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Priming cells for their final destination: microenvironment controlled cell culture by a modular ECM-mimicking feeder film†

Julien Barthes,‡[a,b] Nihal E. Vrana,‡[a,c] Hayriye Özçelik,[a,b] Rabah Gahoual,[d] Yannis N. François,[d] Jalal Bacharouche,[e] Grégory Francius,[e] Joseph Hemmerlé,[a,b] Marie-Hélène Metz-Boutigue,[a,b] Pierre Schaaf[a,b,f] and Philippe Lavalle*[a,b]

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Mammalian cell culture is the starting point in many research studies focusing on biomedical applications. However, researchers have little control over the standardized cell microenvironment parameters. Here a modular ECM-mimicking surface coating for cell culture environment is designed. This substrate is a new and versatile thin film obtained by spin-coating of concentrated gelatin crosslinked by transglutaminase. It can be modified with respect to the biochemical and biophysical needs of the final cell destination, *i.e.* it delivers loaded multi-growth factors and serum components and allows for cell culture in a serum-free culture medium. Also, a well-known cell behavior modulator, the substrate stiffness, is controlled exogenously by addition of nanoparticles. In addition to growth factors, antimicrobial agents such as natural peptides are added to the substrate for limiting the repeated addition of antimicrobial agents to the culture medium and to prevent the increase of resistant bacterial strains in the culture environment. Finally, this substrate contains simultaneously ECM components, growth factors, stiffening elements and antimicrobial agents. It provides a favorable microenvironment and sterile conditions. It is a free-of-maintenance system, as cells will grow without addition of serum or antimicrobial cocktails. This low cost and easy-to-use substrate could emerge as a new standard for cell culture.

1. Introduction

The human body is composed of over 200 different types of differentiated cells spanning a wide range of functions and phenotypes. Since the early days of *in vitro* cell culturing, having a microenvironment that would enable the maintenance of different cell types has been a primary goal. Moreover, future increasing demands in regenerative medicine and drug screening need improvement in culture substrates with well established conditions and with a reasonable production cost.[1]

To this end, three kinds of specific conditions are necessary: (i) a favorable biochemical microenvironment, (ii) an appropriate biophysical microenvironment, and (iii) the sterility of the microenvironment. Aside from having a medium with the right amount of nutrients, pH and essential biomolecules, it is important to provide specific growth factors and cytokines for a given cell type. Recent years have also showed a tremendous increase in studies demonstrating the crucial effects of the biophysical properties of the culture substrate on cell behavior.[2–4] Also, in areas where long-term cultures are necessary such as in regenerative medicine, there is an increased risk of bacterial and fungal contaminations.

[a] *Institut National de la Santé et de la Recherche Médicale, INSERM Unité 1121, Biomaterials and Bioengineering, 11 rue Humann, 67085 Strasbourg Cedex, France. E-mail: philippe.lavalle@inserm.fr*
[b] *Faculté de Chirurgie Dentaire, Université de Strasbourg, 8 rue Sainte Elisabeth, 67000 Strasbourg, France*
[c] *Protip SAS, 8 Place de l'Hôpital, 67000 Strasbourg, France*
[d] *Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes, CNRS UMR 7140, Université de Strasbourg, 1 rue Blaise Pascal, 67008 Strasbourg Cedex, France*
[e] *Laboratoire de Chimie Physique et Microbiologie pour l'Environnement CNRS UMR7564, 405 rue de Vandoeuvre, 54600 Villers-les-Nancy, France*
[f] *Institut Charles Sadron CNRS-UPR22, 23 rue du Loess, 67085 Strasbourg Cedex, France*

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‡ These authors contributed equally.

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There are some inherent problems with the current method of maintaining cells. First, whether it is a serum or a defined mixture of growth factors, such additives are very expensive and can be considered the biggest money sink in cell culture experiments. Moreover, these molecules are fragile chemicals with very short half-lives particularly in the presence of cells, thus their long-term activity in an aqueous environment cannot be ensured. Frequent renewal of the medium is thus necessary and, depending on the frequency, the impact on cell adhesion, proliferation, differentiation, can induce heterogeneities between experiments. Also, as their half-lives and rates of degradation are different, it cannot be said with certainty that at a given time during a cell culture experiment, the constituents of the medium are in the same ratio as in the beginning.[5] For these reasons, introduction of the necessary growth factors through the liquid medium is a simple but imprecise and inefficient method.

In the human body, ECM surrounds cells and most of the growth factors are trapped and associated with proteoglycans from ECM. For example, heparan sulfate, a proteoglycan from ECM, is known to interact with growth factors like VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), and BMP (bone morphogenic protein).[6,7] These interactions have three main consequences: (i) they prolong the half-life of biomolecules by protecting them against degradation; (ii) they control their release and in turn their concentration in the microenvironment; and (iii) they synchronize the cellular response as tethering of the growth factors on ECM directs the respective location of focal adhesion points and expression of membrane receptors for the growth factors.[8] Thus, this downstream simultaneous processing of signals coming from ECM and growth factors has implications on cell behaviour. Most of the growth factors such as FGF, VEGF, PDGF (platelet-derived growth factor), and EGF (epidermal growth factor) are known to interact with extracellular matrix molecules such as collagen, fibronectin, hyaluronic acid, heparin, *etc*. If this environment and this interaction can be reproduced *in vitro*, the quality of cell maintenance and expansion would be increased, while a substantial decrease in the amount of required growth factors in cell medium can be attained. This will mimic the *in vivo* behaviour and moreover it will reduce the overall cost of cell culturing.

The other crucial aspect of cell culture with mammalian cells is the culture substrate.[2] Materials play a key role in cell behaviour and many studies have been devoted to the design of new surface coatings to confer specific properties to surfaces. For example, by playing on surface architecture and composition, self-cleaning and antimicrobial surfaces can be obtained.[9] Two-dimensional nanostructures with a precise control of composition, organisation and thickness can be achieved by layer-by-layer assembly. This process constitutes a versatile and flexible technology useful to functionalize surfaces and is more specifically of great interest for biomedical applications.[10] However although they constitute a substrate of great interest, most of the time these coatings range in thicknesses from tens of nanometers to few micrometers which can be a limitation in applications such as cell culture substrates for long-term studies. Higher thicknesses can be obtained but this needs an increase in the number of deposited layers and thus it becomes a time-consuming process.

In most of the conventional cell culture protocols, gamma irradiated and plasma treated stiff polystyrene surfaces are used. One downside of these synthetic substrates is the absence of any biochemical factors on their surface and their total dependence on passive adsorption of these molecules from the cell culture medium. Moreover, such standardized substrates generally ignore the substantial difference between the natural microenvironments of the cells from different tissues from brain to bone.[11] Substrate stiffness has been shown to have as strong an effect as soluble growth factors, particularly for the direction of stem cell differentiation.[12] Thus having extra control over substrate stiffness during the expansion phase of cell culture would be advantageous.[13]

Matrigel®, a trade name of a protein mixture originating from Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, is often used by biologists as a culturing substrate for a very specific cell culture and with a complete serum in the supernatant.[14] Nonetheless, some drawbacks limit the utilization of Matrigel®, the most serious of which include (i) difficulty of use, (ii) lack of experimental control of composition, (iii) batch-to-batch variability, and (iv) murine origin.

To address these issues we present here a new type of films acting as cell substrates. They are designed with ECM components stabilized with an enzymatic crosslinking step in a dual role of microcarriers for growth factors and substrates with exogenous stiffness control for cell culture. As ECM molecules are natural substrates of the cells, these substrates will be advantageously compared to tissue culture polystyrene dishes which need to be further coated by synthetic poly(lysine) or uncrosslinked ECM components (where the stability of the coating under cell culture conditions is not certain) for several cell types.

Introduction of growth factors through the coating and control of the substrate stiffness by addition of nanoparticles will improve the influence over the cells *in vitro*. Moreover, such a system can be frozen, stored and transferred in a frozen state. The ECM-based substrate designed will ensure the stability of the growth factors for longer periods and also the reproducibility of the cellular microenvironment by keeping the growth factors in an effective and in a non-denatured conformation. The ability of the coating to load and release antimicrobial agents will also be shown. This "gel-feeder" system acts as an "all-in-one" platform containing ECM components, growth factors, stiffening elements and antimicrobial agents, which lead to an optimization of biochemical and biophysical parameters for cell culture (Scheme 1).

This manuscript is innovative in several respects: it describes the design of a gelatin layer (i) using a spin coating method for production of a homogeneous ECM like film; (ii) loaded with a complete serum to contain a cocktail of growth factors; (iii) containing nanoparticles to increase mechanical properties; (iv) functionalized with antimicrobial

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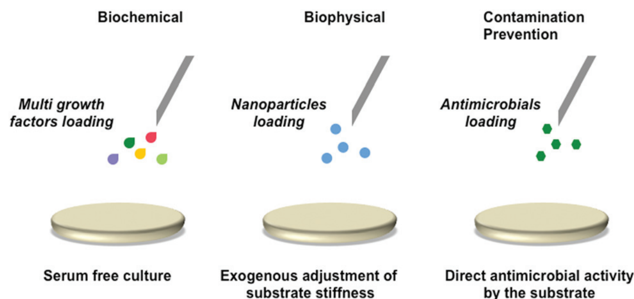

Scheme 1 Concept of the ECM-based substrate "gel-feeder": micro-environment-controlled cell culture *via* a modular cell feeder layer.

peptides; and (v) able to be stored with embedded biological agents for several weeks in a frozen state and remaining active after thawing.

2. Experimental

2.1. Materials

Gelatin type B (M_w = 2–2.5 × 10^4 Da, pI = 4.7–5.2) from bovine skin, fluorescein isothiocyanate labeled Bovine Serum Albumin (BSA^{FITC}, M_w = 6.6 × 10^4 Da, pI = 4.7–4.9) were purchased from Sigma Aldrich (France). Fluorescein-5-isothiocyanate (FITC) and Rhodamine Red-X succinimidyl ester were purchased from Invitrogen (France). Microbial transglutaminase was kindly provided by Ajinomoto (Japan). Polystyrene nanoparticles 100 nm in diameter (Polysphere) and green labeled (Fluorophorex) were purchased from Phosphorex (USA). Recombinant human VEGF (recombinant Human $VEGF_{165}$, M_w = 3.82 × 10^4 Da, pI = 8.5) was purchased from Promocell (Germany). Fluorescein-5-isothiocyanate (FITC) and Rhodamine Red-X succinimidyl ester (Invitrogen, France) were covalently bound to VEGF as described elsewhere.[15]

2.2. Film preparation and characterization

To build up gelatin films, a spin-coater WS-650Mz-23NPP from Laurell (USA) was used. Gelatin was prepared by dissolution of an adequate amount of gelatin powder (15% w/v) in a 0.15 M NaCl/10 mM Tris (tris(hydroxymethyl)aminomethane) solution (pH = 7.4). The solution was heated to 50 °C with constant stirring. Then gelatin solution (200 μL) was deposited on a glass slide previously installed in the spin-coater and the spin-coating program was started. The parameters were 2500 rpm with an acceleration of 1250 rpm for 2 min. Then the film was kept dry at 4 °C for at least 3 h before use. All solutions were prepared using ultrapure water (Milli Q-plus system, Millipore) with a resistivity of 18.2 MΩ cm. For crosslinking of the gelatin films, a solution of transglutaminase (10% w/v) in PBS (phosphate buffer saline) was incubated (100 μL) on a gelatin film for 30 min. Then two rinsing steps with PBS (5 min, 100 μL) were performed.

For release experiments of single proteins BSA^{FITC} solution (1 mg mL^{-1}, 200 μL) or $VEGF^{FITC}$ solution (236 μg mL^{-1}, 50 μL) both prepared in 0.15 M NaCl/10 mM Tris solutions (pH = 7.4) were loaded for 30 min in a gelatin film after the crosslinking step. Then two rinsing steps with PBS (5 min, 100 μL) were performed. For release experiments of multiple proteins, "Endothelial Cell Growth Medium Supplement Mix C-39215" (Promocell, France) was loaded in a gelatin film after the crosslinking step. This mixture (100 μL) was incubated for 30 min and then two rinsing steps with PBS (5 min, 100 μL) were performed.

Before crosslinking with transglutaminase, particle solutions (100 μL) diluted in PBS (initial solutions at a concentration of 1.82 × 10^{13} particles per mL diluted at 1/50 or 1/500 or 1/1000) were incubated on the gelatin film for 30 min. Then two rinsing steps with PBS (5 min, 100 μL) were performed. The distribution of the nanoparticles in the gelatin film was checked by using an Environmental Scanning Electron Microscope (ESEM (Quanta, FEI)) and by confocal laser scanning microscopy. Confocal laser scanning microscopy (CLSM) observations were carried out with a Zeiss LSM 510 microscope using a 40× (Zeiss Achroplan) objective and with 0.4 μm *z*-section intervals. FITC fluorescence was detected after excitation at λ = 488 nm with a cut-off dichroic mirror of 488 nm and an emission band-pass filter of 505–530 nm (green emission). Rhodamine fluorescence was detected after excitation at λ = 543 nm, dichroic mirror of 543 nm, and an emission long pass filter of 585 nm (red emission).

2.3. Release experiments using spectrofluorimeter and CLSM

Release experiments of single fluorescently labeled proteins were carried out with a SAFAS Genius XC spectrofluorimeter (Monaco). A gelatin film was previously crosslinked with transglutaminase solution and incubated with a BSA^{FITC} solution or with $VEGF^{FITC}$. The release experiments were performed at 37 °C in a PBS solution. The supernatant was analyzed with a spectrofluorimeter. A new PBS solution (2 mL) was added after each analysis. For BSA^{FITC} and $VEGF^{FITC}$ the wavelength parameters were respectively $\lambda_{ex}/\lambda_{em}$ = 495 nm/520 nm and $\lambda_{ex}/\lambda_{em}$ = 488 nm/520 nm.

Another release experiment was performed with a cross-linked gelatin film loaded with BSA^{FITC} and seeded with labeled HUVEC cells (red labeling PKH26 $\lambda_{ex}/\lambda_{em}$ = 551 nm/567 nm). Endothelial cell growth medium (2 mL, Promocell) was added and the experiment was carried out for 1 day at 37 °C. The internalization of BSA^{FITC} was visualized using the confocal microscope. The same experiment was performed with $VEGF^{Rho}$ but this time HUVEC cells were labeled with Calcein (green dye). This experiment was performed for 1 day at 37 °C.

2.4. Determination of Young modulus with atomic force microscopy (AFM) nanoindentation

To study the influence on mechanical properties of polystyrene nanoparticles in a gelatin film, AFM nanoindentation experiments were performed to determine the Young modulus. AFM experiments were carried out using a MFP3D-BIO instrument

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(Asylum Research Technology, Germany). The nanoindentation method provides the Young modulus calculated from the force *vs.* indentation curves (FVI). Triangular cantilevers with a colloidal probe (SiO_2 particle with a radius of 300 nm) with a spring constant 10 pN nm^{-1} were purchased from Novascan Technologies (USA). Maps of mechanical properties were obtained by recording a grid map of 32-by-32 force curves at different locations of the film surface. Elasticity maps and the corresponding histograms (statistic distribution) were estimated from the analysis of the approach curves according to the Dimitriadis model.[16] All the FVI were analyzed by means of an automated, home-made Matlab code as described elsewhere.[17]

2.5. Degradation of gelatin films and gelatin quantification

Degradation of gelatin films was observed at 37 °C for 3 days by putting films in PBS (2 mL). These films were analyzed using the "Sirius Red/Fast Green Collagen Staining Kit" provided by Chondrex (USA) to determine the amounts of collagen and non-collagenous proteins in the sample. Sirius Red (OD: seeding cells on 540 nm) specifically binds collagens or derived collagen structures such as gelatin and is used for detecting all types and species of collagen, whereas Fast Green (OD: 605 nm) binds to non-collagenous proteins. To perform the analysis, a dye solution (0.2 mL) was added to the film and incubated at room temperature for 30 min. Then the dye solution was removed and the film was rinsed with water. Finally, dye extraction solution (1 mL) was added to the film to extract the labeled gelatin from the film. Then, the OD was read at 540 nm and 605 nm and the following equation was used as described in the protocol given by the kit: Gelatin (μg per sample) = $(OD_{540} - (OD_{605} \times 0.291))/0.0378$. These results were compared to those obtained with a non-degraded dry gelatin film.

2.6. Characterization of serum release using CE-MS/MS (capillary electrophoresis-mass spectrometry)

2.6.1. Bottom-up proteomic sample preparation. For this experiment, the complex mixture (100 μL) used for HUVEC cell culture (Endothelial Cell Growth Medium Supplement Mix C-39215, Promocell, France) was incubated on a gelatin film for 30 min and then two rinsing steps with PBS (5 min, 100 μL) were performed. Then the release of this mixture composed of many proteins was studied using the capillary electrophoresis-mass spectrometry coupling technique.

Samples were collected each day during the release experiment for bottom-up proteomic analysis. A volume of 700 μL of medium was treated. Prior to analysis the sample underwent tryptic digestion (1 : 20 w/w approx.). Before digestion, the sample was concentrated in a miVac DNA concentrator speed vacuum system (Genevac, UK). Afterwards the sample was diluted in a volume of 65 μL of ammonium bicarbonate (50 mM, pH = 8.0) and then heated to 40 °C to ensure protein solubilization. For protein denaturation and disulfide bond cleavage a volume of 3.9 μL of a solution of 100 mM dithiothreitol (DTT) was added to the sample for 5 min at 95 °C. The mixture was cooled to ambient temperature and a solution of iodoacetamide (IAM) 100 mM was added to a final concentration of 10 mM. The mixture was placed in the dark for 20 min. A volume of 0.5 μL of trypsin was added to the mixture at room temperature for 3 h. Again 0.5 μL of trypsin was added to the sample. Salts were eliminated using C8 spin tips (Proteabio, USA). The sample was finally concentrated using the speed vacuum system and diluted in 40 μL of ammonium acetate (50 mM, pH = 4.0) in order to allow isotachophoresis to occur during the separation.

2.6.2. Capillary electrophoresis-tandem mass spectrometry analysis. Capillary electrophoresis (CE) experiments were carried out with a PA 800 plus CE system from Beckman Coulter (Brea, CA, USA). Hyphenation was realized using a CESI prototype made available by Beckman Coulter. Prototypes of bare fused-silica capillaries (total length 100 cm; 30 μm inner diameter (i.d.)) were used and a second capillary (total length 80 cm; 50 μm i.d.) filled during experiments with background electrolyte (BGE) allowed electric contact. New capillaries were flushed for 10 min at 75 psi (5.17 bar) with methanol, then 10 min with 0.1 M sodium hydroxide, followed by 10 min with 0.1 M hydrochloric acid and water for 20 min also at 75 psi. Finally the capillary was flushed for 10 min at 75 psi with BGE (acetic acid 10%). Hydrodynamic injection was used (0.41 mbar for 1 min) corresponding to a total volume of 100 nL of the sample injected. Separations were performed using a voltage of +20 kV.

The CESI system was coupled to a microTOF-Q II mass spectrometer (Bruker Daltonics, Germany) equipped with a hybrid analyzer composed of a quadrupole followed by a time of flight (TOF) analyzer. Positive mode acquisition was used to detect precursor ions (MS) and fragmented product ions (MS/MS). Concerning the ESI source parameters, the capillary voltage was set to −1.3 kV. Nebulizer gas was deactivated, the dry gas was set to 1.5 L min^{-1} and temperature of the source was set at 180 °C. Spectra were collected at a data acquisition frequency of 2 Hz; for fragmentation spectra, collision energy ranged from 0 to 45 V depending on the m/z ratio and charge state of the precursor ion. For each MS scan, 3 precursor ions were selected for fragmentation, and the total duty cycle was therefore 2 s. Mass range was 100–3000 for MS as well as MS/MS scans.

Data obtained from CESI-MS/MS experiments were processed using the Mascot search algorithm developed by Matrix Science. Tryptic cleavage rules were applied and MS/MS spectra were compared to Swiss-Prot protein database (mammalian species). Carbamidomethylation of cysteine (+57.02 Da) was selected as a fixed modification, N-deamidation of aspartic/isoaspartic acid (+0.985 Da) or succinimide intermediate (−17.03 Da) was selected as a variable modification. Methionine oxidation (+15.99 Da) and N-terminal glutamic acid cyclization (−17.02 Da) were also selected as variable modifications. The mass tolerance for precursor ions was set to ±25 ppm and to ±0.5 Da for fragments. A maximum of 3 missed cleavages was tolerated.

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2.7. Cell culture

For cell experiments, the main work was performed with HUVECs (Human Umbilical Vein Endothelial cells, Promocell). These primary cells were used at passages between 4 and 6. Two other cell types were used: 3T3 (mouse fibroblasts, ATCC) and HGF (human gingival fibroblasts). The culture media used were endothelial cell growth medium (Promocell) for HUVECs, DMEM High Glucose (PAA) for 3T3, and DEM low glucose (GibcoBRL, USA) for HGF. The supplement used for these cells were Supplement Mix C-39215 (mainly composed of heparin, hydrocortisone, fetal calf serum, basic fibroblast growth factor, epithelial growth factor and endothelial cell growth supplement) for HUVECs and fetal bovine serum (FBS) for 3T3 and HGF. For each experiment with HUVECs and 3T3, gelatin films were first crosslinked with a transglutaminase solution and then the supplement (100 μL) was incubated for 30 min and finally the films were UV-treated for 15 min. For frozen samples, after crosslinking with transglutaminase and incubation with the serum, the films were dried for 2 h under vacuum and then put at −20 °C for at least one week before seeding them with HUVECs.

For all cell experiments, the same protocol of seeding on gelatin films was used. Cells, in a 75 cm^3 flask, were first cultivated to a near-confluent state and then they were trypsinized (4 mL of Trypsin and 5 min at 37 °C). After that, culture medium was added (6 mL) to stop the reaction of trypsin and the cells were centrifuged (5 min at 1200 rpm). Then, cells were concentrated in the culture medium (1 mL) and they were counted with a Neubauer chamber. For each experiment 50 000 cells were deposited on top of gelatin films in a 24-well plate and the system was first put for 15 min at 37 °C for adhesion and after that starvation medium (500 mL, without supplement) was added. The plate was then put in the incubator at 37 °C.

To check the metabolic activity of cells, in vitro Toxicology Assay Kit, a resazurin based test (Sigma Aldrich) was used. This test is based on the reduction of resazurin dye which will become fluorescent (red) when incubated with viable cells. The protocol is the following: a solution of resazurin (10% v/v) in culture medium was deposited (500 μL) in each well plate and maintained for 2 h in the incubator. Then the solution was read with the spectrofluorimeter ($\lambda_{ex}/\lambda_{em}$ = 560 nm/590 nm).

To check the viability of cells, Apoptotic/Necrotic/Healthy Cells Detection Kit (Promokine) was used to quantify apoptotic (green fluorescence for FITC-Annexin), necrotic (red fluorescence for Ethidium homodimer III) and healthy cells (blue fluorescence for Hoechst) with a fluorescent microscope. Films with cells were rinsed several times with PBS. Then 50 μL of staining solution (5 μL of each dye for 100 μL of binding buffer provided by the kit) was incubated for 15 min at room temperature and after several rinsing steps the films were visualized with a fluorescent microscope (Nikon Eclipse TE 200, Japan).

For immunofluorescent staining, cells were first fixed with a 3.7% (v/v) solution of paraformaldehyde (PFA) in PBS. Then, cells were incubated for 15 min with a Triton-X solution (0.1% v/v in PBS). Finally cells were incubated overnight at 4 °C with FBS (fetal bovine serum) solution (10% v/v in PBS). PECAM staining (green) was used to visualize the contact point between cells, phalloidin (red) for actin filament and Hoechst 58 (blue) for the nucleus. First, PECAM-1 goat antibody (90 μL, 4 μg mL^{-1} in PBS, Santa Cruz) was incubated on each sample for 90 min. Then, a secondary antibody (300 μL, 4 μg mL^{-1} in PBS, anti-goat@donkey FITC) was incubated for 30 min. Then, phalloidin solution (300 μL, 5 μg mL^{-1} in PBS, Sigma) was incubated for 15 min. Finally, a solution of Hoechst 58 (300 μL, 20 μg mL^{-1}, Sigma) was incubated for 5 min.

To study the effect of substrate rigidity on cell spreading, gelatin films of different rigidities were prepared as described previously with different dilutions of loaded nanoparticles. We used HGF (Human Gingival Fibroblast) cells seeded on the films for 24 h at 50 000 cells per film in a normal medium (culture medium +10% (v/v) FBS). Then, phalloidin staining was performed and cell areas were measured for all conditions with ImageJ software.[18]

2.8. Antibacterial assay

S. aureus (reference 25923, ATCC, France) strains were used to assess the antibacterial properties of the gelatin films loaded with a penicillin/streptomycin mixture or with the antimicrobial peptide, catestatin (in-house synthesis). The bacterial strain was precultured aerobically at 37 °C in a Mueller Hinton Broth (MHB) medium (Merck, Germany). One colony was transferred from the MHB medium (10 mL) and incubated at 37 °C for 18–20 h to provide a final density of 10^6 CFU mL^{-1} (colony-forming unit). To obtain bacteria in the mid-logarithmic phase of growth, OD at 620 nm of overnight culture was adjusted to 0.001 by diluting an overnight culture of bacteria in MHB. To assess the short (1 day) and mid-term (2 days) antimicrobial effects of the penicillin/streptomycin mixture (100 U mL^{-1}) and catestatin (100 μM), each agent was adsorbed for 1 h onto the gelatin films and the mid-logarithmic phase culture of bacteria (250 μL) was incubated. Tetracycline (10 μg mL^{-1}) and cefotaxime (0.1 μg mL^{-1}) were used as positive controls. Antibacterial activity was assessed by measuring absorbance at 620 nm after their respective time period at 37 °C.

3. Results and discussion

3.1. Build up of the spin-coated film

Gelatin type B, a well-known biocompatible material derived from collagen, was used as the main component to build up the film. A gelatin solution heated to 50 °C was deposited on a glass substrate and was spin-coated for 2 min at ambient temperature. The spin-coating method applied to build up gelatin films is an original method, which can be easily upscaled, and has never been used previously for this purpose to our knowledge. The cooling and spinning of the gelatin produce a homogeneous film on the surface. Then, the film

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was crosslinked by a natural enzyme, transglutaminase, to improve its mechanical properties.

Other chemical crosslinking methods such as EDC/NHS or glutaraldehyde exist, however a more natural crosslinking would ensure a better microenvironment for the cells. Transglutaminases are widely available proteins in mammalian tissues for stabilisation of the tissue structure.[19] However, obtaining mammalian transglutaminases at industrial levels is challenging. Microbial transglutaminase works nearly as effectively as mammalian transglutaminase in crosslinking of gelatin molecules *via* the formation of a covalent bond between ε-amino groups from lysine residues and γ-carboxamide groups of glutamine of gelatin molecules.[20] The advantage of using microbial transglutaminase is its Ca^{2+} independency which makes it compatible for use with cell culture media.[21]

A first characterization of the crosslinking of the film was performed with FT-IR (Fig. S1 in the ESI†). We observed an increase in the amplitude of the amide I peak around 1631 cm^{-1} in the case of gelatin crosslinked with transglutaminase. These results are in agreement with the literature and enable us to confirm the crosslinking of gelatin films treated with transglutaminase.[22,23]

Then, characterization of the homogeneity of the crosslinked film was performed by confocal microscopy (CLSM). Before imaging, labeling of the whole film was obtained through 15 min incubation of the film in a poly(L-lysine)^{FITC} (green fluorescent dye FITC, fluorescein isothiocyanate) solution. The 3D image depicts a homogeneous 5 μm thick film (Fig. 1a). The presence of the homogeneous film was confirmed by scratching the film with a needle (Fig. S2 in the ESI†). The remarkable homogeneity of the top surface and thickness of the film were also confirmed by ESEM (Environmental Scanning Electron Microscopy) (Fig. 1b).

By crosslinking the spin-coated gelatin films enzymatically their stability was increased up to 3 days as quantified by spectrofluorimetry through measurement of the total amount of gelatin left after incubation in PBS at 37 °C (Fig. 2a and b). Also crosslinking resulted in a significant increase in the stiffness (Young modulus) of the film layer as quantified by nanoindentation experiments with AFM (Atomic Force Microscopy) (Fig. 2c). These measurements indicate that the film stiffness (elastic modulus or Young modulus) is about 0.9 kPa for non-crosslinked gelatin films and it increases up to about 1.5 kPa. The increase in Young modulus could provide a better surface for initial cell attachment[24] whereas improvement in film stability could ensure a slower release of the growth factors rather than a fast one due to film degradation under culture conditions.

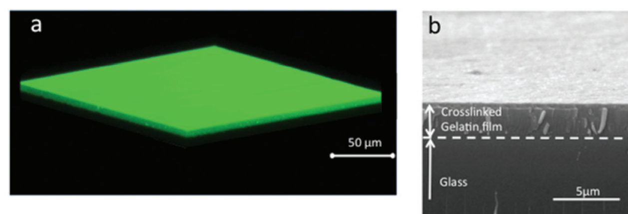

Fig. 1 (a) 3D CLSM image of the spin-coated crosslinked gelatin film labeled with PLL^{FITC}. (b) 3D ESEM images of a spin-coated crosslinked gelatin film under semi-hydrated conditions.

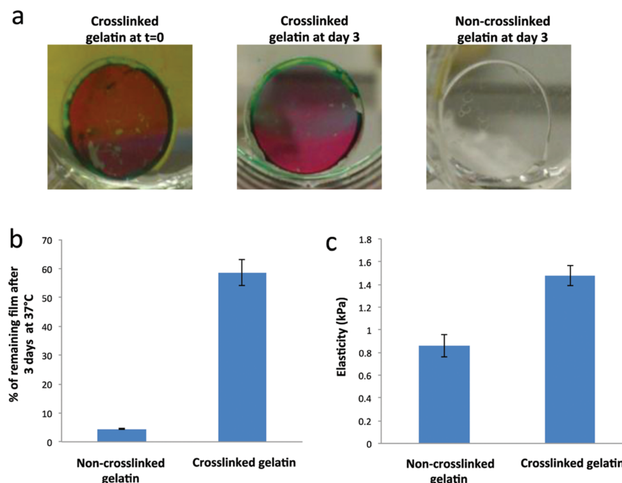

Fig. 2 Stability of the crosslinked gelatin layer and changes in mechanical properties following the crosslinking step. (a) After 3 days of incubation evidenced by Sirius Red/Fast Green staining. (b) Quantification of the degradation of the films after incubation for 3 days at 37 °C in PBS. (c) Measurement of the elasticity of the films performed by AFM nanoindentation experiments.

3.2. Loading and release of serum components

Then, we produced new gelatin films and we added some model proteins under sterile conditions. The loading and release of these model proteins, namely positively and negatively charged proteins were checked. To this end we used FITC-labeled BSA (bovine serum albumin, BSA^{FITC}) and Rho (Rhodamine)-labeled VEGF (vascular endothelial growth factor, VEGF^{Rho}), which are two proteins negatively and positively charged, respectively. Incubations of each protein on crosslinked gelatin films depict a homogeneous distribution through the whole film sections as evidenced by cross-section images obtained by confocal laser scanning microscopy (CLSM) (insets of Fig. 3a and b). The passive release of both molecules mainly occurs during the first 24 h (Fig. 3a and b). When HUVECs are seeded on these films loaded with labeled proteins, delivery was again observed by confocal microscopy: BSA^{FITC} and VEGF^{Rho} were observed in cytoplasm and on membranes. This suggests that the crosslinked gelatin film designed is appropriate to deliver specific proteins and growth factors which are then captured by cells seeded on top of the film.

Multiple growth factor delivery systems are generally limited to the delivery of two factors.[25] In the present study, in order to monitor the loading in the gelatin film and release out of this film of a growth factor mixture and proteins from

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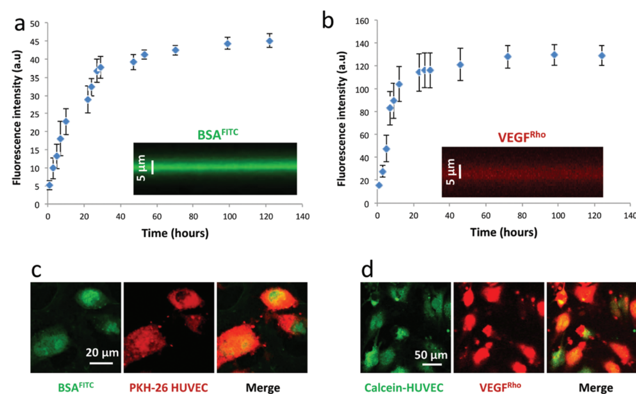

Fig. 3 Loading and release of BSA^{FITC} (a) and VEGF^{Rho} (b) proteins in/from the crosslinked films. Error bars correspond to standard deviations. Cross-section images (*x*,*z*) of the loading were obtained with CLSM (insets of (a) and (b) respectively) and release over time was determined with a fluorescence microplate reader. In the presence of HUVECs after 1 day of growth, the released BSA^{FITC} (c) and VEGF^{Rho} molecules (d) were captured by the cells. HUVECs were previously labeled with PKH26 labeled (c) and (d) calcein-AM.

serum, we performed a bottom-up proteomic analysis using capillary electrophoresis (CE) coupled to electrospray ionization mass spectrometry (ESI-MS)[26] (Table 1 in the ESI†). Commercially available endothelial cell growth supplement has been incubated with the spin-coated crosslinked gelatin film for 30 min. This supplement contains a mixture of endothelial cell related bioactive molecules, mainly a cocktail of growth factors. The film was incubated in PBS for 3 days and the release of proteins at days 1 and 3 was monitored with ESI-MS. The release of proteins (serum albumin, serotransferrin, alpha-2-HS-glycoprotein) and growth factors (FGF, EGF) was detected at days 1 and 3. Moreover, collagen alpha-1-chain and collagen alpha-2-chain were also monitored at days 1 and 3. These two chains correspond to the release of components from gelatin itself. Thus, with this method it is possible to monitor the release of the growth factors and the degradation of the substrate simultaneously, which has not been previously demonstrated.

For certain cell types, such as PtK2 epithelial cells, we have observed a positive effect of the substrate even in the absence of growth factors, *i.e.* with a starvation medium (Fig. S3 in the ESI†). Thus depending on the cell type, doing the cell culture on the specific substrate designed already has significant advantages.

3.3. Gel-feeder as a favorable biochemical microenvironment

The next step was to demonstrate that the loading of a multi-growth factor cocktail can maintain cells under serum free culture conditions. To this end we selected a well-known cell type, namely HUVEC cells. Endothelial cell growth supplement was incubated on the crosslinked gelatin films for 30 min to allow loading of growth factors into the film. Then, cells were seeded on loaded films and they were cultured in 500 µL of starvation medium (SM) for three days (condition called "SM + serum released"). This starvation medium corresponds to a medium without serum supplements, free of proteins and growth factors. As a negative control, a similar cell culture was performed on unloaded films (condition called "SM") and as a positive control, cells were grown on unloaded films but under normal medium conditions with a serum supplement ("NM" condition). After 1 day of culture, cell viability under the "SM + serum released" conditions was nearly identical to NM conditions: about 85% of cells were alive under "SM + serum released" conditions, whereas under SM conditions, 45% of cells were necrotic or apoptotic cells (Fig. 4a). At day 3, alive cells represent 55% of the total cells under "SM + serum released" conditions whereas under SM conditions they decreased to 15% (Fig. 4b). Alive cell number in the positive control is slightly higher than that under "SM + serum released" conditions (70% *versus* 50% but without statistically significant differences). This clearly indicates that the loaded gelatin films are efficient to maintain cells up to 3 days in a favorable environment without the need of using a serum or a supplemented medium. From cell staining with a live/apoptotic/necrotic cell kit, significantly more necrotic cells under SM conditions were observed compared to "SM + serum released" conditions, whereas the alive cell numbers were similar for normal medium and release conditions (Fig. 4c).

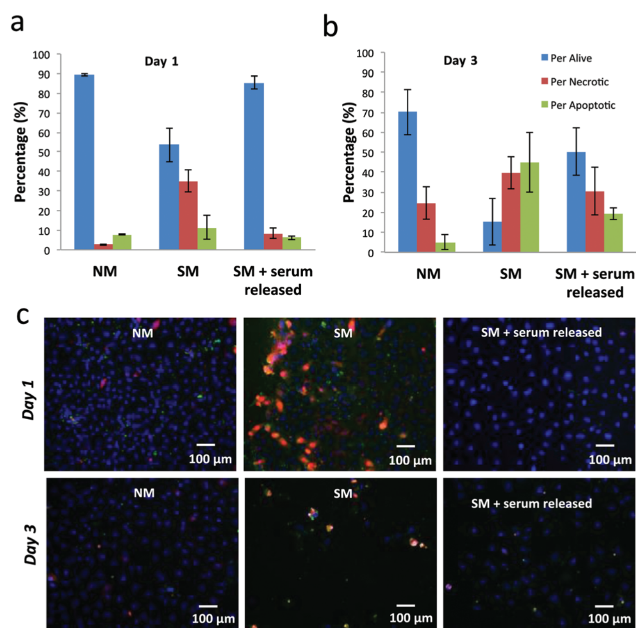

Fig. 4 Maintenance of HUVECs on the gel-feeder substrate. (a) Percentage of HUVECs alive (blue), necrotic (red) or apoptotic (green) at day 1 on the gelatin substrate under normal medium conditions with serum supplement (NM), or under starvation conditions (SM), or on the gelatin film pre-incubated with growth factors ("SM + serum released" condition). (b) Same as (a) but at day 3. Error bars in (a) and (b) correspond to standard deviations. (c) Microscopy observations of alive (blue), necrotic (red) and apoptotic (green) cells for the three conditions (NM, SM, SM + released serum) at day 1 and day 3.

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To demonstrate the potential of the crosslinked gelatin substrate compared to more conventional substrates for cell culture, we measured metabolic activity of cells on a glass slide in starvation medium (Fig. S4 in the ESI†). For cells cultured on a glass slide under serum-free culture conditions, a weak metabolic activity was monitored at day 3. However when glass was replaced with non-loaded crosslinked gelatin films (SM condition), metabolic activity increased by a factor of four. A gelatin film loaded with medium ("SM + serum released" condition) shows the highest metabolic activity, about 5 times higher compared to a glass slide. This suggests that the gelatin crosslinked substrate by itself is already a satisfactory environment for cell metabolism in starvation medium. Moreover cell–cell contacts between the HUVEC cells were checked with F-actin and PECAM labeling (Fig. S5 in the ESI†). PECAM is an important cell–cell adhesion molecule for endothelial cells and also it is an important modulator of endothelial cell function.[27] After 3 days of culture, "SM + serum released" conditions show near confluent, well spread layers of HUVECs with a high number of cell–cell contacts (PECAM in green) whereas in the case of SM conditions, there were only occasional islands of cells.

To emphasize the importance of the release, we performed an experiment where the enzymatic crosslinking step was done after the loading of the growth factors. Most of the cells were apoptotic or necrotic after 1 day of culture (Fig. S6 in the ESI†). Probably most of the growth factors have been crosslinked and consequently no release occurred and/or their activity was lost.

Another important aspect of loading growth factors into the culture substrate is the prevention of growth factor degradation. Under aqueous conditions, most of the growth factors are highly fragile and even at 4 °C they slowly degrade. Finally, a supplemented culture medium has a gradually decreasing bioactivity due to this degradation. In order to see whether growth factor activity can be kept in the gelatin substrates after prolonged storage times, films were dried and stored at −20 °C for a week. Afterwards, cells were seeded for 3 days on thawed gelatin substrates with ("SM + serum released") or without (SM) growth factors under starvation medium conditions. There was a significantly higher number of cells on growth factor containing films and the number necrotic and apoptotic cells were two times smaller (Fig. S7a and b in the ESI†). Metabolic activity was also strongly increased under "SM + serum released" conditions compared to SM conditions (Fig. S7c in the ESI†). This underlines the potential of the designed substrate in terms of improvement of cell attachment.

In order to see whether this system can be generalized, one more cell model was selected, a fibroblastic one, to show that the concept can work with undefined serum too. 3T3, known as the standard fibroblast cell line (mouse fibroblasts) was seeded on FBS (Fetal Bovine Serum) loaded films. After three days there was a significant difference in 3T3 cell metabolic activity under "SM + released conditions" compared to SM. Moreover, most of the cells under SM conditions were necrotic (Fig. S8 in the ESI†). Finally, we also demonstrated that the release of the supplement from the film was necessary to get focal adhesion with epithelial cells PtK2 by using vinculin immunostaining (Fig. S9 in the ESI†).

3.4. Gel-feeder as a favorable biophysical microenvironment

We demonstrated that the biochemical properties of the feeder layer can be adjusted by loading individual proteins, growth factors or complete media. The next step consisted of the modulation of physical properties of the designed feeder layer. Mechanical properties of the cellular environment play a major role in cell attachment, proliferation and differentiation.[2,28] Moreover each cell type needs substrates of specific Young modulus to grow optimally. The addition of the nanoparticles in a gel-feeder system could be advantageous as this would not intervene with the release and could confer novel mechanical properties. Polystyrene nanoparticles with a diameter of 100 nm were incubated for 30 min at a 1/500 dilution (corresponding to 3.6×10^{10} particles per mL) on the spin-coated gelatin films before crosslinking with transglutaminase. Then, distribution of nanoparticles in the gelatin film was checked using ESEM and CLSM and homogeneous distribution of nanoparticles on and in the film was observed (Fig. S10 in the ESI†).

To quantify the changes in adhered cells with respect to particle loading, 3 different cell types were tested: Human Gingival Fibroblasts (HGF), 3T3 fibroblasts and HUVECs (Fig. S11 in the ESI†). Primary fibroblasts HGF were the most sensitive cells to the presence of nanoparticle concentration and the primary endothelial cells were the least sensitive with respect to cell density and cell area (Fig. S11 in the ESI†). In order to determine optimum attachment for cells with respect to the changes in nanoparticle concentration, surfaces loaded with various amounts of nanoparticles were established and HGF cells were tested at different nanoparticle concentrations (Fig. 5). When the nanoparticle concentration in gelatin increases, we observe a strong increase in cell spreading (Fig. 5a and c) and a moderate increase in cell density (Fig. 5b). Gelatin enzymatically crosslinked without loading any nanoparticles allows HGF cells to attach but is not sufficient to promote the spreading of the cells.

We have also shown that the gel-feeder substrate incubated with nanoparticles had an effect on cell proliferation (Fig. S12 in the ESI†). The effect is particularly important for cell types such as epithelial cells which are hard to culture and known to be very sensitive to mechanical properties of the substrate.

To determine the origin of the effect of nanoparticles and if it can be attributed to a change in mechanical properties of the film, nanoindentation experiments with AFM were performed (Fig. 6 and S13 in the ESI†). These measurements indicate that film stiffness (elastic modulus or Young modulus) is about 1.5 kPa for enzymatically crosslinked gelatin films and loading of nanoparticles further improves this Young modulus to 3 kPa (gelatin crosslinked with nanoparticles deposited at a dilution 1/1000) up to 15.5 kPa (gelatin crosslinked with nanoparticles deposited at a dilution 1/50). Finally a direct correlation between the increase in elastic modulus of the film and

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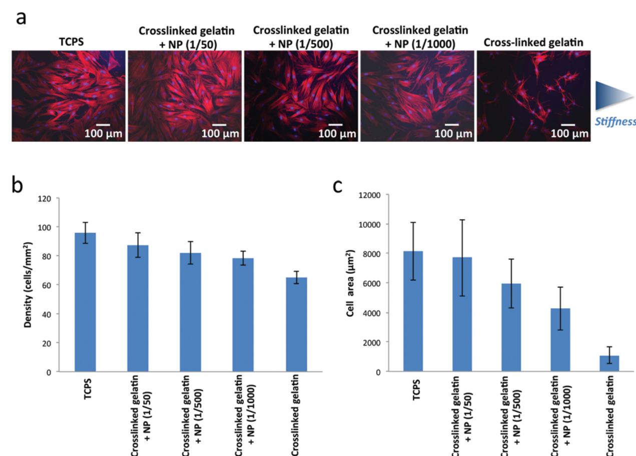

Fig. 5 Differential attachment of primary fibroblasts HGF on gelatin films loaded with various concentrations of nanoparticles (loading from solutions diluted at 1/50, 1/500, 1/1000). (a) HGF actin filaments (phalloidin labeling, red) and nucleus (Hoechst labeling, blue) observed on various substrates TCPS used as control is considered as the more rigid substrate of the series and gelatin (enzymatically crosslinked) is the softest one. (b) Cell density and (c) cell area determined for HGF on various substrates. Error bars correspond to standard deviations.

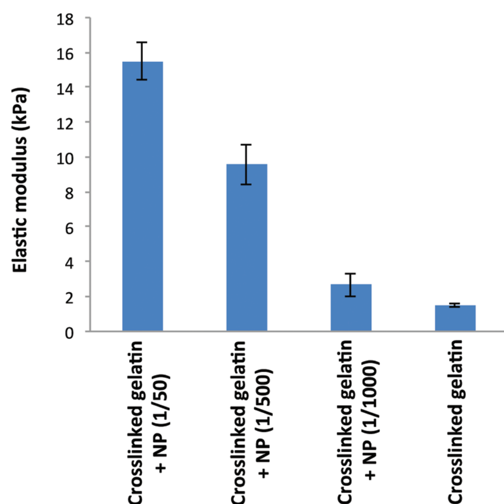

Fig. 6 Average elastic moduli measured by AFM for gelatin films enzymatically crosslinked non-loaded or loaded with various amounts of nanoparticles (loading from solutions diluted at 1/50, 1/500, 1/1000). Error bars correspond to standard deviations.

the improvement of spreading and adhesion of cells can be made.

In order to see whether concomitant utilization of nanoparticles and growth factors has a synergistic or antagonistic effect on cell behavior, 2^2 factorial experimental systems were used to determine the interaction between these two parameters by Design Expert program (StatEase, USA) (Fig. S14 in the ESI†). From the model, it could be seen that initially the presence of the particles governed the cell behavior (the presence of the particles had a positive effect on day 1), whereas the latter behavior was mainly governed by the growth factor presence with a small synergistic effect between the particles and the growth factor.

3.5. Gel-feeder to ensure sterility of the microenvironment

Another crucial aspect in cell culture concerns the sterility of the microenvironment. A cocktail of antibiotics like penicillin/streptomycin is currently added to the conventional cell culture medium to avoid bacterial or fungal contaminations. In addition to growth factors, antimicrobial agents as antibiotics or synthetic antimicrobial peptides[29] have been added to the sterilized crosslinked gelatin substrate for limiting the constant addition of antimicrobial agents to the culture medium and to prevent the rise of resistant bacterial strains in the culture environment. A penicillin/streptomycin mixture as an antibiotic or catestatin as an antimicrobial peptide[30] were loaded on crosslinked gelatin films. Then, *S. aureus* strains were incubated on the films for 1 or 2 days and efficiency of the films towards bacteria proliferation was checked (Fig. 7). After one day, bacteria growth is strongly inhibited by the presence of antibiotics in the substrate and to a lesser extent catestatin has a similar effect. After two days, the efficiency of the film to prevent bacterial growth is reduced probably because the loading rate is not sufficient to ensure the presence of antibiotics and antimicrobial peptides for more than one day. However, the number of incubated bacteria in these experiments was huge compared to what occurs for contamination during a cell culture (seeding of bacteria was done at a final density of 10^6 CFU mL^{-1} while contamination of a cell culture well generally starts with one colony). Our gel-feeder system should thus be optimized to prevent local contaminations during cell culture protocols for several days or weeks.

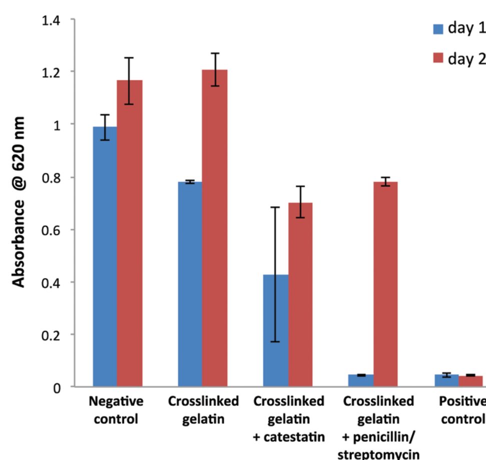

Fig. 7 Absorbance at 620 nm to monitor *S. aureus* growth in solution at days 1 and 3: (i) without gelatin (negative control); (ii) with a crosslinked gelatin film without any loading; (iii) with a loading of either catestatin or penicillin/streptomycin. Positive control corresponds to a cocktail of tetracycline and cefotaxime in solution. Error bars correspond to standard deviations.

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4. Conclusions

An enzymatically crosslinked ECM-based substrate which is amenable to cell growth was shown to be able to contain and release multiple growth factors at the same time. The gel-feeder designed provides direct linkage of the growth factors to the ECM structure and offers cells a better microenvironment. Moreover, stiffness of this substrate is adjustable due to the presence of nanoparticles without damaging the activity of the loaded growth factors. This gel-feeder system can be used as a generic, storable, growth factor containing substrate for biotechnology applications. This would not only decrease the cost of the growth factors used but also provide a different means to study cellular activity without the presence of exogenous growth factors and also a high level of control over substrate stiffness. We can also easily envision having a plate with different types of films (loaded with different bioactive molecules) to cultivate distinctive cell types using a unique common basic medium without any need to add new specific growth factors for at least 3 days. Moreover this film layer can be used for phenotype modulation and differentiation. Finally, such a system could be applicable for cell amplification, cell sheet engineering and also to design kits with specific cell selective surfaces for preferentially binding of tumor cells, or sub-cell phenotypes.

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