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Creating Biomimetic Surfaces through Covalent and Oriented Binding of Proteins

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This manuscript describes a novel method for the biofunctionalization of glass surfaces with polyhistidine-tagged proteins. The main innovation of this methodology consists of the covalent binding between the nitrilotriacetic acid (NTA) moiety and the proteins, ensuring not only orientation, but also stability of the recombinant proteins on NTA-covered surfaces. In this work, as C-terminal polyhistidine tagged cadherin extracellular fragments have been used, this methodology guarantees the proper orientation of these proteins, by mimicking their insertion into cell plasma membranes. These biofunctionalized surfaces have been characterized by confocal microscopy, X-ray photoelectron spectroscopy, contact angle, and atomic force microscopy, showing a high density of cadherins on the glass surfaces and the stability of the linkage. The prepared materials exhibited a high tendency to promote cell spreading, demonstrating the functionality of the protein and the high utility of these biomaterials to promote cell adhesion events. Interestingly, differences in the cytoskeleton organization have been observed in cells adhering to surfaces with no cadherins or with nonoriented cadherins, in comparison to surfaces functionalized with well-oriented cadherins. This method, which allows the robust immobilization of polyhistidine tagged proteins due to their covalent binding and with a defined orientation, may also find particular usefulness in the making of protein biochips, for analysis of protein–protein interactions, as well as structural and single-molecule studies.

Introduction

Biofunctional surfaces are extensively used in therapeutics and diagnostics for cell-based assays,^{1,2} tissue-engineering applications,^{3,4} biosensor developments,^{5,6} bioelectronics,⁷ and, more generally, medical devices.^{5,6,8} The ideal biofunctional surface should be easy to prepare and characterize, be amenable to design, and be biocompatible and efficient. In this regard, the incorporation of relevant biomolecules has already been reported going

from proteins,⁹ peptides,^{10,11} carbohydrates,^{12,13} oligonucleotides,¹⁴ and others.^{15,16} Several strategies have been followed for the chemical modification of these biomaterials using covalent or noncovalent chemistry. However, both approaches have advantages and disadvantages. Covalent binding ensures a strong linkage between the biomolecules and the surface, but it requires surface modification, and most importantly, it does not generally guarantee the right orientation of complex molecules such as proteins.¹⁷ Such chemical binding of unmodified proteins most often leads to their random orientation onto reactive surfaces. On one hand, unspecific adsorption constitutes a simple strategy for the biofunctionalization of surfaces,¹⁸ but it constitutes a binding

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very dependent on the media conditions (pH, ionic strength, temperature, etc.).¹⁹ Moreover, such uncontrolled interactions often induce protein unfolding and thus a decrease of their biological activity.

The study of cell adhesion to biomaterials is a very important topic in tissue engineering.²⁰ The interactions between cells and materials modulate the cellular response to implanted devices as well as cell culture supports.^{7,21} Biochemical data highlight the necessity of extracellular matrix (ECM) proteins in promoting cell adhesion through specific interactions.²² Cells, via integrins, bind to specific amino acid sequences on ECM proteins such as RGD present in fibronectin, vitronectin, and thrombospondin.^{23,24} These proteins provide an attachment network for the adhesion and growth of specific cells in vivo. Furthermore, adhesive receptors named cadherins are also involved in cell–cell adhesion events. Classical cadherins are transmembrane glycoproteins essential in controlling the specificity, organization, and dynamics of cell adhesion, which is crucial for the development and maintenance of tissue architecture and function.^{25–27} Cadherins interact with other cell surface cadherins on neighboring cells through their extracellular subdomain repeats named EC1 to EC5.^{28–30} Their adhesive engagement initiates intracellular signals ranging from cytoskeletal organization to cell polarity, proliferation, or apoptosis that are communicated through the conserved cadherin tail domain to cytoplasmic pathways.^{25,31,32} The role of adhesion forces in the signaling process is yet to be understood due to difficulty in the interpretation of biological responses involving dynamic and complex multimolecular organizations.³³ On the biomaterial surfaces in vitro, the same mechanisms also apply. When foreign materials come into contact with body fluid or cell culture medium, the initial response is protein adsorption at the material surfaces. Thus, the materials interact with the cells

through the adsorbed protein layer. The composition and structure of this protein layer play critical roles in determining subsequent cell behaviors.^{34–37}

We describe in this paper an efficient, simple, and straightforward procedure to prepare as a model system functionalized surfaces with cadherins in an oriented fashion, mimicking the cadherin presentation on cell surfaces. The developed protocol uses modified cadherin fragments with a polyhistidine tail at the C-terminal end of the proteins and silanized surfaces incorporating a metal chelator complex such as nitrilotriacetic acid (NTA) molecules. A two-step protocol has been developed. The chelation of the polyhistidine tails to the Ni²⁺-NTA complexes located at the surfaces through its high binding affinity will ensure a correct orientation of the proteins incorporated to the surface. However, the polyhistidine–NTA interaction is highly dependent on pH, ionic strength, and other media conditions.^{38,39} In order to guarantee a stable linkage to the surface, a second step has been proposed by an amide formation between amino groups from the histidine lateral chains and the carboxylated groups presented in the NTA. The prepared biofunctional surfaces have been characterized by fluorescent microscopy, contact angle measurements, and XPS. In AFM studies, the stability of the protein immobilization process was probed at the single-molecule level by the height of the covering layer. These surfaces have also been proven as excellent adhesive platforms for cells presenting cadherins on their surfaces, confirming their potential capabilities as biomaterials to study cadherin–cadherin interactions using cellular models. Indeed, the total control over the orientation of the immobilized proteins on such surfaces without affecting their conformation and function optimizes the accessibility of proteins' active sites. Interestingly, differences in cell adhesion behavior are obvious when comparing random versus oriented cadherin immobilization through the method reported here. Because the polyhistidine tag is one of the most generically used affinity tag for ease of purification, this method provides the basis for numerous further applications for studies focusing on research targets at single-molecule levels and for nanobiotechnology. The homogeneous orientation and distribution of immobilized proteins demonstrate the high quality of the designed surface and qualify its application in nanoscaled protein chip architectures.

Experimental Section

Materials. Water used in the described reactions and for cleaning was obtained from a water purification system, MILLI-Q A10 from Millipore. For the activation of glass supports, sulfuric acid (ACS reagent, 95–98%) and hydrogen peroxide (30%) were purchased from Sigma-Aldrich. For the functionalization of glass supports with NTA, (3-glycidyloxypropyl)trimethoxysilane ($\geq 98\%$), *N*-(5-amino-1-carboxypentyl)iminodiacetic acid ($\geq 97\%$, TLC), dry toluene (99.8%), sodium carbonate ($\geq 99\%$),

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and sodium bicarbonate (99.7%) were purchased from Sigma-Aldrich.

For protein immobilization, nickel II chloride hexahydrate, Hepes ($\geq 99.5\%$), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride crystalline, imidazole (ACS reagent, $\geq 99\%$) were purchased from Sigma-Aldrich. *N*-Hydroxysuccinimide ($\geq 97\%$) was purchased from Fluka. Sodium chloride (ultrapure, 5 M) was purchased from Gibco. Ethylenediaminetetraacetic (EDTA, $\geq 99\%$) was purchased from Panreac.

For scanning electron microscopy, glutaraldehyde (25% in water) and hexamethyldisilazane (HDMS, $\geq 99\%$) were purchased from Sigma-Aldrich. Sodium cacodylate and osmium tetroxide were purchased from Agar Scientific. Tannic acid was purchased from Fluka. Methanol (Spectralanal, $\geq 99.9\%$) was purchased from Riedel-de Haën. Uranyl acetate was purchased from Eloïse SARL.

For immunocytochemistry, formaldehyde, Triton X-100, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Mouse monoclonal anti-E-cadherin antibodies (Clone 36) were purchased from BD Transduction Laboratories (BD Biosciences, Pharmingen). Actin filaments were stained with Rhodamine-phalloidin (Molecular Probes, Invitrogen). The mounting medium was SlowFade Gold antifade reagent with DAPI (Molecular Probes, Invitrogen).

All the chemicals were used as received.

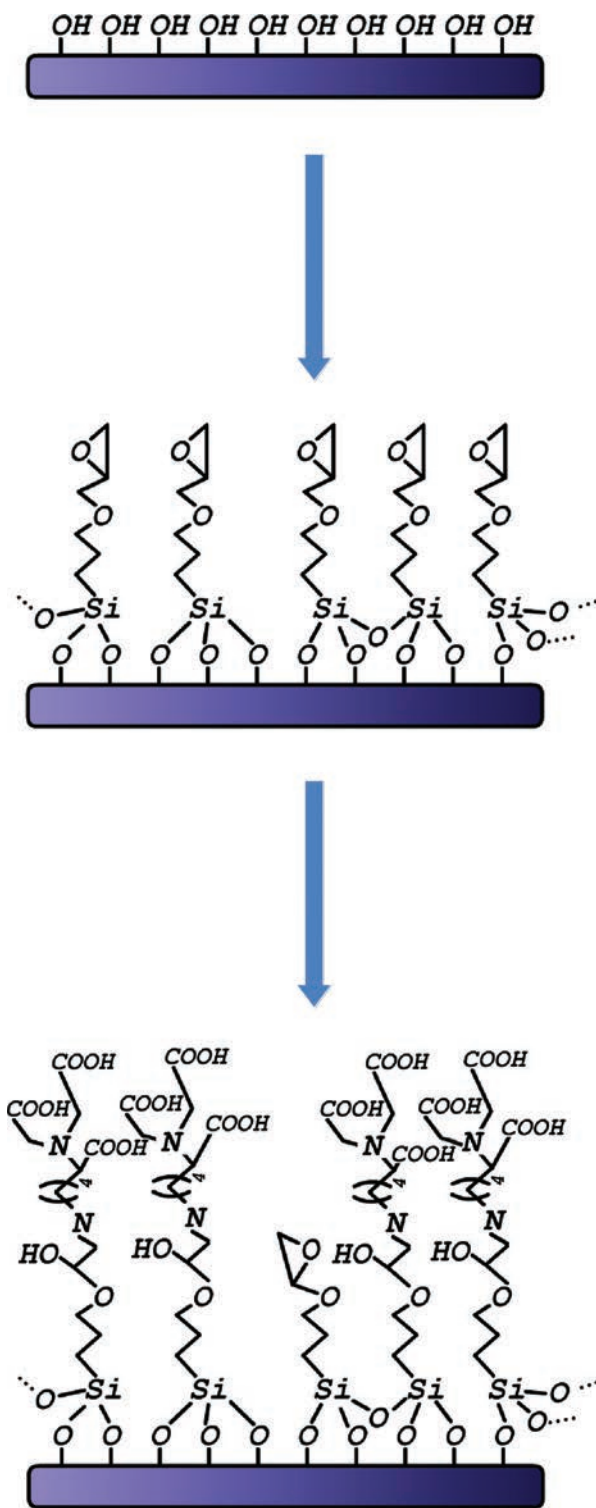
Activation of Glass Supports. Glass supports were cleaned with piranha solution (H_2SO_4 and 30% H_2O_2 , 3:1) for 30 min at room temperature (RT). The slides were then rinsed several times with Milli-Q water and dried under nitrogen.

Functionalization of Glass Supports with *N*-(5-Amino-1-carboxypentyl)iminodiacetic acid (NTA-NH₂). A 2% solution of 3-(2,3-epoxypropoxy) propyltrimethoxysilane in dry toluene was added overnight (ON) at RT onto the activated glass supports. After washing extensively with toluene and 10 mM carbonate buffer pH 10.8, glass supports were incubated ON at RT with 25 mM NTA-NH₂ in carbonate buffer pH 10.8. The supports were then washed and dried.

Expression and Purification of the Hexahistidine Tagged Cadherin Fragments (E/EC12). The mouse E-Cadherin extracellular fragment (MIEGR-E/EC12) corresponding to the two outermost cadherin modules bearing a C-terminal hexahistidine tag was produced as already described.⁴⁰ Briefly, the IEGR sequence was introduced in the plasmid construct of the cadherin fragment, in order to obtain the correctly cleaved cadherin fragment at the N-ter end. For production of this chimeric protein, 500 mL of Terrific Broth supplemented with 50 $\mu\text{g}/\text{mL}$ kanamycin were inoculated with a transformed colony and protein synthesis induced by IPTG. Cell pellet was resuspended in lysis buffer [4 M urea, 50 mM Na_2HPO_4 (pH 7.8), 20 mM imidazole, and 20 mM β -mercaptoethanol]. The resulting lysate was cleared by centrifugation, and the supernatant was incubated for 2 h with Ni-NTA Superflow agarose resin beads (Qiagen). The beads were then thoroughly washed and subjected to stepwise dialysis against PBS. Factor Xa protease (Qiagen) digestion of MIEGR-E/EC12 was performed for 60 h at 16 °C and stopped by removing the enzyme with Xa Removal Resin (Qiagen). Finally, the resulting E/EC12 fragments were then dialyzed against 150 mM NaCl and 20 mM Hepes (pH 7.0) in order to remove Ca^{2+} ions and the cleaved peptide. Protein purity was checked by SDS-PAGE, Western blotting, and mass spectrometry (not shown).

Covalent Oriented Protein Immobilization. The NTA-surfaces were loaded with 100 mM NiCl_2 in aqueous solution to form the complexation environment for the hexahistidine motif. Cadherin fragments (0.1 mg/mL) were incubated at RT for 1 h, and then, the surfaces were extensively washed with 150 mM NaCl, 20 mM Hepes (pH 7.0).

Scheme 1. Functionalization of Glass Surfaces with NTA



Cadherin fragments were conjugated to the glass supports by cross-linking carboxyl groups of the NTA moiety to amino groups of the proteins, mediated by 50 mM EDC, 75 mM NHS, 20 mM Hepes (pH 7.0) solution, for 45 min at RT.

Noncovalently linked proteins were eluted by 1 M imidazole, 10 mM EDTA in 150 mM NaCl, 20 mM Hepes (pH 7.5). Samples were extensively rinsed with 150 mM NaCl, 20 mM Hepes (pH 7.0).

Covalent Nonoriented Protein Immobilization. The NTA-surfaces were loaded with 50 mM EDC, 75 mM NHS (20 mM Hepes, pH 7.0) for 45 min at RT for further carboxyl group

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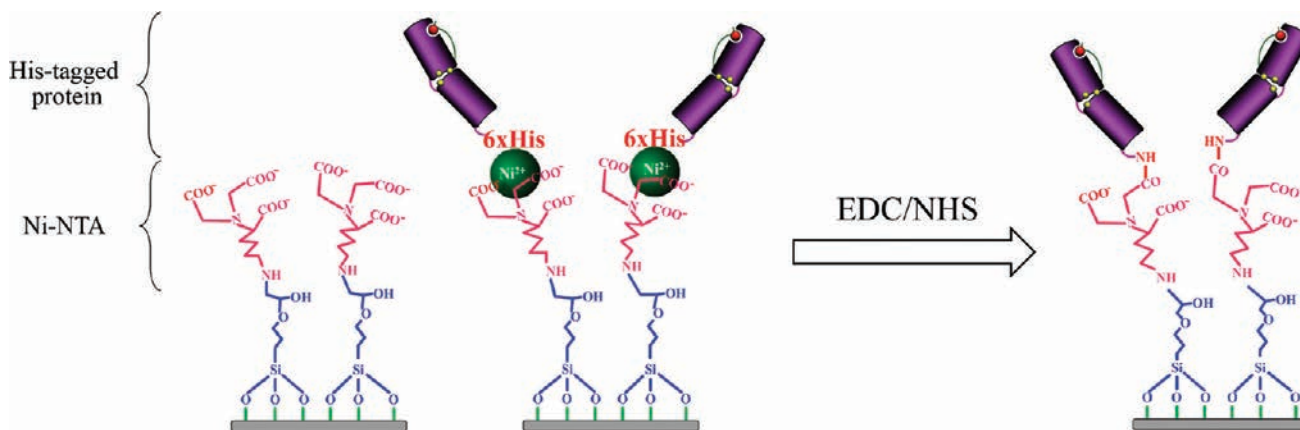


Figure 1. Covalent and oriented cross-linking of Histidine-tagged cadherin fragments onto NTA surfaces. The C-ter hexahistidine-tagged cadherin fragments were first immobilized in an orientated manner using the well-known Ni-NTA technology. Then, the proteins were cross-linked to the carboxylic groups of the NTA moiety, mediated by EDC and NHS.

activation. Cadherin fragments (0.1 mg/mL in 150 mM NaCl, 20 mM Hepes pH 9.0) were incubated at RT for 1 h, then surfaces extensively washed as described. Amino groups from lysine residues are more reactive at the working pH than the terminal polyhistidine moieties⁴¹ due to the differences in the pK for histidine and lysine amino groups, thus favoring the random orientation of the protein immobilization.

Contact Angle Measurements. Contact angle measurements were carried out using an easy drop commercial instrument (Krüss, Germany). Milli-Q water (3 μ L) was added onto each surface using a Hamilton syringe at 25 °C. Three measurements were done per sample, and the contact angle was calculated using the Sessile Drop Fitting Method.

X-ray Photoelectron Spectroscopy (XPS). XPS measurements were made using an ESCALAB 220-iXL spectrometer (Thermo-Electron, VG Company). Photoemission was stimulated by monochromatized Al K α radiation (1486.6 eV). An area of about 150 μ m diameter was analyzed for each sample. Surveys and high-resolution spectra were recorded and then fitted with the *Avantage* processing program provided by ThermoFisher Scientific.

AFM Characterization. The atomic force microscopy analysis was performed using a commercial instrument (www.nanotec.es) operated in dynamic mode in liquid (PBS+Ca²⁺ or milli-Q H₂O). To characterize the samples functionalized with covalently bound cadherins, we used cantilevers with a nominal force constant of 2.8 N/m (www.nanosensors.com). To be able to image the proteins in the samples where cadherins were not covalently bound to the surface, we needed softer cantilevers with a force constant of 0.03 N/m (BioLevers, www.olympus.com).

Cell Culture. The normal mouse mammary epithelial cell line HC11 has been described⁴² and was kindly provided by Dr. L. Raptis (Kingston, ON, Canada). These cells were used to assay the cell attachment onto cadherin-coated surfaces. Cells were grown in RPMI (Gibco) supplemented with 10% FCS, 100 U/mL penicillin G, and 100 mg/mL streptomycin, 5 μ g/mL insulin, and 10 ng/mL EGF. Cell culture was conducted at 37 °C in a humidified atmosphere of 5% CO₂ in air.

For cell attachment assay, the cadherin-coated samples were cultured in 24-well tissue-culture test plates. Cells were seeded on the coated surfaces at a density of 1.2×10^6 cells/mL and cultured for up to 2 h 30 min. At each culture period (0.5, 1, 1.5, and 2.5 h), samples were taken out to new tissue-culture plates.

Scanning Electron Microscopy. Cells were cultured on treated glass coverslips for the indicated periods of time before cells were washed with cacodylate buffer and fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M Na cacodylate pH 7.4 for 90 min at 4 °C. Samples were then treated with 1% osmium tetroxide and 1% tannic acid, then dehydrated with a series of graded methanol/water baths, stained in 2% uranyl acetate in 70% methanol before critical-point drying with HMDS. The samples were sputter-coated with gold and observed with a Jeol JSM 6700F field emission scanning electron microscope operated at 5 kV.

Immunocytochemistry. HC11 cells were seeded on coverslips at a subconfluent density and incubated for different periods of time. Cells were fixed in 4% formaldehyde in PBS for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed in PBS, blocked for 10 min in PBS containing 0.2% BSA, and stained with the appropriate antibodies. The cells were incubated for 1 h with primary antibodies, washed, and incubated with secondary antibodies for 1 h. Monoclonal anti-E-cadherin antibody against the cytoplasmic tail (BD Biosciences) was used at a 1:25 dilution made with PBS containing 0.2% BSA. Images were obtained with a laser-scanning confocal microscope (Leica DMR upright TCS SP2 AOBs).

Results and Discussion

Surface biofunctionalization is a very important and competitive field in biochemistry. Several routes have been designed for modifying different types of materials and/or linking biomacromolecules to these surfaces. However, most of them fail to link these molecules covalently and in an oriented manner. Here, we present a simple glass surface modification, using the well-known Ni-NTA technology, which for the first time fulfills these double criteria, and furthermore could be extended to a broad type of different materials.⁴³

Glass Support Modifications with Nitrilotriacetic Acid (NTA). Glass has been used as the model material due to extensive use for cell culture. The first step of this functionalization was to remove all the organic residues from the glass supports, in order to correctly graft the epoxysilane on the surface, as described in the Experimental Section. As shown in Scheme 1, a two-step method has been used: (i) first, coating of the glass support with 3-(2,3-epoxypropoxy) propyltrimethoxysilane; (ii) then, linkage, through the epoxy group, of the (*S*)-*N*-(5-amino-1-carboxypentyl)iminodiacetic acid (NTA-NH₂). The grafting of the epoxysilane was performed with dry toluene to avoid gel formation of the

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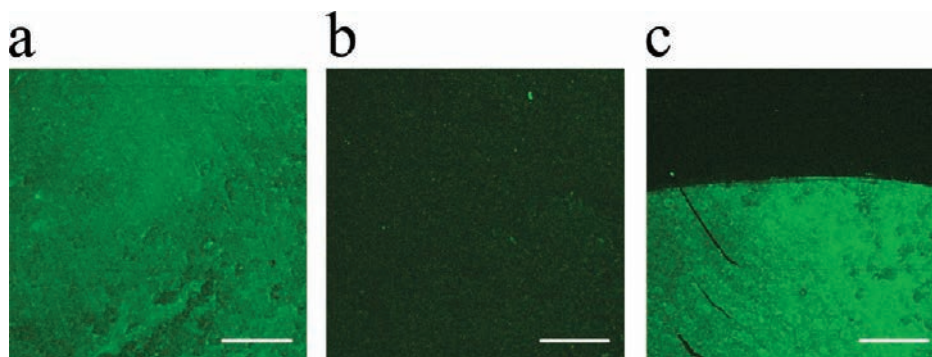


Figure 2. Immunodetection of covalently linked cadherin fragments onto NTA surfaces. Confocal microscopy of glass supports either covalently (a) or noncovalently (b) coated with hexahistidine-cadherin fragments. Both types of surfaces were treated with imidazole/EDTA to eliminate noncovalently bound proteins. Only covalently immobilized cadherin fragments remained bound. Finally, glass support covered half with and half without cadherins (mask was made with polymerized PDMS) indicating the labeling specificity (c). Scale bar = 50 μm .

Table 1. Average of XPS Results^a

	C	O	N
glass slide	12.4	60.3	0.4
glass-NTA-NH ₂	18.9	52.95	1.9
glass-NTA-EC12 covalent link	36.9	36.95	4.85

^a Slides were cleaned, silanized, and covered with NTA-NH₂. These surfaces were then biofunctionalized with hexahistidine-tagged E/EC12 fragments that were not covalently linked. Following E/EC12 incubation, surfaces were extensively washed with [Imidazole 1 M/EDTA 10 mM], in order to eliminate noncovalently bound proteins.

silanes. The epoxy groups on the surface guarantee an efficient reaction with the amine group of the NTA at basic pH (10.8). At this pH, the amine of the NTA easily opens the epoxy group in a high molar ratio. Finally, samples were incubated with a 100 mM NiCl₂ solution to chelate the metal ion into the NTA moiety.

Contact angle measurements have been carried out to monitor the functionalization process. These data can provide useful information about the structure and composition of a surface by examining their wettability after the different chemical treatments. After cleaning the glass samples with piranha solution, contact angle changed from 62° to near 0° due to the appearance of hydroxyl groups on the glass surface using these oxidative conditions, as has been previously reported in the literature.⁴⁴ After the silanization using 3-(2,3-epoxypropoxy) propyltrimethoxysilane, the contact angle changed to 54°.⁴⁵ Finally, incorporation of NTA moieties originated the modification of the contact angle to 36° due to the appearance of carboxylic groups on the glass surface, and therefore the increase of the hydrophilicity of the surface.⁴⁶

Hexahistidine Tagged Proteins Immobilization. A very convenient and common way for purifying recombinant proteins is to modify their terminal end with a polyhistidine tag and immobilize them onto a Ni-NTA support. We developed the same strategy for attaching 6 \times His cadherin recombinant fragments to glass supports (Figure 1). The orientation of the proteins on the supports will be driven by the position of the histidine tag in the protein sequence. Cadherin amino groups in the polyhistidine tag proximity were then cross-linked to the NTA carboxyl groups mediated by the EDC/NHS, resulting in a simple and novel

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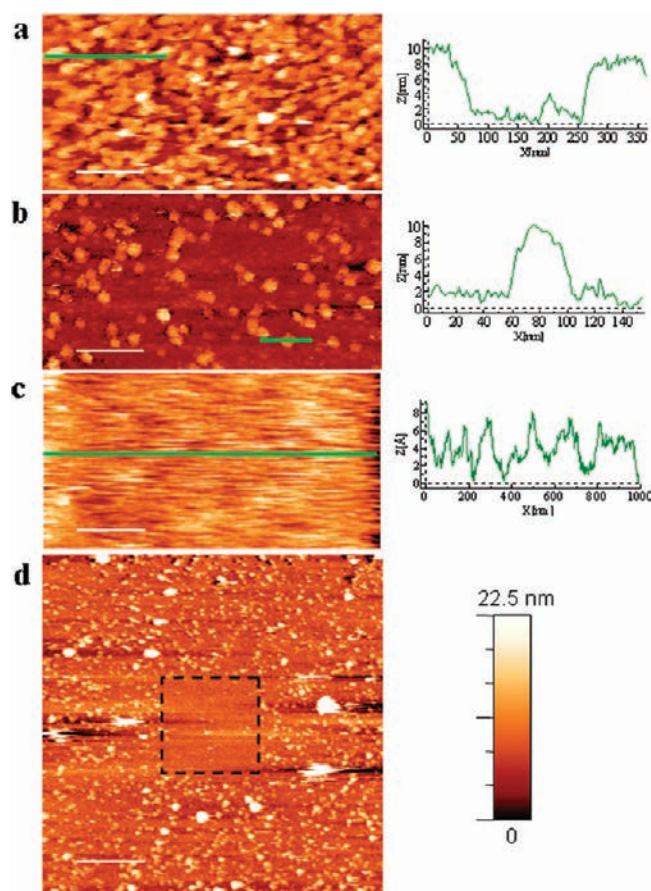


Figure 3. Dynamic atomic force microscopy analysis of surfaces. Images of the functionalized surfaces were taken under the same buffer conditions (PBS + Ca and Mg). (a) Coverage of the covalently bound protein is around 80%, with a coverage height of a single protein (~9 nm), as shown in the profile. The coverage of noncovalently bound protein (b) is about 15%, with a height also corresponding also to that of a single protein. By slightly increasing the interaction force between tip and sample on the noncovalently bound protein sample, the protein is easily swept away, as can be observed in the dashed square marked in (d) which appears depleted from protein after just one scan under these somewhat lower amplitude operating conditions. (c) Control sample, NTA+Ni. Such a surface exhibits a flat profile within a few angstroms of height. Scale bar = 200 nm (a, b, c); 600 nm (d).

method for binding 6 \times His-tagged proteins in an oriented and covalent manner. An EDTA/imidazole mixture was used to remove

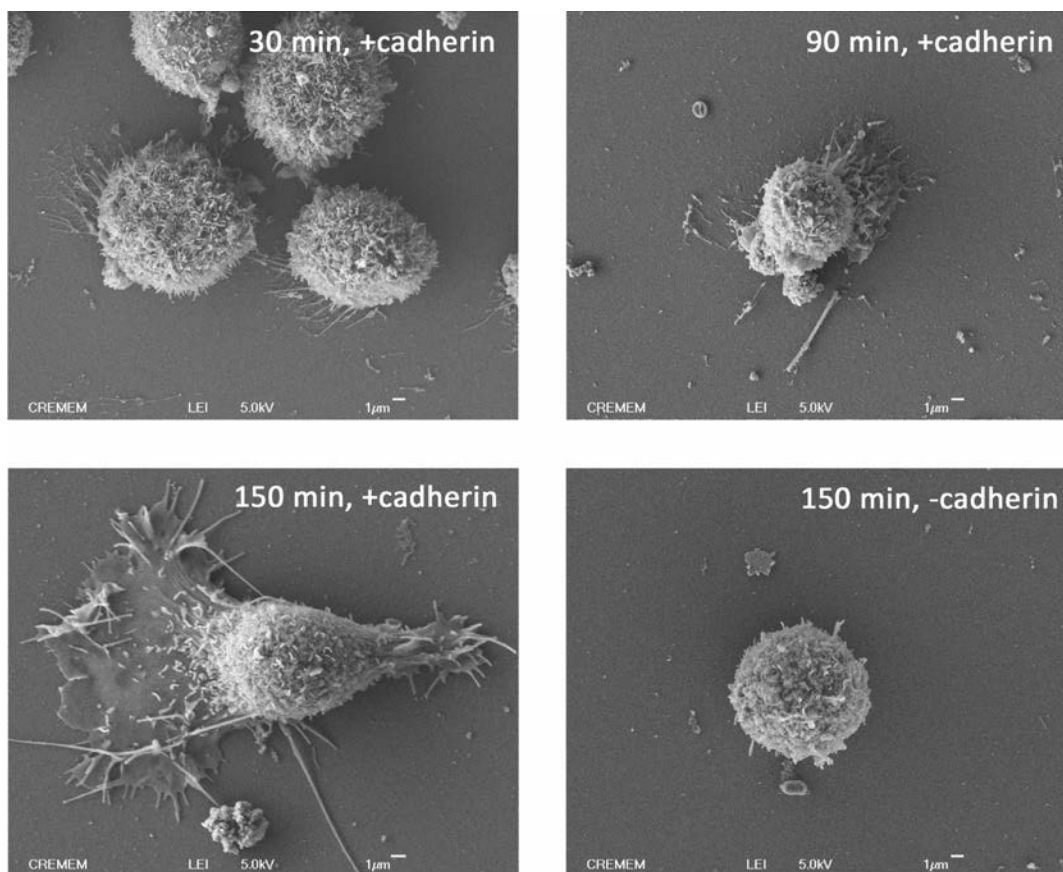


Figure 4. Dynamic cell spreading depends on surface biofunctionalization. HC11 cells were plated for different periods of time onto NTA-coated glass slides covalently decorated or not with cadherin fragments, as indicated on the images. Extent of adhesive contacts was visualized by scanning electron microscopy.

Ni^{2+} ions and subsequently eliminate the noncovalently linked proteins (Figure 2a,b).

In order to check the cadherin binding efficiency, we hid half of the glass support with a fresh and clean piece of polydimethylsiloxane (PDMS). Cadherin fragments were incubated on the non-hidden part of the supports, and then, the noncovalently linked proteins eliminated as was previously described. The protein remaining on the surface was visualized by immunofluorescence using a fluorescently labeled anticadherin antibody and confocal microscopy. As shown in Figure 2c, cadherin fragments were indeed detected only on half of the glass supports.

XPS analysis was performed to determine the surface elemental composition for each of the modification steps used in this study. The analysis confirmed the coupling of NTA to the glass surface (addition of carbon and nitrogen) and the cadherin incorporation (addition of carbon and nitrogen) (Table 1) (for more details in the XPS spectra, see Supporting Information Figure S1).

Atomic force microscopy (AFM) (Figure 3) has confirmed the formation of covalent bonds using this strategy, by the height of the protein layer. Figure 3a shows an image of the covalently bound cadherins. The surface protein coverage is high (about 80% of the surface area) and shows the expected height of the cadherin layer with respect to the Ni^{2+} -NTA surface (8–9 nm) as can be observed in the profile (more examples of surfaces profiles could be found in Supporting Information Figure S2). However, when we tried to image the noncovalently bound protein samples, we always wiped out the protein from the surface even for operation conditions in which we tip exerted minimum interaction force. We were not able to observe the protein in the noncovalently bound surface (Figure 3b) until we used a cantilever about

100 times softer than the ones used for the covalent sample. The surface coverage was low (about 15%), but the measured protein height corresponded to the expected one (8–9 nm), as shown in the profile. Still, if we increased the tip–sample interaction by just a few percent of the amplitude reduction we managed to sweep away the protein from the scanning area (dashed square marked in Figure 3d) as can be observed when an enlarged image is taken (Figure 3d). Images of control samples NTA+Ni (Figure 3c) and bare glass slide surface in purified H_2O showed the expected roughness, and no agglomerates adsorbed.

Adhesion of Cells to Biofunctionalized Glass Supports.

Cadherins participate in cell–cell contacts, especially in the specific cell adhesion mechanisms mediated by the highly homophilic interactions they establish and which define cell–cell selectivity. We choose the E-cadherin fragment E/EC12 to cover our glass surfaces and promote the adhesion of HC11 cells, which also express E-cadherin³⁴ on their plasma membrane.

For this purpose, we seeded HC11 cells, a mouse mammary epithelial cell line, on glass slides modified or not with our mouse E-cadherin fragments in an oriented manner and followed their kinetics of attachment from 30 min up to 2 h 30 min. We observed by phase contrast microscopy that, after 30 min, cells seem to gently adhere to the surfaces. Cell density was low at early time points and increased with time. The attachment of HC11 cells was similar for both surfaces (not shown), which is most probably due to the dynamic properties of cadherin trans interaction. Indeed, E/EC12 adhesive interactions that last for 0.1 to 1 s^{10,30} do not appear to have a major influence on attachment efficiency.

We then investigated the influence of cadherin-decorated surfaces on cell spreading. By phase contrast microscopy, we observed

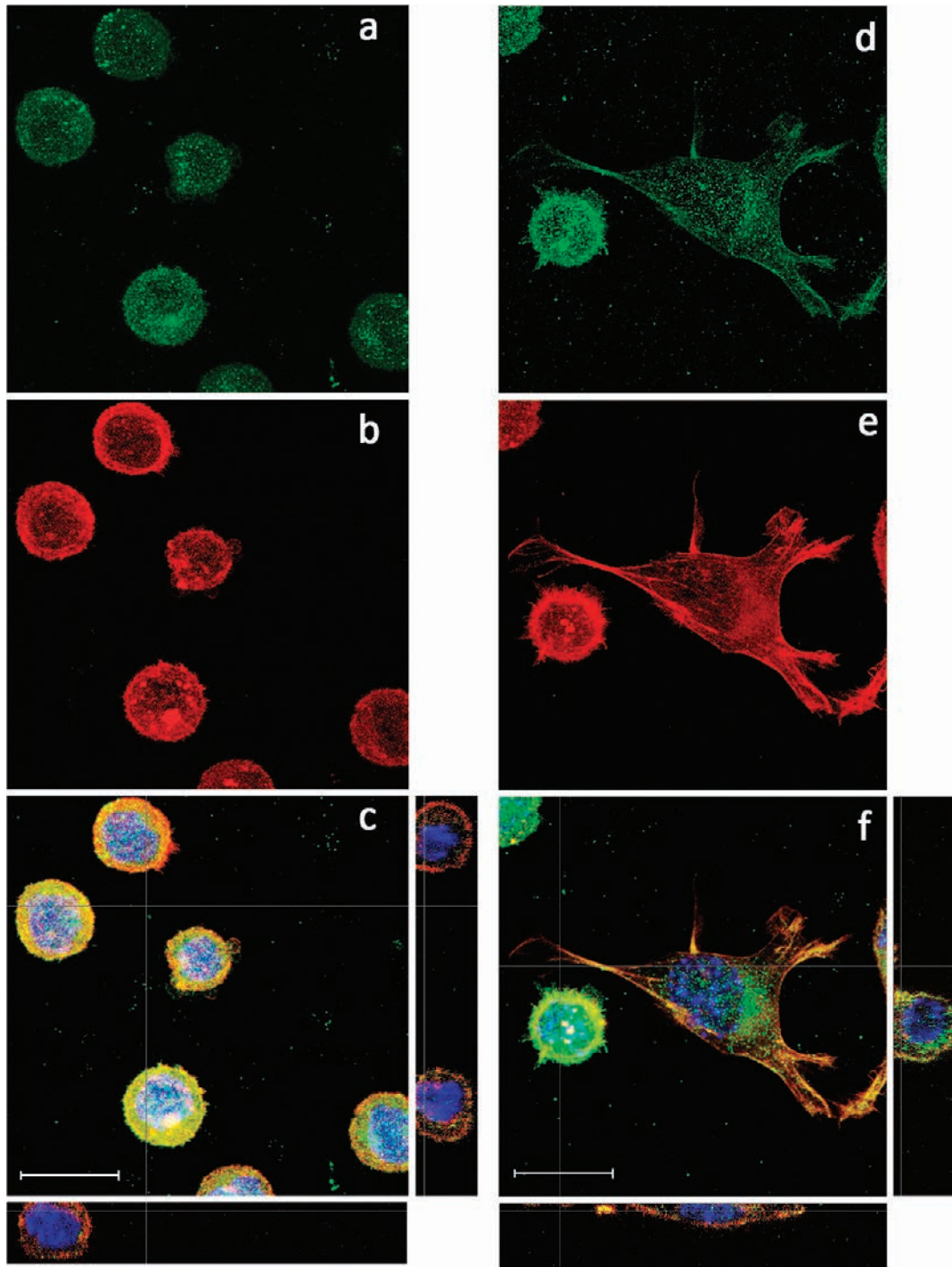


Figure 5. Characterization of cadherin adhesion topography. HC11 cells were allowed to adhere on NTA-coated (a,b,c) or covalently coated E-cadherin (d,e,f) coverslips for 1 h 30 min in the absence of serum to avoid integrins and growth factor receptor activation. Cells were fixed, permeabilized, and then stained for cadherin (green, a,d), actin (red, b,e), and DNA (blue, c,f in the merge images) and observed by confocal laser scanning microscopy. On cadherin-surfaces, many cells are well-spread and exhibit a large number of membrane tethers involving cadherin contacts. Most cells still appear spherical on NTA-surfaces (a,b,c). Corresponding orthogonal sections in the $x-z$ and $y-z$ axis are shown alongside, clearly indicating differences in spreading and height of cells on different surfaces. Round cells and spread cells are about 13–15 and 6–8 μm height, respectively. Scale bar = 15 μm .

that cells plated onto cadherin-decorated glass slides were mostly round at early time points then developed membrane extensions with time (30% and 38% of cells exhibited extensions after 90 and 120 min, respectively). These extensions were clearly less numerous on control NTA-surfaces (6% and 8% of cells exhibited extensions after 90 and 120 min, respectively). To gain information about these cell-surface contacts, cell morphology was analyzed using

scanning electron microscopy (SEM). After different incubation times, cells were fixed with glutaraldehyde and treated with contrast agents. As shown in Figure 4, analysis at higher magnification revealed that, after 30 min plating on cadherin-coated surfaces, cells began to establish contacts with some membrane extensions. After 1 h incubation, although most cells were still round, we could also observe that some adhered in a stronger

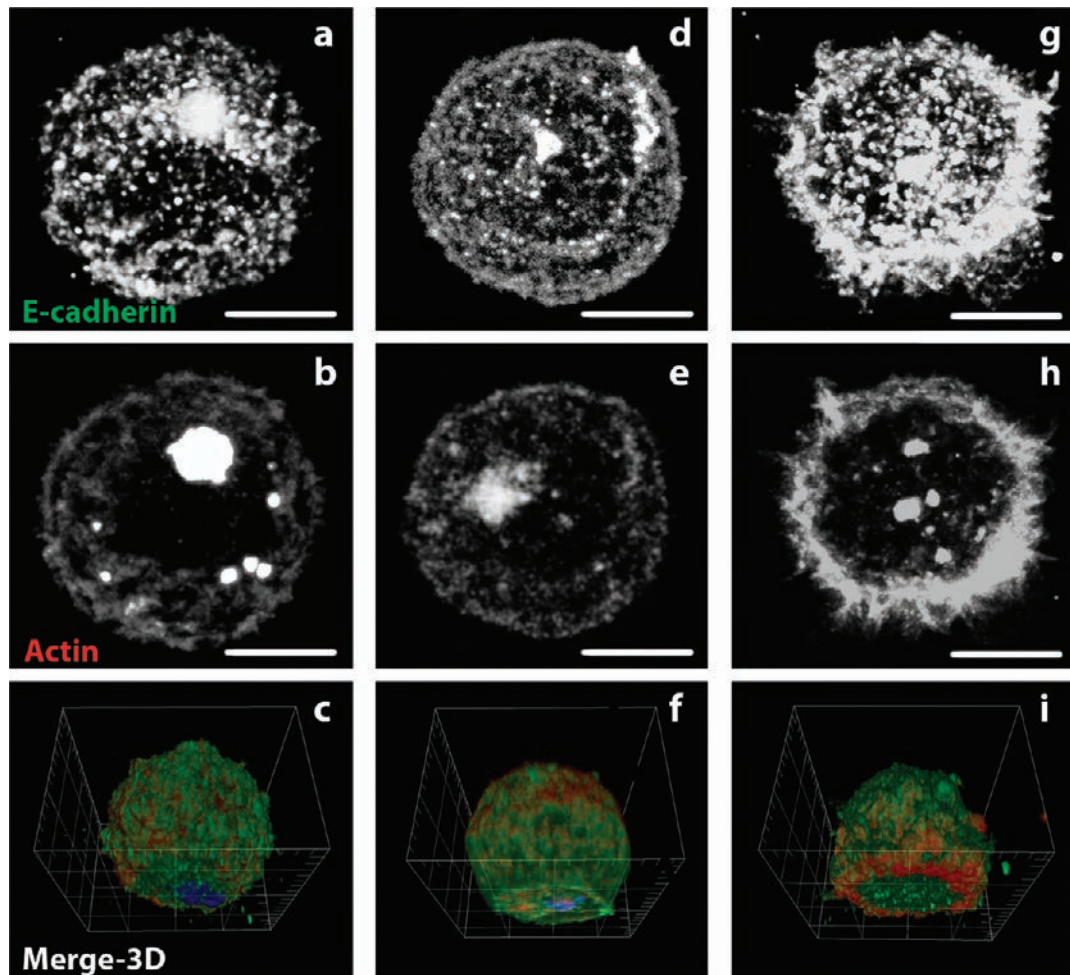


Figure 6. Effect of surface biofunctionalization on the recruitment of cadherin and actin at the contact zone. HC11 cells were plated on NTA (a–c), covalently but nonoriented E-cadherin (d–f), or covalently and oriented E-cadherin (g–i) coated surfaces for 1 h 30 min. The ventral zone of two round cells was compared for the distribution of cadherin and actin filaments. Panels show for each condition matched three-dimensional projections of $4\ \mu\text{m}$ confocal stacks. On all types of surfaces, E-cadherin appeared as puncta (a,d,g). However, oriented cadherin-decorated surfaces promoted a peripheral organization and lamellipodia protrusions (g). Interestingly, F-actin exhibited the typical radial organization driven by cadherin attachments (h) but a diffuse distribution on NTA- and nonoriented cadherin decorated surfaces (b,e). This difference in topography is emphasized in the merge 3D images of the corresponding cells (c,f,i).

manner on the surfaces. After 1 h 30 min and 2 h 30 min plating, many cells were well-spread even though some still exhibited a round shape. As shown in Figure 4, cells responded to our cadherin-decorated surfaces, as large membrane extensions were visible after 2 h 30 min, indicating that cells interact strongly with the substrate. Cells on control supports without cadherins behave in a different manner. After 2 h 30 min, few cells began to spread, and most of them showed a round shape. Higher magnification shows that cells did not engage significant membrane extensions to enhance their adhesiveness to the surface.

To further evaluate the functionality of the cadherin fragments after our protocol and analyze the impact of such surface biofunctionalization, we used confocal microscopy. We compared E-cadherin and actin distribution in HC11 cells adherent on surfaces covalently decorated with oriented and nonoriented cadherin fragments or NTA-covered surfaces (Figures 5 and 6 and Supporting Information Figure S3). We observed after 1 h 30 min plating similar topography as described in the SEM experiments: when the cells are cultured on cadherin substrates, many HC11 cells were well-spread and few still adopted a round shape. On these spread cells, the distribution of actin cytoskeleton was most dense at the cell periphery, whereas cadherins were more uniformly distributed on the cell surface as shown in the merge

image (Figure 5f). On cells attached to cadherin surfaces and exhibiting a round shape, fluorescent labeling highlighted that the actin cytoskeleton was characterized by a circular network of filaments surrounding the nucleus (Figure 5e), together with radial fibers directed toward and ending in the edge of the cell, as already described.⁴⁷

On substrates with no cadherin functionalization and as observed by SEM, cells did not spread (Figure 5a,b,c). Nearly all cells were round with uniform cadherin distribution. Actin did not form typical cytoskeleton patterns controlled by cadherins as described in Figure 5e.

To better analyze the formation of cadherin-mediated adhesions, we have focused on the ventral cell surface of E-cadherin expressing cells still in a round shape after 1 h 30 min plating, in contact with NTA (Figure 6a,b,c), covalent nonoriented (Figure 6d,e,f), or covalent oriented (Figure 6g,h,i) cadherin-decorated surfaces. E-cadherin exhibited a punctate distribution on all substrates. Near the cell edge where new contacts likely form, E-cadherin and actin filaments (circular and radial) colocalized, essentially on oriented

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cadherin surfaces. Finally, the topography, visualized by 3D reconstruction (Figure 6e,f), clearly showed different actin–cadherin distributions driven by cadherin fragments linked in an oriented fashion onto the surfaces. Such patterns were documented for N-cadherin expressing cell contacts.⁴⁸

Conclusions

Covalent, stable, and oriented attachment of proteins on surfaces is a very challenging field of biochemistry and cell biology. We have designed a fast and inexpensive method for the immobilization of oligohistidine-tagged proteins by exploiting the high recognition of this motif to the metal chelator nitrilotriacetic acid (NTA). Our technology could also be adapted to a broad type of different materials. The use of NTA technology may be extended to any type of 6×His tagged proteins, and could be a great development for biochips, biosensors, substrates for cell adhesion, and so forth. Most importantly, this method is compatible with a large set of fusion proteins while maintaining their integrity, native conformation, and biological function.⁴³ The protein immobilization on the surface is controlled with respect to selectivity: indeed, the orientation of the biomolecules on the surface is preferentially driven by the position of the histidine tag. With this manuscript, we have also highlighted the importance of

the orientation of adhesion proteins for their incorporation in functional biomaterials. As has been demonstrated in this work, different protein orientations/presentations drive different cytoskeleton organization, modifying cellular response against them.

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