability). These Z potential values indicate that *lactis* and *coli*-derived nanoparticles tend to aggregate and precipitate.

5.1.2 Atomic Force Microscopy

Atomic Force Microscopy in dynamic mode was used in this practice to analyze the surface structure of the maleimide-containing SAM substrates to check the monolayer topography before and after incubation with IBs. Each AFM picture result is composed of 3 outcomes: topography, phase and amplitude. Surface roughness, IB bonding and impurity adhesion to the surface are the main subject to be studied.

Bare Au surface results (Figure 12 A, B and C) show an homogeneous granulated surface, and roughness analysis measures topography to be in average 7.2nm. Such texture is representative of gold roughness and it is similar to other described gold-evaporated surfaces [23].

IB AFM results (Figure 12 D, E, F and G, H and I) show 1µm average-sized bulges bound on the monolayer (section 4.6), which match with *L. lactis* IB proportions (see section 5.1.1) and were not wash away during the rinsing procedure.

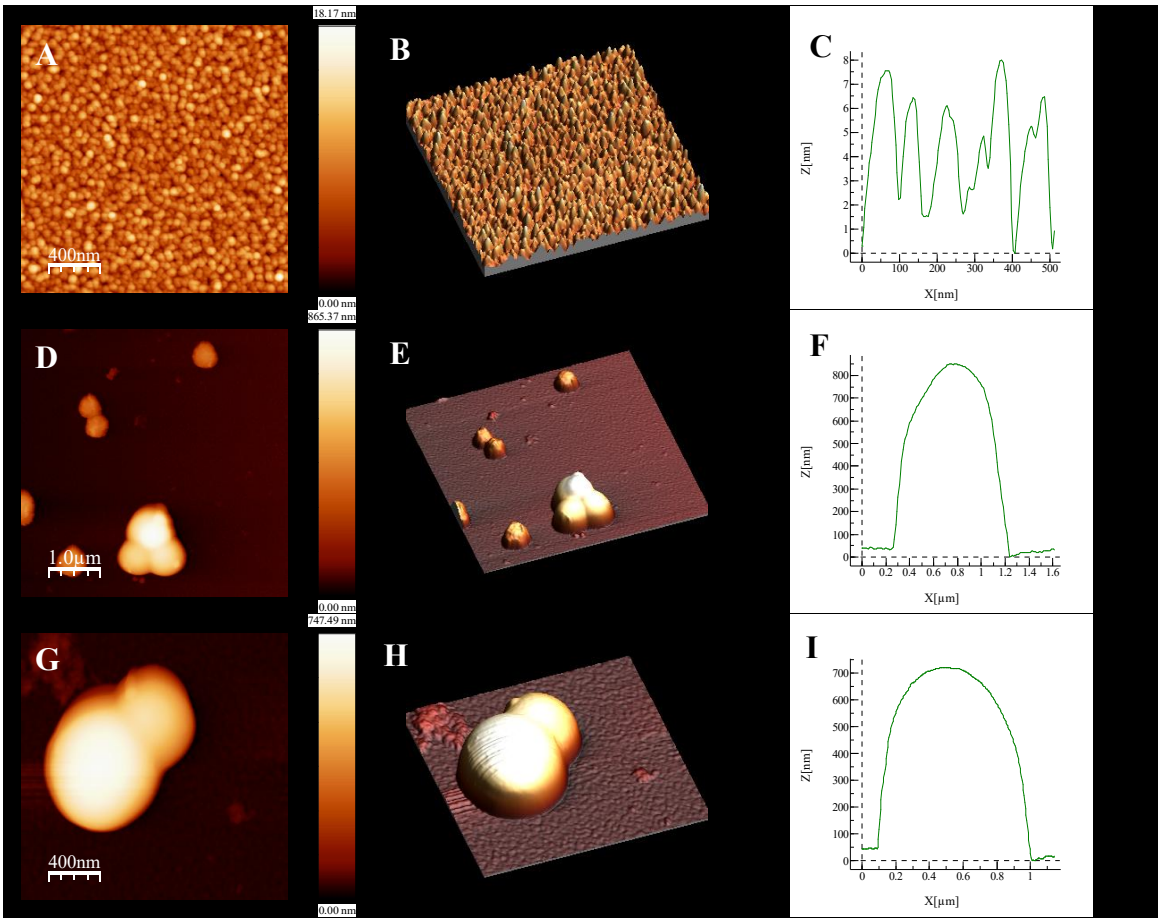


Figure 12. AFM results. Each row displays tree pictures: first one is a topography picture, second one is its 3D representation and last one shows the respective topography profile of each surface. First row displays bare gold surface results. Second row is a maleimide *L. lactis* IB-incubated substrate showing their adhesion to the SAM. Third row is a closer look to IBs.

5.2 Study of surface coverage of IBs on pre-modified surfaces with SAMs

5.2.1 Influence of IBs suspension media: PBS vs. Milli-Q

In order to check if salts on phosphate-buffered saline (PBS) were interacting with IBs and causing them to adhere nonspecifically to the SAM functionalized substrates, two GFP IB pellets were resuspended in 10ml of two different media; PBS and ultrapure water (Milli-Q). Both suspensions were then incubated for three hours at room temperature on SH-PEG-OH-functionalized substrates. Ultrapure water results showed no IB binding and PBS suspension exhibited the usual adhesion to the SAM.

To shed some light to this, PBS pH was raised from pH 6,7 to 7,59 adding H_2SO_4 until it reached ultrapure water's pH to discard this parameter's influence on IB binding. Results (Figure 13) show equal adhesion on both substrates proving pH does not have an important role on *L. lactis* IB binding to SAMs.

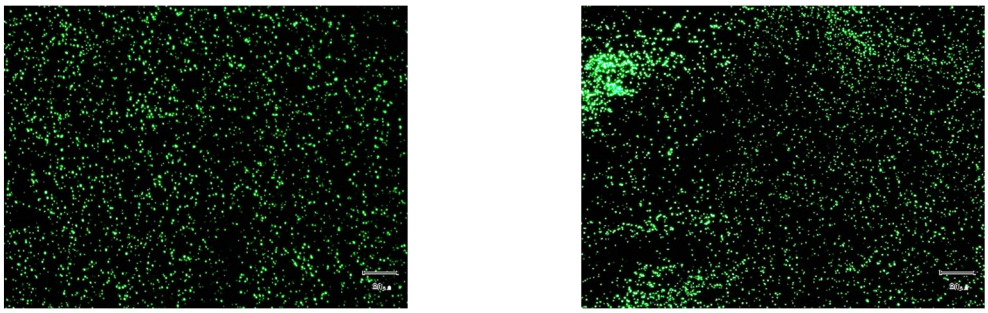


Figure 13. Fluorescence microscopy images of SH-PEG-OH SAM after IB incubation with PBS at pH=7,59. Left picture was taken before intense rinsing and right picture immediately after the rinsing. No fluorescence loss is detected.

5.2.2 IB specific interaction with maleimide SAMs

Fluorescence microscopy was used to analyze the interaction of IBs on 100% SH-PEG-OH and 100% maleimide-terminated SAMs. Specifically, *E. coli* and *L. lactis* IBs adhesion were studied separadetly on SH-PEG-OH and LA-PEG-MAL SAMs. Results (Figure 14) demonstrate no *E. coli* IB adhesion selectivity regardless the different SAM termination. However, *L. lactis* IBs show higher adherence on maleimide SAMs than on OH-terminated SAMs even after four rinsing steps.

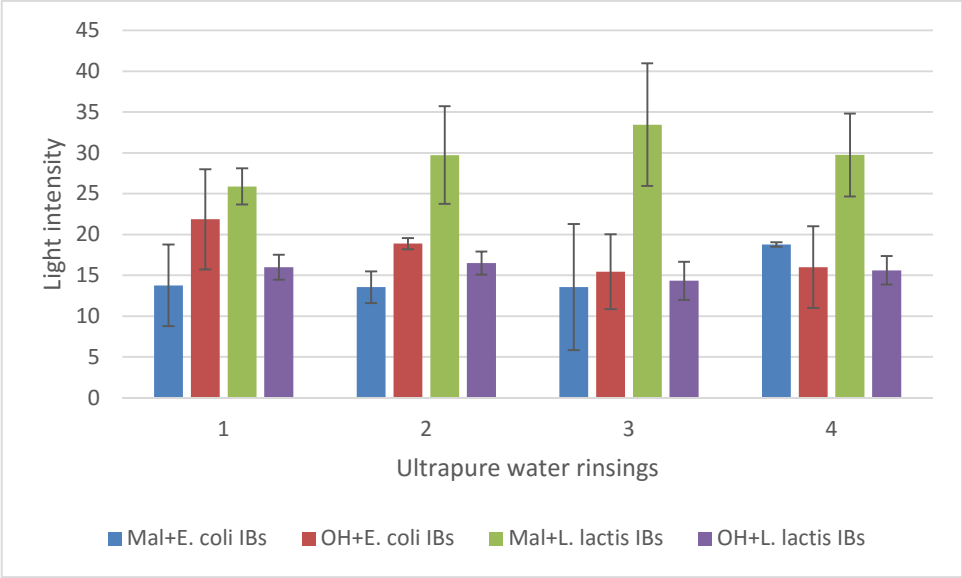


Figure 14. ImageJ luminescence study of the fluorescent light (proportional to IB concentration) emitted by *E. coli* and *L. lactis* IB after being adhered to maleimide and OH-terminated SAMs, after four rinsing procedures using Milli-Q water.

In order to quantify the IB adhesion of SAMs we have analyzed the luminescent images of the microscope and integrated the fluorescent light emitted from the IBs for each one of them. Even there is a higher adhesion of IBs from *L. lactis* on the maleimide SAM, Figure 14 indicates that IBs adhere quite well on both; maleimide and –OH SAMs, even we expected much higher adhesion on the maleimide than OH.

5.2.3 Contact angle analysis

Surface's hydrophobicity, which is characteristic of the most external groups of the SAMs, can be studied by performing a contact angle test. By comparing SAMs' hydrophobicity and considering IBs and terminal groups' polarity, the presence of SAMs and bionanoparticles can be characterized.

SH-PEG-OH and LA-PEG-MAL SAMs packing have been evaluated using contact angle test, showing different hydrophobicity characteristics depending on the different terminal group of the

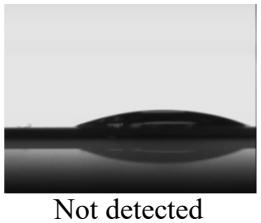
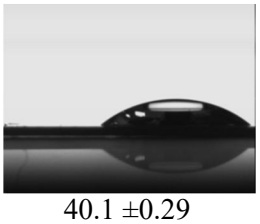
	SAM	SAM+IBs
LA-PEG-MAL		
Angle	44.1 ±1.11	60.5 ±0.16
SH-PEG-OH		
Angle	Not detected	40.1 ±0.29

Table 3. Contact angle values of a 5µl droplet. An increasing contact angle is detected when IBs are added in maleimide surface. The most notable hydrophobicity increase is detected at LA-PEG-MAL SAM surface, increasing 26.9% when IBs are added.

molecules at the outer surface. Results (Table 3) showed that the contact angle value is higher for the maleimide-functionalized surface compared to the SH-PEG-OH SAM. This is consistent with the fact that maleimide surface shows higher hydrophobicity than and hydroxyl-terminated surface which is capable of forming H-bonds with water, which was found to be very hydrophilic as expected. In fact, contact angle of SAMs of SH-PEG-OH couldn't be determined due to its low value. The value found for the maleimide SAM is 44º which matches perfectly the value previously described for this kind of SAMs [24]. The contact angle also increased when IBs are added to the functionalized surfaces, showing that hydrophobicity properties on the surface have incremented because the bioparticles adhere on the SAM. These results are coherent with literature, that states that GFP has an hydrophobic nature [25]. Thus, homogenous distribution and adhesion of IBs on both functionalized surfaces can be deducted.

5.2.4 Cyclic voltammetry analysis to study the reactivity of the maleimide SAMs

As we did not see a clear better attachment of IBs on the maleimide SAM, we decided to perform a test in order to make sure that the maleimide SAM was well formed. Thus, cyclic voltammetry (CV) provided us understanding regarding IB unspecific adhesion to maleimide and OH-terminated SAMs since thiolated ferrocene is expected to bind covalently to maleimide motifs on SAMs (Figure 15).

First we have performed a CV using a bear gold substrate as working electrode and unsubstituted ferrocene as electroactive species in the electrolyte solution. In this control experiment ferrocene peaks were detected at the expected potential (Figure 16.A), $E_{1/2}$= 0,44V and the difference between the anodic and cathodic peak is $\Delta E_{A/C}$= 100mV.

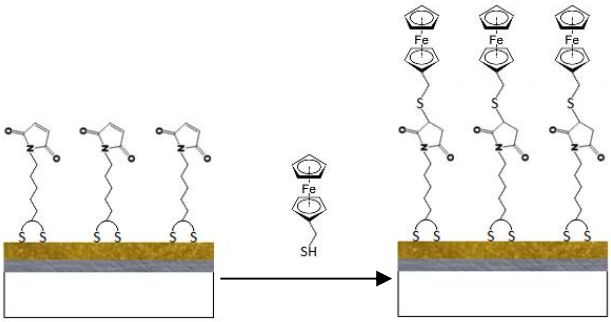


Figure 15. Thiol-ferrocene binding to maleimide SAM.

Then a maleimide and OH-terminated SAM (2:98) was treated with thiolated ferrocene and the resulting substrate was then used as working electrode for the CV (Figure 15). Results (Figure 16.B) clearly show the presence of an electroactive unit on the SAM substrate ($E_{1/2}$ = 0,49V) demonstrating ferrocene adhesion to maleimide-functionalized monolayer.

The fact that the anodic and cathodic peak are closer ($\Delta E_{A/C}$=20mV) than for the non binded ferrocene (Figure 16.A) is a clear indication of the non needed diffusion phenomena to get the molecules electroactive and thus, it is an indication of the covalent binding of the thiolated ferrocene on the maleimide surface.

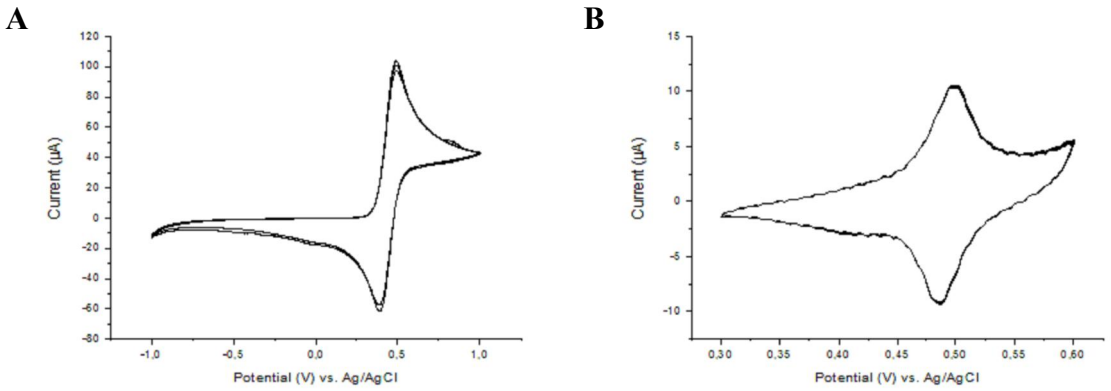


Figure 16. A: cyclic voltammetry of ferrocene in solution. B: maleimide-ferrocene SAM was used as the working electrode.

5.3 Micro structuration of IBs on SAMs: microcontact printing

As SH-PEG-OH unexpectedly showed fine IB binding outcomes (see section 5.2.3 figure 14), we performed microcontact printing of *E. coli* and *L. lactis* IBs on OH-terminated SAMs and proved its strong adhesion by rinsing intensely and repeatedly. Results (figure 17) demonstrate we achieved well-defined IB patterns whose fluorescent intensity did not decrease over time, proving a firm attachment to the surface. *L. lactis* IBs displayed poorer adhesion to substrate in all µCP trials compared to *E. coli* IBs.

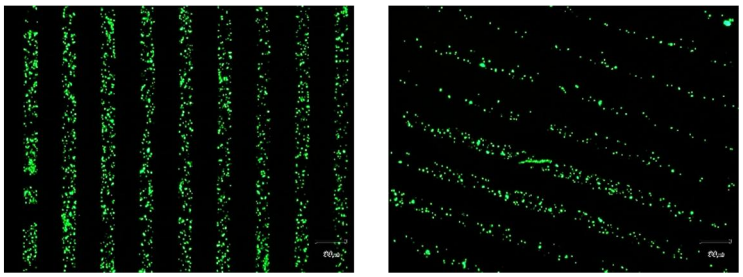


Figure 17. Representative fluorescent microscopy images of 20µm striped µCP patterns of GFP IBs on prefunctionalized surfaces. Left: *E. coli* IB patterns. Right: *L. lactis* IB stripes. *L. lactis* pattern displays poorer adhesion compared to the *E. coli* IBs substrate. Scale bar indicates 30µm.

5.4 Study of cellular focal adhesions on IB patterned surfaces

Having successfully achieved IB patterns on SH-PEG-OH SAMs, we proceeded to study the influence of IBs on cell growth through 20µm IB-printed patterns (Figure 17). Since cell-matrix junctions are strongly influenced by local ligand density [26], we examined focal adhesion formation[27]. SH-SY5Y INc neuroblastoma cells were attached to the surface and fixed and dyed after a 22-hour incubation. Cells were grown with a triple staining where paxillin (a focal adhesion-associated protein that recruits signaling molecules to focal adhesions [28]) was dyed in green, cell nuclei in blue and actin (cytoskeletal protein) in red (Figure 18).

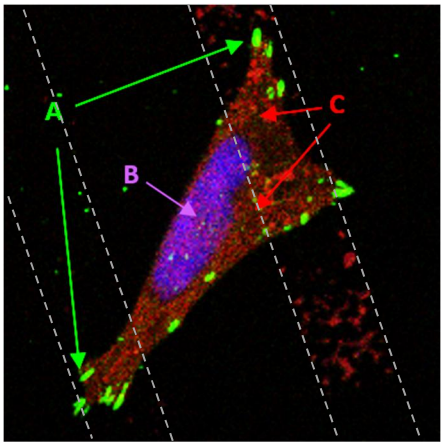


Figure 18. Example of a stained cell using (A) green dye for paxillin (focal adhesions), (B) blue dye for nuclei and (C) red dye for actin. IBs pattern area is marked with dot lines.

Cellular disposal on IB patterns (Figure 19) was observed and photographed using confocal microscopy. *E. coli* IB-patterned surface images show neuroblastoma cells' focal adhesions perfectly bind on IB stripes causing them to grow aligned or perpendicular (forming a bridge) to the lines. *L. lactis* results demonstrated a poorer IB adhesion to the substrate (section 5.3) and, therefore, a reduced cell adhesion to the poor-defined stripes compared to *E. coli* were obtained. However, cell guidance can still be observed: some cells' orientation align to IB stripes and some other develop focal adhesions on two consecutive lines, forming a cell bridge between stripes.

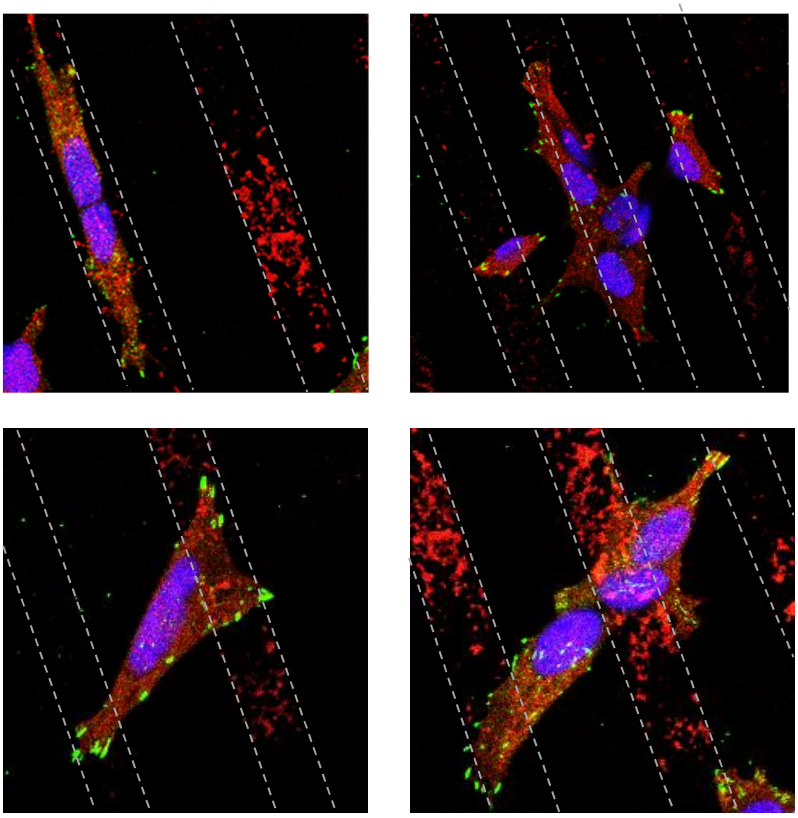


Figure 19. Confocal microscopy pictures of *E. coli* IB-patterned substrates where cells are stained as represented in Figure 18. Superior pictures show perfectly aligned cells to the IB pattern and inferior pictures show a cellular bridge between two IB stirpes. Cells of *L. lactis* patterned substrates are not shown. Pictures were taken at the Servei de Microscòpia of UAB. IB patterned area is marked with dot lines.

In order to compare the different tendency of forming focal adhesions in *E. coli* and *L. lactis,* we have counted the focal adhesions stablished per cell. Total count of focal adhesions was divided by the number of cells and then represented in Figure 20. Results suggest *L. lactis* IB substrate even the lower density of IBs on the surface present more beneficial effect on focal adhesion development since the average of focal adhesions per cell is higher.

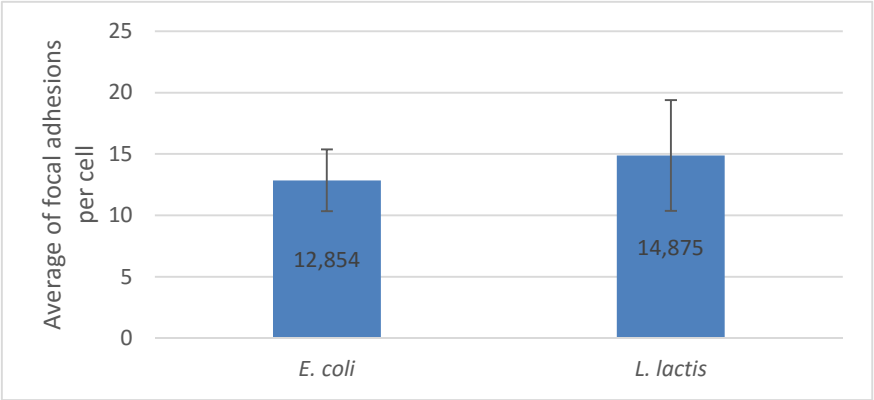


Figure 20. Average number of focal adhesions per cell in *E. coli* and *L. lactis* IB patterned substrates. The focal adhesions of 35 *E. coli* and 16 *L. lactis* cells were counted.

6. DISCUSSION

We successfully produced and purified *E. coli* and *L. lactis* GFP inclusion bodies and characterized their adhesion to SAMs using fluorescence microscopy, AFM and contact angle. 700nm to 1µm diameter protuberances are observed by AFM on maleimide IB-incubated SAMs which can be identified as inclusion bodies. DLS analysis results show that the *L. lactis* IB suspension has an homogeneous size dispersion and average particle size of 911.2µm, indicating that IBs are the adhered bioparticles to the maleimide-functionalized surface. IBs produced in *E. coli* show a more heterogeneous particle size dispersion than *L. lactis* IBs.

Moreover, both SH-PEG-OH and LA-PEG-MAL SAMs packing have been evaluated using contact angle test, showing different hydrophobicity characteristics depending on the different terminal group of the molecules at the outer surface. Results showed that the contact angle value is higher for the maleimide-functionalized surface compared to the SH-PEG-OH SAM. This is consistent with the fact that maleimide surface shows higher hydrophobicity than hydroxyl-terminated surface which is capable of forming H-bonds with water, which was found to be very hydrophilic as expected. Consequently, uniform SAM formation for both PEGilated molecules is deducted. Furthermore, the contact angle also increased when IBs are added to the functionalized surfaces, showing that hydrophobicity properties on the surface have increased because of the bioparticles adhered on the SAM. These results are coherent with literature, that states that GFP has an hydrophobic nature [25]. Thus, from these data, homogenous distribution and adhesion of IBs on pre-functionalized substrates can be deducted.

On the other hand, for SH-PEG-OH surface the same angle was expected for SAMs before and after incubation with IBs since these bioparticles were expected to show no adhesion to this SAM (Figure 9), but surprisingly, as optical fluorescent microscopy (Figure 13) suggest, IBs are capable of binding with OH-terminated surfaces via unidentified interactions. Such unidentified interaction can be due to the high adhesion properties of the IBs itself that adhere similarly to the OH-terminated SAM where they were not expected to adhere than on the maleimide SAM on which we expect to obtain an specific binding through the SH cysteine unit of the IBs (Figure 9).

Cyclic voltammetry confirmed the well formation and reactivity of the maleimide SAM, since a model thiolated ferrocene (instead of our thiolated IBs) was successfully bound on it confirming the expected thiol-maleimide covalent bond.

Due to the high adhesion of IBs on OH-terminated SAMs we decided to print IBs directly on them (which are easier to obtain and process than maleimide SAMs) using the µCP technique. The performance of µCP has been better for the *E. coli* than the *L. lactis* IBs as *E. coli* patterns display a better IB confluence. This might be due the resuspension heterogeneity of *E. coli* as can be seen in the DLS analysis (section 5.1.1). It is worth mentioning that IBs purified from *E. coli* microorganism can contain other components which enhance their binding properties as it is a gram-negative microorganism and thus has more membrane remains that might remain on the sample.

Stripes were posteriorly used as scaffolds for neuroblastoma cell attachment and growth as shown by confocal microscopy pictures (Figure 19). From these images it is clearly seen for the first time that focal adhesions attached specifically on IB patterns and consequently, cells aligned perfectly or formed bonds from one stripe to another. This observation is a clear indication of the mechanism of cell guidance of IB functionalized substrates. However, the *L. lactis* IB functionalized substrates, as mentioned before, displayed less IB confluence, and thus, less number of cells growing on them. Moreover, the staining protocol of focal adhesions, actin and nuclei for these substrates was not optimized. These facts make difficult to validate these experiments for the *L. lactis* samples. Even with this adversity, we have performed a preliminary counting of the number of focal adhesions per cell and found that IBs from *L. lactis* are promoting more focal adhesions per cell than *E. coli* IBs, which can be an indication of its better biocompatibility and interaction with the cell. In fact, as already stated in section 2.4.1, lactic acid bacteria (LAB) as production strain has been classified as a GRAS group of microorganisms and therefore, *L. lactis* are a safer alternative for pharmaceutical and tissue engineering applications.

With these interesting preliminary results, future work is clearly necessary to perform a further optimization of the IB patterning in order to develop a robust comparison between the cell guidance, proliferation and differentiation capacity of *E. coli* and *L. lactis* IBs quantifying focal adhesion points.

6.1 Ethics and sustainability

The studies of cellular interactions with the different systems depicted in the project were made using always cell lines from commercial collections (never primary cultures). For this, such laboratories have the corresponding permissions from the biosafety committees of their institutions which are available for checking. Specifically, we used human neuroblastoma cells obtained from commercial sources and therefore, there is no need to ask for approval to the Ethical Committee of Clinical Investigation.

The work developed in this project will provide elemental knowledge for the improvement in the field of tissue engineering and regenerative. Cell proliferation, guidance and morphogenesis is orchestred by multifaceted signaling pathways of the surrounding environment, which enable assembly of cells to form more complex structures. Elucidating developmental mechanisms like the ones studied in this work and appropriate cell assembly schemes could have immense savings and implications for engineered tissue, repairing ischemic wounds or bone defects [29]–[33]. Therefore, this work is essential to face limitations like implant rejection, cost and the inability to integrate with the surrounding host tissue. Consequently, the savings in the optimization of artificial implants that this project will provide in the future justify sustainability in this work. As a result, less resources will be misused on implants, and human organ demand will decrease since they will be created *de novo*.

7. CONCLUSION

We have successfully produced and purified *E. coli* and *L. lactis* IBs and used them as a new nanbiotechnology strategy to study the interaction with biocompatible SAMs mimicking the ECM. We have found that IBs have very high adherence properties and attach unexpectedly to all the prefunctionalized substrates (SAMs) used in this work. Covalent binding to the maleimide SAMs has been described for the pegilated-OH SAMs. Surface patterning with IBs using μCP was carried out on OH-terminated SAMs obtaining well-defined 20μm stripes with strong IB adhesion to the substrate. Finally, SH-SY5Y INc neuroblastoma cells were successfully cultured on IB patterned substrates for the first time. A focal adhesion study of cells was performed to determine that SH-SY5Y INc cells align and preferentially attach on IB-patterned stripes and that cells growing on *L. lactis* IB patterned substrates tend to develop more focal adhesions than cells on *E. coli* IB-functionalized substrates. Further work must be executed in order to optimize culture parameters such as IB pattern width and concentration to improve cell attachment and alignation to IB stripes on the substrates. With this work we have given a step forward towards the development of a new generation of innovative biotechnologically engineered biomaterials capable of bone healing. It has been proved that IBs are advantageous nanomaterials for the control of cell culture through the formation of focal adhesions to regulate cell behavior and guidance as well as promising biomaterials for tissue engineering and regenerative medicine.

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