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Specific capture of mammalian cells by cell surface receptor binding to ligand immobilized on gold thin films

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Abstract

Aldehyde-terminated self-assembled monolayers (SAMs) on gold surfaces were modified with proteins and employed to capture intact living cells through specific ligand-cell surface receptor interactions. In our model system, the basic fibroblast growth factor (bFGF) binding receptor was targeted on baby hamster kidney (BHK-21) cells. Negative control and target proteins were immobilized on a gold surface by coupling protein primary amines to surface aldehyde groups. Cell-binding was monitored by phase contrast microscopy or surface plasmon resonance (SPR) imaging. The specificity of the receptor-ligand interaction was confirmed by the lack of cell binding to the negative control proteins, cytochrome C and insulin, and by the disruption of cell binding by treatment with heparitinase to destroy heparan sulfate which plays an essential role in the binding of bFGF to FGF receptors. This approach can simultaneously probe a large number of receptor-ligand interactions in cell populations and has potential for targeting and isolating cells from mixtures according to the receptors expressed on their surface.

Introduction

Array technology is an important tool for the parallel analysis of biomolecules. (1–3) Examples include DNA arrays for gene expression measurements(3), protein arrays for evaluation of protein-protein interactions(1,2), and peptide(4,5) and carbohydrate arrays(6,7) for the evaluation of ligand-receptor interactions and for screening enzymatic activities. Microarray technology has been expanded to cell microarrays which can be used to study gene activity, protein expression and interaction between cell surface receptors and their ligands(8). In one kind of cell microarray, a small molecule, antibody or peptide microarray is generated and an assay is performed with living cells.

We distinguish two steps in cell adherence to a substrate; these are cell binding and cell attachment. Cell binding is necessarily the first step in cell adherence to a substrate. This binding can be either specific or non-specific. Specific binding of a cell-surface molecule to a substrate-coupled ligand would constitute a specific interaction; in contrast, non-specific binding of a cell to a plastic such as a microtiter plate would constitute a non-specific interaction. In either case, the initial interaction between cell and substrate may or may not be followed by cell attachment, in which the bound cell spreads, flattens, and possibly proliferates by cell division. To date, mainly protein and peptide arrays which test cell attachment to a predefined surface pattern have been devised (see (9–13)). For example, Falsey et al. printed

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peptide and small molecule arrays and performed adhesion assays with living cells.(12) There are a few examples of microarrays probing specific cell surface molecule-ligand interactions that do not necessarily involve subsequent cell attachment. Most are antibody microarrays, typically prepared on glass or nitrocellulose bound to glass, which selectively recognize and bind cell surface antigens.(14–16) For example, bacteria (*Escherichia coli* O157:H7) were captured on antibodies stamped onto silicon oxide substrates and detected using optical diffraction.(16) Belov et al. developed an antibody array which allows the determination of more than 50 clusters of differentiation (CD) antigens on leukocytes or leukemia cells in a single experiment.(15)

Here we present an example of cell binding, in which baby hamster kidney (BHK-21) cells are captured via the interaction between their bFGF receptors and basic fibroblast growth factor (bFGF) immobilized on a gold substrate. We have sought to minimize interference from non-specific cell binding and to have the cell binding be mediated solely by specific interactions between the surface-bound ligand and the cell surface receptor proteins. We have employed gold thin films on glass as substrates. The advantage of using gold is that the well characterized gold-alkanethiol chemistry facilitates the creation of versatile and well-defined substrate surfaces to control cell-substrate interactions, minimizing undesired non-specific interactions. Arrays prepared on gold thin films are compatible with many detection methods such as conventional phase contrast bright field microscopy, fluorescence microscopy, mass spectrometry (MALDI), and surface plasmon resonance (SPR) imaging, as well as electrochemical detection methods.(17–19) We also demonstrate the difference between cell binding and cell adhesion assays.

bFGF is an ~ 18 kDa, 155 amino acid protein, and is a member of the fibroblast growth factor family (FGF1–20).(20) Fibroblast growth factors have diverse biological activities including regulating cell growth, survival, differentiation and migration. Their biological response is mediated by binding to and activating cell surface tyrosine kinase FGF receptors (FGFRs). The phosphorylation of FGFRs triggers the activation of cytoplasmatic signal transduction pathways.(20) There are four fibroblast growth factor receptors and their binding affinity for various fibroblast growth factors is determined by their immunoglobulin II and III domains. (20) At least two of the receptors, FGFR1 (the *flg* gene product) and FGFR2 (the *bek* gene product) are high affinity receptors for bFGF.(20) bFGF interacts with both specific high affinity receptors and low affinity heparan sulfate proteoglycans on the cell surface.(21) The density of high affinity binding sites has been reported to be 120,000 sites/cell on BHK-21 cells.(22) The binding constants for the high and low affinity binding sites are 20–270 pM and 2 nM, respectively.(21,22) We tested the specificity of the receptor-ligand interaction in two ways. First, we used cytochrome C and insulin, which have been shown not to compete with bFGF for binding at the FGF receptor on BHK-21 cells, as negative controls.(21) Second, we were able to eliminate cell binding by treatment of the cells with heparitinase, an enzyme that degrades heparan sulfate which is required for the high affinity, specific interaction between bFGF and FGFR.(23)

Materials and Methods

bFGF was obtained from Invitrogen or was the generous gift of Drs. Mark Levenstein and James Thomson. Anti-bFGF monoclonal antibody that binds to residues 14–40 of bFGF was obtained from Oncogene Research Products (GF23L) and was labeled with amine reactive fluorescein (fluorescein 5 EX succinimidyl ester (F-6130) from Molecular Probes). Insulin, cytochrome C and heparitinase were obtained from Sigma.

Cell culture

BHK-21 cells were obtained from the American Type Culture Collection (Manssas, VA). BHK-21 cells were cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM) and F-12 Ham medium supplemented with 5% (v/v) fetal bovine serum (FBS), penicillin and streptomycin at 37°C in a humidified incubator containing 5% CO_2.

Array fabrication

Proteins were covalently immobilized on aldehyde or N-hydroxy succinimidyl (NHS) ester terminated gold surfaces using reaction schemes previously described for the attachment of amine-bearing biomolecules (Figure 1).(24)

Surface attachment chemistry for bright field microscopy: the gold coated glass substrate (1 nm chromium and 25 nm gold) was prepared using chemical vapor deposition yielding a thin layer of gold transparent to visible light and thereby facilitating the use of an inverted optical microscope.(25) Alkanethiol self-assembled monolayers were formed on the gold surface by soaking the gold surface in 1–10 mM di(10-decanal) disulfide ($(CHO-(CH_2)_9-S-)_2$)/ethanol solution for 24 hours.(24) After rinsing the surface with ethanol and water, we spotted three different proteins, bFGF, cytochrome C and insulin onto the aldehyde terminated surface using a homebuilt robotic spotter and incubated overnight at room temperature in a humid chamber. The concentration of the deposited protein solutions was 1 mg/mL bFGF, 5 mg/mL cytochrome C and 10 mg/mL insulin, respectively, dissolved in HEPES buffer (10 mM HEPES, 1mM Ca^{2+}, 0.1% NaN_3, pH 8.5). In the attachment reaction, the free aldehyde groups of the SAM react with the primary amine groups present on the lysine residues of the proteins. (24) Since the imine formed in this reaction is not air stable, $NaBH_3CN$ was added to the protein solution (50 mM final concentration) just before deposition to reduce the imine to a secondary amine.

Incubation of the protein-modified surface with cells for microscopic imaging

The protein modified surfaces were blocked with 1% BSA/1× PBS pH 7.3 solution. Then BHK-21 cells were trypsinized, washed twice with 1× PBS pH 7.3 and incubated with surfaces on ice (0°C) for 30 min at a density of ~ 10^6 cells/mL unless otherwise noted. After incubation the surface was washed three times with 1× PBS and imaged with an Olympus IX81 phase contrast microscope.

Surface attachment chemistry for SPR imaging: growth factor arrays were fabricated on SpotReady chips (GWC Technologies, Madison, WI). These chips are manufactured with 1mm circular gold spots in a 4×4 spot format surrounded by a hydrophobic barrier which allows for manual spotting. Proteins were immobilized on gold spots via their lysine-NH_2 groups using DSP (Dithiobis[succinimidylpropionate], Pierce) linker (Figure 1B). SpotReady chips were first soaked in a 10mM DSP solution in DMSO for 1hr. Following a DMSO wash, proteins (bFGF 1mg/ml and 0.2 mg/mL, insulin 50 mg/mL and cytochrome C at 5 mg/ml in HEPES buffer, pH8.3) were manually deposited (0.4–0.5 μl/spot) on the DSP activated gold spots and were allowed to react for 2 hours at room temperature in a humid chamber. The protein arrays were rinsed with PBS and mounted on the SPR imager sample cell with some buffer still clinging to the gold spot so as to avoid protein drying and denaturation.

Heparitinase treatment

BHK-21 cells were treated with different concentrations (5, 10 and 30 μl of a 0.1unit/100 μl stock) of heparitinase, for 30 minutes at 37°C. The treated cells were added to a fresh bFGF array immobilized on aldehyde terminated SAMs. After 30 min incubation on ice (at 0 °C) the chips were washed with PBS and the binding was monitored on the microscope.

SPR imaging measurements

All SPR data was collected on an SPR imager II (GWC Technologies, Madison, WI). The SPR imaging apparatus has been previously described.(17,26) Briefly, light from a collimated polychromatic source passes through a polarizer and impinges on the sample cell at a specific angle near the SPR angle. The reflected light passes through a narrow bandpass filter and is then detected at a fixed angle with a CCD camera to produce an SPR image. The SPR image captures the variation in reflected light intensities due to differences in film thickness (mass) or index of refraction that occur when unlabeled analyte binds to probes. The sample cell consists of a prism, a gold coated glass chip to which the proteins are tethered (an array), and a flow cell that delivers unlabeled analyte to the array.

The SPR imaging experiment was performed by taking two measurements: first the “reference” image was collected for a chip exposed to PBS, pH 7.4. Unlabeled BHK21 cells at known concentrations were then introduced in the flow cell and allowed to bind for 15 min. A “post binding” image was collected after washing the array with 2 mL of PBS. The “reference” image was subtracted from the “post-binding” image to generate a “difference” image (for example Figure 5A). Cell binding to particular probe spots results in increased reflectivity in the difference image due to the increase in mass on the gold surface. Images were collected and the reflectivity changes for each spot were quantified using V++ image analysis software (Digital Optics, New Zealand).

Results and Discussion

The presence of the proteins on the surface was confirmed using fluorescein labeled anti-bFGF and SPR (Figure 2B and 2C). Three different proteins, bFGF, cytochrome C and insulin were delivered onto an aldehyde terminated surface in the pattern shown in Figure 2A using a robotic spotter. The protein array was blocked with 1% BSA/1× PBS pH 7.3 solution, then incubated with fluorescently labeled anti-bFGF antibody, washed and imaged with a fluorescence scanner (Figure 2B). Comparison of Figure 2A and 2B confirmed the pattern of bFGF spots in the fluorescence image of the surface. No fluorescence was observed from cytochrome C or insulin spots. Attachment of all three proteins was also confirmed using SPR imaging. In SPR imaging, spatial differences in reflectivity due to differences in film thickness or index of refraction are measured across the sensor surface. Since the proteins were deposited onto a uniform aldehyde terminated surface, increased reflectivity from the background is a result of protein attachment. In Figure 2C, all the protein spots are brighter than the background, indicating that protein immobilization was successful. There is an intensity variation between spots which is consistent with the varying masses of the proteins (bFGF 18 kDa, cytochrome C 12 kDa, insulin 5 kDa).

When the protein-modified surfaces were incubated with BHK-21 cells, the cells only attached to the spots where bFGF protein was deposited (Figure 3). We used surfaces modified with three proteins, bFGF, cytochrome C and insulin deposited in a pattern shown in Figure 3A. Cytochrome C and insulin were shown not to compete with bFGF for binding at the FGF receptor on BHK-21 cells.(21) Cytochrome C is a protein similar in size (12 kD) and isoelectric point (pI = 10.7) to bFGF (18 kDa and pI = 10). Insulin, a smaller protein, has a mass of 5 kDa and its isoelectric point 7.4.(21,27) Figure 3B and 3C show that the cells (white dots) are bound to the surface in well defined spots and minimal binding on the aldehyde background is observed. From comparison of Figure 3A and 3B, it is clear that BHK-21 cells specifically bound to spots where bFGF was deposited. On the neighboring spots, where cytochrome C and insulin were spotted, no cells are bound. This observation confirms that we are probing a specific interaction between the high affinity receptors on the BHK-21 cell surface and bFGF.

We further tested the specificity of the cell capture by treating the cells with heparitinase prior to incubation with the bFGF-modified surface. Cell surface heparan sulfate proteoglycans play a critical role in the FGF-FGF receptor interaction.(23) The heparan sulfate acts as a bridge in the formation of FGF/FGFR complex and thereby increases its stability. Thus, it is expected that destruction of heparan sulfate would decrease the specific capture of BHK-21 cells to bFGF modified spots. To test this hypothesis, BHK-21 cells were treated with different concentrations of heparitinase to degrade the heparan sulfates on the cell surface. The treated cells were then washed with PBS and added to the aldehyde terminated SAMs spotted with bFGF. The number of BHK-21 cells captured on the immobilized bFGF decreased with increasing concentrations of heparitinase (Figure 4). Heparitinase treatment at 0.01 units/mL abolished binding of BHK-21 cells to bFGF modified spots (Figure 4D). Treatment with concentrations lower than 7×10^{-5} units/mL had no effect on binding. This experiment further confirms that capture of BHK-21 cells is indeed mediated by a specific interaction between immobilized bFGF and its receptor on the cell surface.

We also explored the use of SPR imaging to detect cell capture on the surface. SPR technology is widely used to monitor binding events on chips because biomolecular interactions can be measured in real time without need to label the analyte.(17) SPR imaging combines the advantages of SPR measurements (i.e. real time, label free and surface sensitive) with spatial resolution.(17,28) In an array format, analysis is very rigorous as spots that carry control probes are exposed to the analyte at the same time and under the same experimental conditions. Since its invention it has been applied to many biomolecule-modified surfaces such as DNA, protein and carbohydrate microarrays to study interactions between biopolymers (for a recent review see (17,26,28). SPR images of the protein array prepared as described in the experimental section were collected in PBS before and after cell binding. The reflectivity change that occurs on each protein spot in the “difference image” (Figure 5A) is indicative of the mass of material bound at that position, within the vertical SPR-sensitive distance of approximately 100 nm. (17,29) The signal is greatest on the bFGF spots, consistent with the binding specificity evident from visible microscopy observations as shown in Figures 3 and 4. Interestingly, signal corresponding to non-specific binding is evident for the insulin and cytochrome C spots in the SPR imaging results, whereas no such non-specific binding is observed by visible microscopy. This is likely a reflection of the ability of SPR to detect low levels of surface-adsorbed proteins or other species present in solution. Such surface-adsorbed species do not generate sufficient contrast to be detectable by visible microscopy. The SPR signal change was very reproducible among spots that carried the same proteins (Figure 5). The spatial resolution of these SPR imaging experiments is of the order of tens of microns (for example, for a SPR wavelength of 676.4 nm and 44 nm thick gold, the spatial resolution is 14 μm) which is enough to resolve spots, but not individual cells.(17) However for a binding assay, high resolution is not necessary and SPR imaging provides a simple and compact experimental design.

To further probe the specificity of the cell capture, we performed a competition experiment (data not shown). BHK-21 cells (10^6cells/ml) were incubated with bFGF at 50 μg/ml for 30 min. These cells were then washed with PBS and added to a chip with bFGF and insulin spots. The binding of these bFGF preincubated cells to the immobilized ligands was monitored by SPR imaging. We observed that exposing BHK-21 cells to bFGF prior to adding the cells to the protein array, decreased but did not abolish cell binding to immobilized bFGF. There are up to 120,000 bFGF receptors on the surface of BHK21 cells and we do not know how many receptors need to interact with the immobilized ligand for a cell to be captured. Although cell binding was not completely abolished, we believe this data supports that cell capture on the array is a result of a specific interaction between the bFGF on the array and FGFR on the cells.

To test whether cell binding to bFGF led to cell attachment and growth, we exposed cells captured on the protein array to conditions conducive to cell growth (Figure 6). First, a bFGF-

modified surface was incubated with BHK-21 cells suspended in PBS as described previously and as shown in Figure 3 (Figure 6A). Note that no cells were observed attaching to the aldehyde background and that the cells captured on the bFGF-spots are rounded in shape. The surface shown in Figure 6A was then placed in medium (1:1 DMEM and F-12 Ham supplemented with 5% (v/v) FBS, penicillin and streptomycin) and incubated for 2 days at 37 °C and 5% CO_2 (Figure 6 B). The cells on the bFGF spots were still rounded in shape showing that they did not attach and grow. We thus conclude that binding to bFGF doesn't support cell growth. This result is surprising since there have been previous reports that bFGF can promote cell attachment.(30–32) On the other hand, cells did start to attach and spread over the background aldehyde terminated surface. Furthermore these cells had a different morphology; they had flattened, a sign of surface attachment and multiplied, as the number of cells increased with time of incubation.

One hypothesis to explain these observations is that BHK-21 cells attach to the aldehyde-modified surfaces because proteins which support attachment and growth are adsorbed to the surface from the fetal bovine serum present in the cell growth media. (33,34) This adsorbed protein layer may then facilitate cell attachment. The bFGF-mediated cell binding experiments presented here target a specific cell surface receptor and are thus at present better defined chemically than are the binding and attachment processes observed on the aldehyde-modified surfaces.

In summary, a protein modified surface was prepared using aldehyde/amine chemistry and was used to specifically capture BHK-21 cells through their bFGF receptor. The specificity of the receptor-ligand interaction was confirmed by the lack of binding to cytochrome C and insulin and decreased bFGF-binding ability of BHK-21 cell via destruction of heparan sulfates on the cell surface. The gold substrate allowed use of conventional phase contrast microscopy or SPR imaging as a detection method. This approach can simultaneously probe large numbers of receptor-ligand interactions in whole cell populations. A natural extension of the present work is the demonstration of this method with a wide range of receptor-ligand pairs. Indeed, we have successfully used this approach to investigate carbohydrate-lectin interactions(35) and are currently investigating cell surface receptors involved in stem cell self-renewal and angiogenesis of endothelial cells. The approach presented here also has potential for targeting and isolating cells from mixtures according to the receptors expressed on their surfaces. Work investigating this possibility is in progress.

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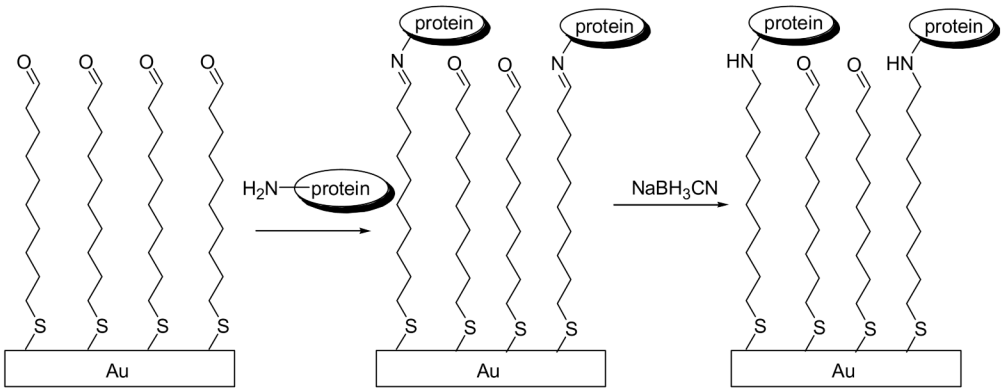
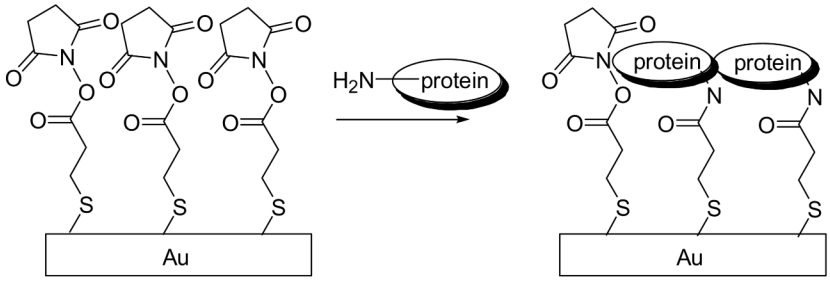


Scheme A



Scheme B

Figure 1.

The reaction of primary amines with aldehyde and N-hydroxy succinimidyl (NHS) ester functionalities on the gold surface. Scheme A) An aldehyde terminated gold surface is reacted with amine groups in proteins. The imine formed in the first step is then reduced to a secondary amine using $NaBH_3CN$. Scheme B) DSP (Dithiobis[succinimidylpropionate]) activated gold surface is reacted with protein; NHS ester reacts with the free amines of the lysine residues to form a stable amide bond.

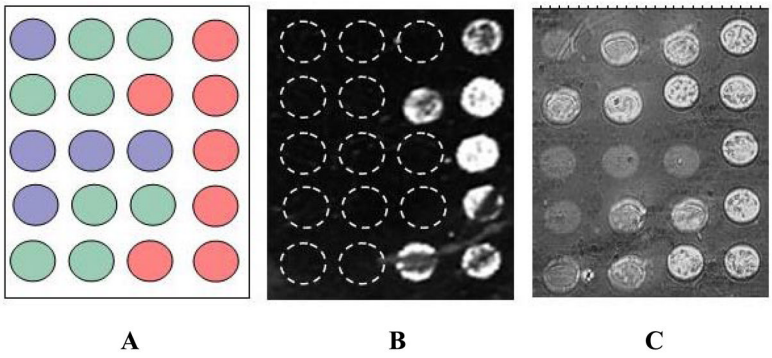


Figure 2.

Gold/(CHO-(CH2)$_9$-S-)$_2$ linker/protein surfaces used in cell capture experiments. A) Pattern of proteins: bFGF spots are shown in red, cytochrome C in green, insulin in blue. B) Fluorescence image of the surface after incubation with labeled anti-bFGF antibody C) SPR image of the protein modified surface

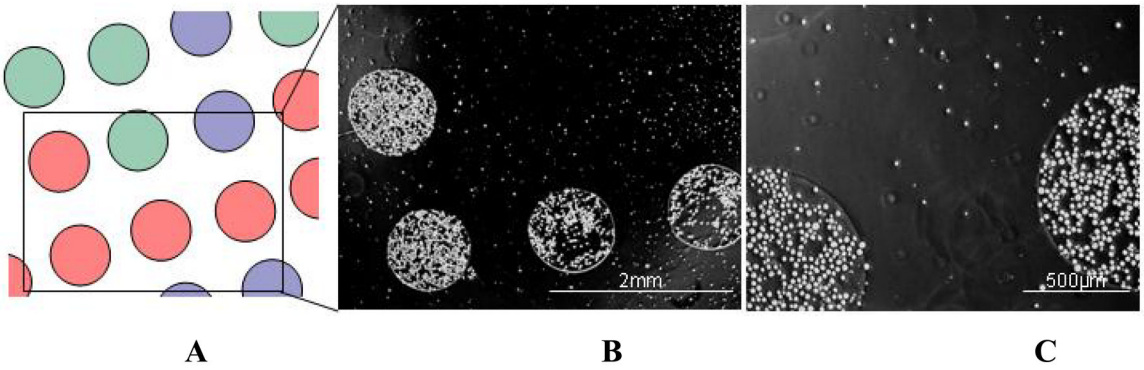


Figure 3.
Photomicrograph of cells bound to bFGF immobilized on aldehyde-terminated gold surfaces. A) Array pattern: bFGF (red), cytochrome C (green), insulin (blue) B) Image of protein modified surface after incubation with BHK-21 cells. C) Magnification of a section of B

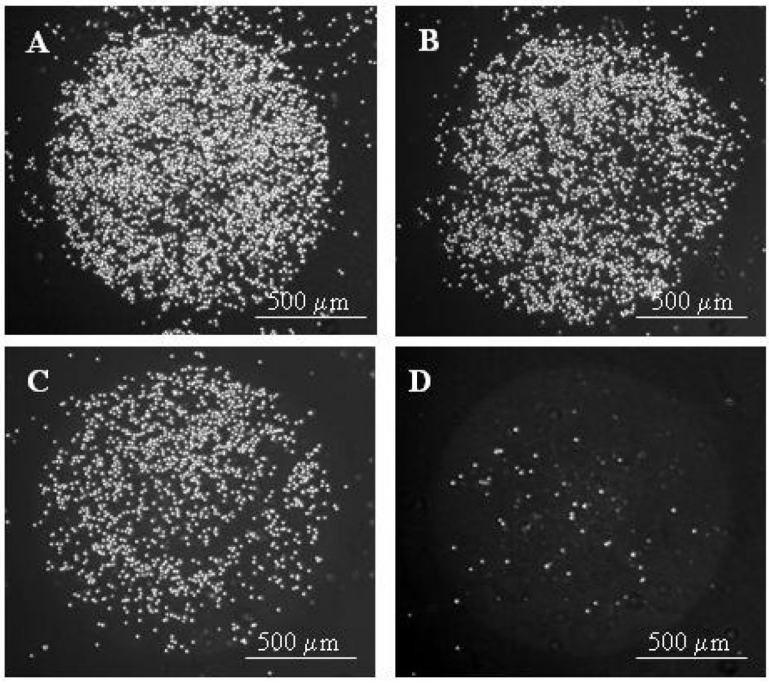


Figure 4.

Capture of heparinitase treated cells to immobilized bFGF. bFGF was immobilized on an aldehyde-terminated gold surface. Surfaces were incubated with BHK-21 cells at 3×10^5 cells/mL which had been treated with A) no heparitinase B) 0.0017 units heparitinase/mL C) 0.0033 units heparitinase/mL D) 0.01units heparitinase/mL

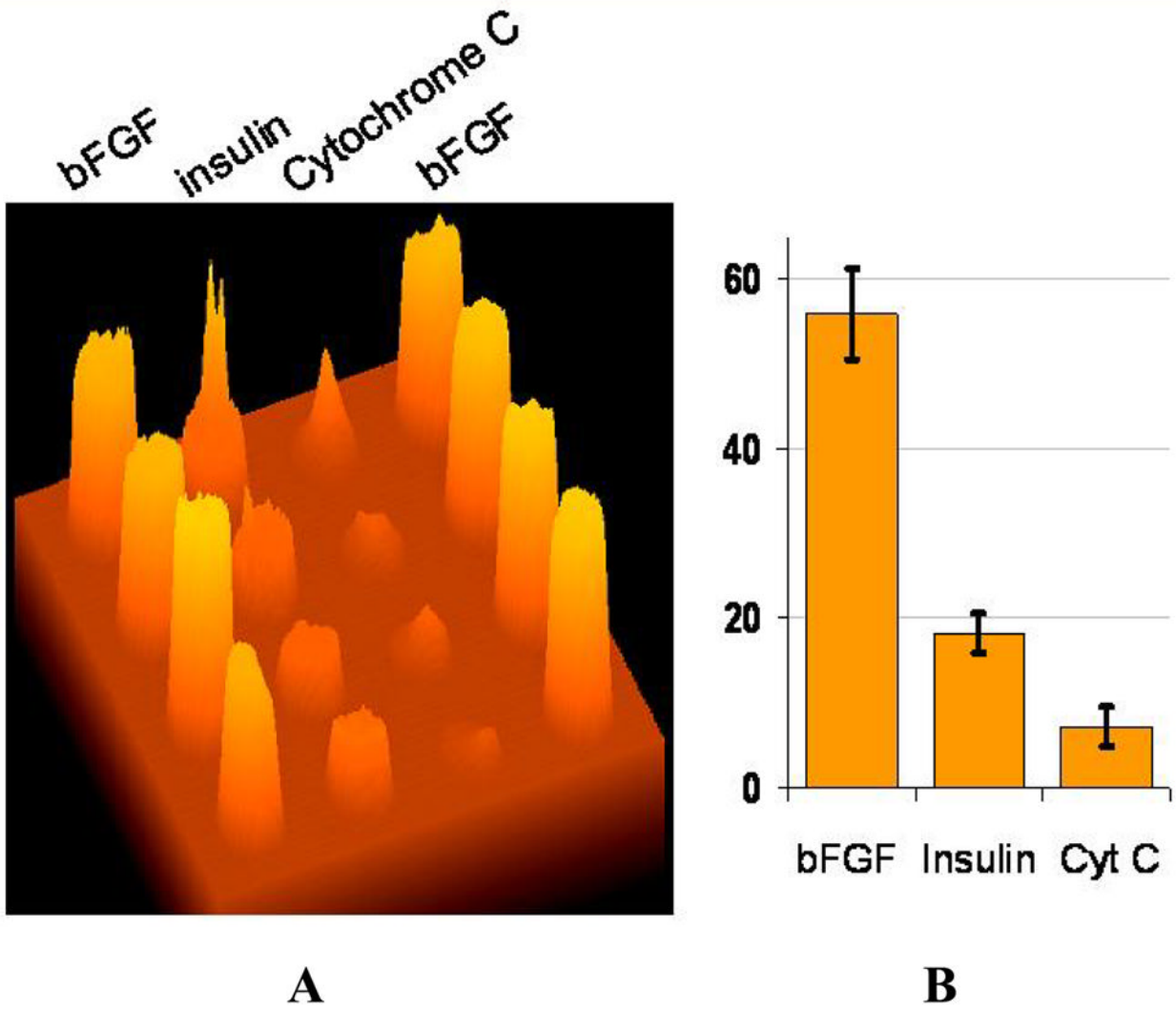


Figure 5.

Cell capture monitored with SPR imaging. Proteins were immobilized on a DSP-activated gold surface (Scheme B in Figure 1). A) Color enhanced surface plot of a difference image of cells captured on a protein array. The spotted protein concentrations were: bFGF (3 mg/mL, column 1 and 0.2 mg/mL, column 4), insulin (50 mg/mL, column 2), and cytochrome C (5 mg/mL, column 3). The SPR difference image was obtained by subtracting the reference image taken before addition of BHK-21 cells from the image taken after incubation with BHK-21 cells. B) Average and standard deviations of SPR signals obtained in A.

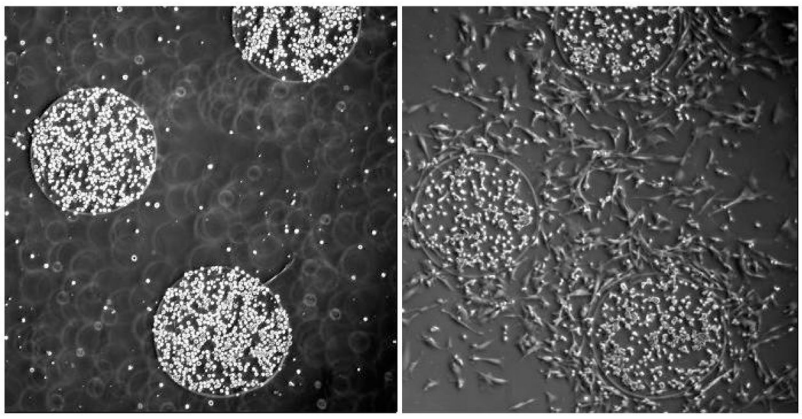


Figure 6.

Cell capture and cell adhesion on bFGF-modified surfaces under different experimental conditions. bFGF was immobilized on an aldehyde-terminated gold surface. A) BHK-21 cells captured on bFGF (4°C on ice in PBS for 30 min. as described in the experimental section) B) the same surface shown in A) was placed in medium (1:1 DMEM and F-12 Ham supplemented with 5% (v/v) FBS, penicillin and streptomycin) and incubated at 37C, 5% CO_2 for 2 days.