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Ligand engagement on material surfaces is discriminated by cell mechanosensoring

Edmondo Battista [a], Filippo Causa [a, b, c], Vincenzo Lettera [a, b], Valeria Panzetta [a], Daniela Guarnieri [a], Sabato Fusco [a], Francesco Gentile [d], Paolo A. Netti [a, b, c, *]

[a] Center for Advanced Biomaterials for Healthcare@CRIB, Istituto Italiano di Tecnologia (IIT), Largo Barsanti e Matteucci 53, 80125 Naples, Italy
[b] Interdisciplinary Research Centre on Biomaterials (CRIB), University "Federico II", Piazzale Tecchio 80, 80125 Naples, Italy
[c] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale (DICMAPI), University "Federico II", Piazzale Tecchio 80, 80125 Naples, Italy
[d] Istituto Italiano di Tecnologia (IIT), via Morego 30, 16163 Genova, Italy

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ABSTRACT

Peptide or protein ligands can be used for molecular decoration to enhance the functionality of synthetic materials. However, some skepticism has arisen about the efficacy of such strategy in practical contexts since serum proteins largely adsorb. To address this issue, it is crucial to ascertain whether a chemically conjugated integrin-binding peptide is fully recognized by a cell even if partially covered by a physisorbed layer of serum protein; in more general terms, if competitive protein fragments physisorbed onto the surface are distinguishable from those chemically anchored to it. Here, we engraft an RGD peptide on poly-ε-caprolactone (PCL) surfaces and follow the dynamics of focal adhesion (FA) and cytoskeleton assembly at different times and culture conditions using a variety of analytical tools. Although the presence of serum protein covers the bioconjugated RGD significantly, after the first adhesion phase cells dig into the physisorbed layer and reach the submerged signal to establish a more stable adhesion structure (mature FAs). Although the spreading area index is not substantially affected by the presence of the RGD peptide, cells attached to chemically bound signals develop a stronger adhesive interaction with the materials and assemble a mechanically stable cytoskeleton. This demonstrates that cells are able to discriminate, *via* mechanosensoring, between adhesive motives belonging to physisorbed proteins and those firmly anchored on the material surface.

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1. Introduction

The encoding of biomolecular cues by chemical conjugation of protein or peptides has been widely proposed to extend the biological performance of synthetic materials [1–4]. This approach has been validated as viable and effective in enriching cell-specific recognition on otherwise bioinert materials [5–9]. The outcome of such an approach strongly depends on how the ligands interact with surfaces in term of adhesion strength and presentation [10]. In this context, concerns exist on the practical use of these bioactivated materials in *in vitro* and *in vivo* contexts since the proteins present in biological fluids (i.e. serum culture media or *in vivo* serum) might adsorb at the interface inducing a partial -if not total- screening of the conjugated signal [11,12]. However, the same adsorbed serum provides RGD-like adhesion motifs and others signals with a proactive role in mediating cell attachment; as a whole, it affects cell behavior [13]. It has been reported that, when surfaces are exposed to culture serum, soon a proteinaceous layer of up to 20 nm is formed [14] at the interface submerging the actual material surface and masking its chemical features [15–20]. To date, little is known about the influence of absorbed protein layers on the effective presentation of bound molecular signals and whether, or to what extent, the bound signal is still available to cell receptors in a practical *in vitro* or *in vivo* context. In fact, cells cultured on peptide-activated materials are exposed to two populations of matricellular cues: one associated with the physisorbed serum protein layer that is more directly available to the cell membrane and another firmly bound to the material surface, but

* Corresponding author. Center for Advanced Biomaterials for Healthcare@CRIB, Istituto Italiano di Tecnologia (IIT), Largo Barsanti e Matteucci 53, 80125 Naples, Italy.
E-mail address: nettipa@unina.it (P.A. Netti).

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submerged by the protein layer, hence less accessible to the cell membrane. Conventional methods to assess surface bioactivation through ligands are represented by cell counting and cell spreading area; however, such methods often fail to provide information about the cell–material interaction. It has recently been highlighted that cells communicate with the surrounding environment exerting forces by means of ligands-integrins-cytoskeleton chain [21–23]. The ability of cells to react to the mechanical properties of substrata has been mainly investigated in relation to the "global" stiffness of materials, rather than to the ability of surface ligands to withstand the pulling forces of adhesion plaques.

Here we assess the cellular availability of bound *vs*. physisorbed matricellular signals represented by complex protein media and evaluate the efficacy of peptide conjugation on cell recognition over different spatial and temporal scales. In particular, the evaluation of the adhesion footprints from RGD bound or adsorbed on polymeric surfaces is performed along the ligand-receptor-cytoskeleton chain through the investigation of focal adhesion growth, actin bundle organization and mechanical properties of the whole cell body. Furthermore, in order to investigate the dynamics of cells in multiple signal recognition and their competitive selection, we compare the mechanism of integrin-receptor recognition actuated by living cells to that actuated by specifically functionalized RGD-binding polymer microparticles.

2. Materials and methods

2.1. Materials

All reagents were ACS grade or better and purchased from Sigma–Aldrich (Milano, Italy), unless otherwise specified throughout the text. Poly-ε-caprolactone (PCL) pellets of Mw = 65,000 g/mol, 1,6-hexanediamine (DEA), hexylamine, diethylene glycol diglycidyl ether (DGDGE), glycerol, tritolyl phosphate (mixture of isomers, 90%), ethanolamine, tween 20, QuantiPro BCA assay kit, Kaiser test kit, phosphate buffered saline (PBS), Tris–HCl buffer (Tris–HCl 50 mmol/L, 150 mmol/L NaCl, 0.1% V/V Tween-20, pH = 7.5), dichloromethane (DCM), tethraydrofuran (THF), water (CHROMASOLV Plus), ethanol, 2-propanol (IPA). The peptides GYGRGDSP and GYGRGESP were purchased from INBIOS S.r.l., Naples, Italy. For the microparticle assay the mimic of RGD-binding integrin site, CWDDGWLC-biotin, was purchased by Celtek Biosciences, (Nashville, TN), Streptavidin Fluoresbrite® YG Microspheres (d = 2.18 μm (SD 0.01), 1.9 ng/mL biotin binding capacity, 1.37% solid) were purchased by Polysciences. For protein adsorption, fetal bovin serum (FBS) was purchased by BioWhittaker, Walkersville, MD.

2.2. Surface bioactivation and characterization

PCL was spin casted onto glass slides from a 3% (W/V) PCL in THF solution by (2500 rpm for 30 s). Subsequently, spun polymer coated slides were placed onto a hot plate at 95 °C for 30 min and then in a vacuum desiccator overnight to remove organic solvent. An even polymer film of 300 nm (±30 nm) was formed as evidenced by profilometer (Dektat 150, Veeco). Afterwards, the polymer film was aminated by means aminolysis in a 10% (W/W) 1,6-hexanediamine in 2-propanol solution at 37 °C for 30 min followed by copious rinsing steps in deionized water (H_2Odd).

The aminolyzed polymer was first activated in 5% (V/V) of DGDGE in carbonate buffer (50 mM, pH 8.5) for 2 h on an orbital shaker at room temperature. Then, a peptide solution (0.1 mg/ml in carbonate buffer, 50 mM, pH 8.5) was added to thoroughly rinsed samples for 4 h at room temperature. In the bioactivation step GYGRGDSP sequence was used as positive control, and GYGRGESP as negative control [5,24]. Samples were then rinsed with H_2Odd and treated with a 0.2 mM ethanolamine solution for 30 min in the same condition as for the peptide. Finally, samples were extensively washed with H_2Odd, dried and stored in a vacuum desiccator until use.

For simplicity the samples will be indicated as follows: PCL for the un-treated polymer surface; PCL-NH_2 for the aminated poly-ε-caprolactone surfaces; PCL-RGD/-RGE (or alternatively RGD/RGE) for the peptide engrafted polymer surfaces.

Surface characterizations were performed directly onto spin casted films after each step of modification by colorimetric assays and PM-IRRAS spectroscopy, AFM and contact angle.

As previously detailed [7], Micro-BCA assay was used to assess peptide density directly bound onto the PCL surface. PM-IRRAS measurements were performed on a Fourier Transform Infrared NICOLET 8700 step-scan spectrophotometer equipped with a dual channel electronic unit and a PhotoElastic Modulator (PEM) module of ZnSe. The polymer samples were mounted onto the holder with an angle of incidence of 83° and the PEM module was operated at 50 kHz. The detector output was sent to the dual channel electronic that generated two interferograms processed and Fourier-transformed to obtain the PM-IRRAS signal $\Delta R/R = Rp - Rs/Rp + Rs$, where Rp and Rs are the p-polarized reflectivity and the s-polarized reflectivity [25,26]. All the PM-IRRAS spectra reported throughout the paper were recorded at 8 cm^{-1} resolution by co-adding 300 scans.

Topological features of surfaces were obtained with BioAFM NanoWizard II, (JPK, Germany) in intermittent contact mode in air at room temperature with a TESP (Veecoprobes) silicon tip tapping the surface at 280 kHz with a spring constant around 40 N/m. Multiple measurements were taken in different scan directions, to prove the avoidance of artifacts, and on different regions of sample surfaces. Height and phase images of different sizes were captured at a scan rate of 0.8 Hz, with a resolution of 256 × 256 lines, and processed by resident software using either flattening or plane fit according to the relief characteristics, with the minimal polynomial order needed, to obtain roughness parameters. Ra, RMS and R were calculated on images of 50 × 50 μm^2, the R parameter is defined as the ratio between the surface area of the sample measured and the projected area of the analyzed window.

Wettability tests by using a Theta T200 (KSV/Attension) were performed dropping 2 μL of ultra-pure water and calculating the static contact angle from the images, at least five measurements were taken on different areas of each samples and averaged. In order to consider the roughness effect onto surface wettability, the Wenzel relation was used, where a correction parameter R due to the apparent surface was applied to the Young equation [27].

Surface free energy components were calculated by applying the Chaudhury-Good-van Oss model at the contact angles of three different liquids (ultra-pure water, Glycerol and Tricresyl phosphate).

2.3. Ligand presentation at interface

The presence of the RGD residues on the surface of the treated PCL surfaces was visualized using an integrin mimicking RGD-binding peptide (CWDDGWLC-biotin) (Celtek Biosciences, Nashville, TN) [28,29] and fluorescent streptavidin coated microparticles (Streptavidin Fluoresbrite® YG Microspheres, Polysciences, d = 2.18 μm (SD 0.01), 1.9 μg/mL biotin binding capacity, 1.37% solid). The extent of particle functionalization with RGD-binding peptide, on the basis of biotin-binding capacity, was around $3.0*10^5$ molecules per microparticles, with a maximum lateral spacing of streptavidin molecules of about 9 nm. A stock solution of Streptavidin microparticles was suspended in TRIS buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% V/V Tween-20, pH = 7.5, used as washing and binding buffer) and coupled with the biotinilated peptide as per the protocol of the supplier. Briefly, the microparticle suspension was centrifuged at 5000 rpm, washed three times with buffer and subsequently incubated for 30 min in a solution of 0.5 μM of biotinilated peptide with a gentle shaking at rt. Finally, another washing and centrifugation cycle allowed obtaining microparticles coated with RGD-binding peptide. Neat PCL and PCL-RGD surfaces, with and without preadsorption of FBS, in a 24 well-plate were incubated for 45 min with 500 μL of microparticles coated with an integrin-mimicking peptide (13.7 ng/mL) onto an orbital shaker. Finally, the surfaces were gently washed three times with buffer and allowed to dry at room temperature. To count the fluorescent microparticles, an inverted confocal microscope LSM510 Zeiss equipped with a Zeiss 20×/3 NA objective and an Ar laser (λex = 496 nm, λem = 518 nm; pinhole ≈ 1.5 Airy unit, 20% power) was used. For each substrate a large number of images (>40) was taken for statistical analysis. Each image was acquired over a region of interest of 924 × 924 μm^2 (pixel size = 1.8 μm) at a resolution of 512 × 512 and exported into MatLAB® and Matematica® for deconvolution using an in-house developed software [30]. The particle density per unit area was obtained dividing the average of microparticles adhered to each sample surface by the area analyzed through the images.

2.4. Cell-adhesion tests

Cell-adhesion was monitored over 24 h and performed in serum free conditions and in medium supplemented by 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD). Mouse embryo fibroblasts NIH3T3 were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine (Sigma, St. Louis, MO), 1000 U/l penicillin (Sigma, St. Louis, MO) and 100 mg/l streptomycin (Sigma, St. Louis, MO) and maintained at 37 °C and 5% CO_2. For the experiments 70–80% confluent cells were used. About 1.0 × 10^4 cells/cm^2 cells were seeded on PCL, PCL-RGE and PCL-RGD materials, incubated at 37 °C and observed at 0, 2, 4, 6 and 24 h from the seeding. Samples were washed twice with PBS to remove non-adhered cells and fixed with 4% paraformaldehyde for 20 min at RT.

For the monitoring of density and cell spreading areas, cells were stained with CellTracker (Red CMTPX, Invitrogen), while for focal adhesion analysis Paxillin was stained by using an Anti-Paxillin antibody (Y113) (ab32084, from RabMAbs, UK) and a secondary anti-mouse Alexa Fluor 488 labeled (Alexa Fluor 488, Lifetechnologies). The cell number was calculated from a set of 7 images, acquired by an inverted microscope in bright field (Olympus IX 80, 40X air); at least 30 cells per sample were analyzed. Cell areas were calculated from 10 cells for each time point and sample.

For focal adhesions (FAs) studies, cells were imaged on an inverted microscope system (Cell-R, Olympus, Japan) equipped with a 60× oil-immersion objective. Images of green paxillin structures were captured from cell samples obtained from immunostaining and size of FAs was quantified. Fluorescent images were acquired at

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