Subproject E2.2

Nanostructured Templates with Cadherin Specific Adhesive Properties

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Introduction and Summary

During the recent years the cellular microenviroment has become of growing interest in the stem cell research. Stem cells are characterized by their ability to self-renew and to differentiate into a broad set of specialized cell types. Specific properties of the cellular microenvironment control maintenance of the stem cell fate while others promote stem cell differentiation [1]. Continuously proliferating stem cells are housed in a microenvironment called the stem cell niche. Cells leave the stem cell niche to enter the amplification zone, another microenvironment that stimulates the production of progenitor cells. When the latter settle in new microenvironments they start differentiation [2]. The specific biochemical and biophysical conditions of the new cellular surrounding decide about the cell type [3, 4].
The ability of a certain microenvironment to control stem cell renewal or stem cell differentiation relies on its molecular components like extracellular matrix molecules, adhesion proteins and growth factors but also on physical properties like stiffness and elasticity of the extracellular scaffolds and their topography. Reproducing this natural organization with tools developed by nano- and microtechnology is the challenging question addressed in E2.2. Several micro-engineering tools have already been applied to create an appropriate cellular surrounding with promising results in manipulating cell behavior summarized by Liu and Chen [5].
In order to control cell shape and spacing we made use of tailored surface structures generated by different techniques like micro contact printing (μCP), chemical etching or lithography techniques. To ensure that cells undergo specific biological interactions with the inanimate surfaces we developed novel immobilization methods for adhesion proteins. Adhesion processes play a key role in microenvironments. Each of them - stem cell niche, amplification or differentiation zone - consists of a certain combination of cell-cell and cell-substrate adhesion molecules [6]. The bottleneck in surface functionalization is the lack of smart linker molecules that provide multiple immobilization procedures in parallel. Another drawback is the lack of biologically inert surface materials, a prerequisite for specific ligand localization. To overcome these disadvantages we followed different surface functionalization strategies, covalent coupling of peptides as well as non-covalent and covalent binding of His- or Snap-tagged proteins. The method to use the His-tag for surface immoblization was developed by Robert Tampés group [7,8]. An elegant method to bind proteins covalently to surfaces provides the SNAP-tag [9], and more recently also the CLIP-tag [10], both designed by Kai Johnssons group. We have selected these tag-systems because we also can control the surface orientation of the adhesion proteins. In comparison to the widely used Fc-tagged cadherins [11,12] we aim to reduce the number of bridging elements like protein A or antibodies. Cadherins are important proteins in stem cell niches [13]. Apart from their adhesive function they influence cell differentiation and control cell migration [14]. These different functions can be assigned to individual cadherin subtypes but also depend on specific cell types. Murine stem cells, for example, maintain stem cell fate when plated on E-cadherin [15]

1. Design of Surface Structures

To succeed in the preparation of peptide or protein decorated nano- or microstructures both, materials and the chemistry (1) used for structuring the surface and (2) used for immobilizing the peptides or proteins have to be compatible. The chemical functionalization of oxidic surfaces (e.g. glass, SiO_2 wafers, ITO etc.) is usually carried out by silane chemistry wheras the silanes are solved in organic solvents. However, these strategies can not be applied for polymer substrate, as the

solvent would solve the substrate. Therefore, one goal of the project E.2.02 is the development of alternative surface structuring techniques which are compatible with the coupling strategies for (1) gelatin, (2) laminin-, fibronectin- or collagen-specific peptides, and (3) SNAP-tag and His-tag based coupling procedures to cadherin ectodomains.

In the last funding period we followed different pathways of surface structuring and functionalization with results, briefly summarized in the following:

- We tailored established structuring techniques to develop a highly reproducible method for the covalent and non covalent coupling of peptides and proteins [16, 17, 18, 19], to surfaces patterns to enable the specific adhesion of stem cells on the patterns. As a new result the site selective surface functionalization of polymer surface patterns with gelatin was successfully carried out [20]. A European patent for the UV-light lithography based structuring of polymers could be achieved [21]

- In cooperation with the group of Prof. Ron Naaman at the Weizmann-Institute (Rehovot, Israel) the new method of magneto lithography [22] could be successfully applied for a SNAP-tag based coupling of a fusion protein.

- The SNAP-tag based coupling strategy of model proteins could be transferred to functionalized quantum dots

1.1 Using UV-light lithography for specific stem cell adhesion on polymer patterns

By the irradiation of polymer surfaces with UV-light the physical, chemical and biological surface properties are changed as *e.g.* wettability or biocompatibility [23, 24, 25, 26]. In the case of conventional optical lithographic techniques which are based on a light induced chemical modification of the surface, the different chemical behavior of the irradiated sample areas compared to that of the non irradiated surface areas (e.g. against solvents) is used to immobilize gelatin site selectively for stem cell adhesion. This method which was patented and published recently [27, 20, 21,] is characterized by two new aspects compared to hitherto existing methods: (1) The dimensions of the structures (lateral and vertical dimensions) can be controlled merely by the irradiation dose and (2) the structures are chemically functionalized during the irradiation so that structure formation and chemical functionalization are both achieved in one step without further processing. The schematic preparation process for the site selective immobilization of gelatin on UV-irradiated polystyrene areas and stem cells adhesion is shown in Figure 1a. More details for the preparation are published in [20].

Results

When PS is irradiated by UV light in an oxygen-containing atmosphere three phenomena can be observed: (i) the hydrophilization of the surface due to the oxidation induced by irradiation, (ii) the radical initiated cross-linking of the polymer chains causing a reduced solubility against solvents of the non cross-linked polymer and (iii) the degradation of the polymer surface yielding gaseous oxidation products *e.g.* CO_2 (photo etching) [25, 26]. As the irradiation of the sample for gelatine based stem cell adhesion was limited to 3 minutes, the degradation of the polymer can be neglected. However, it was found by time of flight secondary ion mass spectrometry (TOF-SIMS) that the irradiated areas are functionalized with OH-groups (Figure 1b), while those surface areas which

were covered by the mask are non oxidized and show the signal of the molecular fragment CH in the negative TOF-SIMS spectrum, which is significant for non modified polystyrene. Those oxidized polystyrene areas can be functionalized by the immobilization of gelatin. To prove the site selective functionalization with gelatine, in cooperation with the group of Dr. M. Bruns (IMF-III / KIT) XPS was carried out by using the N1s peak as the characteristic element for the gelatin. In Figure 1c the XPS spectra of a sample are shown which was coated with polystyrene and which was irradiated through a TEM grid with UV light. The increase in the N 1s signal intensity going from non-irradiated to irradiated areas is significant and proves the selective adsorption of gelatin on the irradiated surface areas. The reason that the non-irradiated areas show a weak signal for N 1s is the limited lateral resolution of the XPS which is with 30-40 µm within the size of the created structures so that the signal which is averaged always contains information of both, the irradiated and the non irradiated areas.

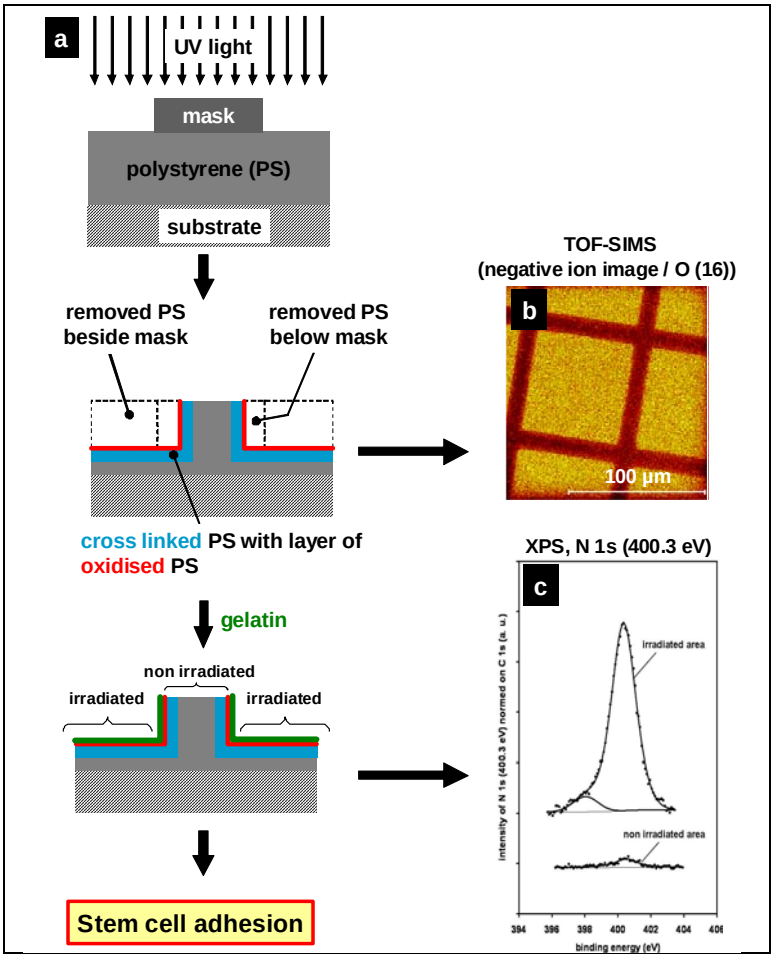


Figure 1
Scheme of the preparation of gelatin coated UV light irradiated polystyrene areas for selective stem cell adhesion (a). (b) shows the lateral distribution of oxide functional groups determined by TOF-SIMS and (c) proves the site selective immobilization of gelatin on the irradiated areas by XPS [20].

The site selective adhesion of stem cells on the irradiated and gelatin functionalized surface areas is shown in Figure 2a-d. Images 2c and 2d were obtained by zooming into the surface areas displayed in 2a and 2b, respectively. For the sample shown in Figures 2a and 2c no Pluronic (-PL) was used as additive during the cell adhesion, while for the samples shown in Figures 2b and 2d Pluronic was added(+PL). Without Pluronic a specific cell adhesion on the irradiated areas can be observed, however, some cells also adhere on the non-irradiated areas. In presence of Pluronic an increase of the selectivity to nearly 100% can be observed and the adhesion of cells on the non-irradiated areas of the polymer can be avoided. The statistical figures in Figure 2e and 2f summarize the results quantitatively.

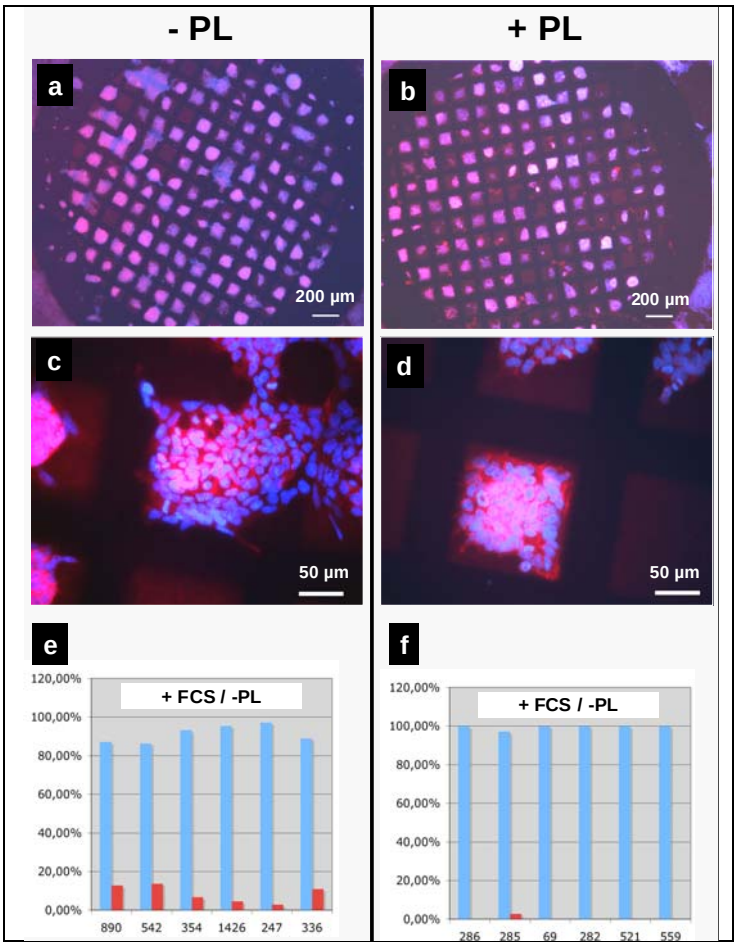


Figure 2
Fluorescence images of murine stem cells immobilized on the UV-light irradiated and gelatin coated areas of a polystyrene film (small squares). (a) and (c) show the cell attachment without Pluronic. Although a clear site selective cell adhesion can be recognized, some cells also adhere on those areas, which were not irradiated (c). The selectivity of cell attachment is increased by using Pluronic ((b) and (d). (e) and (f) show the quantification of selectivity with and without Pluronic, respectively. Each blue/red bar pair represents a new experiment [20].

1.2. SNAP-tag based coupling of proteins on surfaces

As described above both, silane based chemistry and the fuctionalization of UV-irradiation polymer substrates with gelatin were developed and applied for site selective cell attachment. Although, the gelatin adsorption is site selective, the coupling of gelatin to the substrate is not specific. Therefore, a SNAP-tag based coupling strategy was applied to immobilize poteins specifically on substrates while the His-SNAP-GFP fusion protein is used as a model and “easy-to-detect” protein. The goal is to use this coupling strategy to immobilize a His-SNAP-E-cadherin. In the following the results of the

1. site selective coupling of the HIS-SNAP-GFP fusion protein on planar, bezylguanine terminated surface areas by applying a new kind of structuring technique called magneto lithography and

2. coupling of the HIS-SNAP-GFP fusion protein to benzylguanine (BG) functionalized nanodots

are summarized.

Results of HIS-SNAP-GFP coupling by magnetolithography

The schemes for the magneto lithography process and for the coupling mechanism of the fusion proteins are shown in Figure 3. The experiments were carried out in the labs of Prof. Ron Naaman at the Weizman-Institute in Rehovot, Israel where the method of magneto lithography was developed [22]. For the site selective immobilization of the benzylguanin on a cover glass, the substrate was positioned above a metal wire which bundels the magnetic field of the permanent magnet. Then the suspension of magnetic nanobeads was dropped on the glass, while the o-ring avoids the spreading of the liquid over the whole substrate. The nanoparticles arrange along the longitudinal axis of the magnetized wire on the substrate and cover the substrate from further chemical reaction. Then a silanization step with octadecyl-trichloro-silane (OTS) follows where all glass areas, which are not covered by the nano beads are terminated with a CH_3-group . Then the magnet and the wire are removed and the nanoparticles are washed away. The non-functionalized surface areas are then functionalized by a NH_2-terminated silane. Benzylguanine which is terminated with an N-hydroxy-succinimide (NHS) ester group is coupled to the aminated surface areas. In the final step the thiolate group of the fusion protein (here green fluorescing protein, GFP) reacts with the BG by forming a thioether group and cleaving off the BG group.

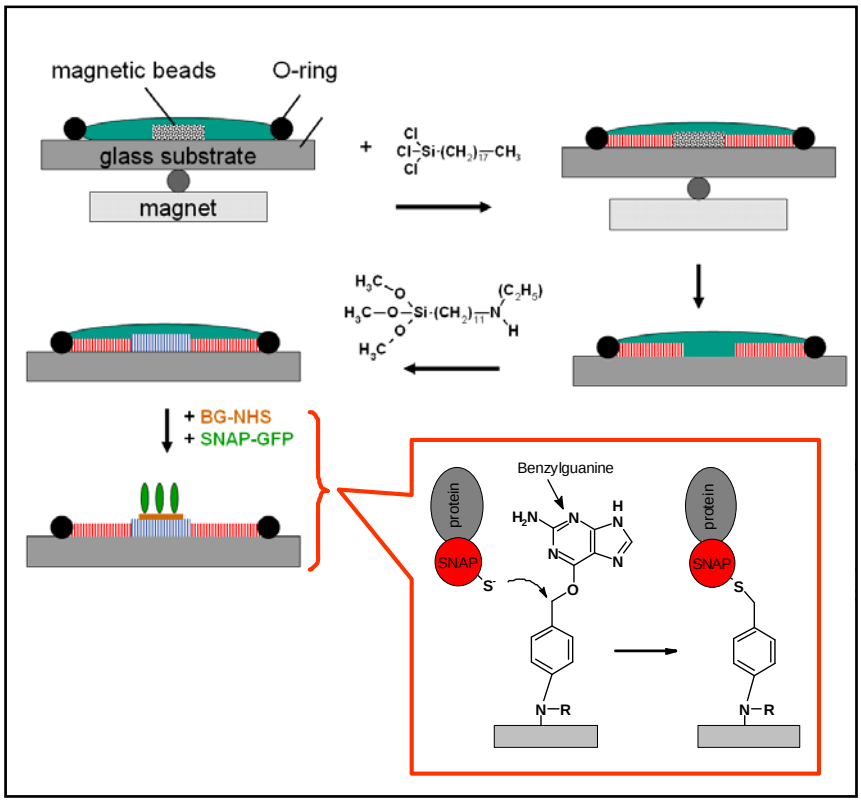


Figure 3
Scheme of the site selective coupling of SNAP-Tag fusion proteins by applying magneto lithography. The chemical coupling of fusion proteins shown in the red box is also applied for nanodots.

In Figure 4 the fluorescence images of areas on a glass substrates are shown which were homogeniously functionalized with the His-SNAP-GFP fusion protein in different ways. In Figure 4(a)/4 the positive experiment shows a bright spot indicating the successful coupling of the His-SNAP-GFP by the reaction with BG, which was covalently coupled to the aminosilane functionalized glass substrate. The samples shown in Figures 4(a)/1-3 are negative control experiments, where the solution of the protein was dropped on non functionalized glass (Figure 4(a)/1), on aminosilane functionalizd glass (Figure 4(a)/2) and on BG, physisorbed on a glass substrate (Figure 4(a)/3), respectively. Only in the case of the aminosilane functionalized glass a weak fluorescence can be detected, which is the result of the unspecific adsorption of the His-SNAP-GFP on the silane layer. In Figure 4(c) a 400 µm wide line of chemically bound of His-SNAP-GFP protein can be detected. The line was prepared according to the scheme shown in

Figure 3. As negative control, fusion protein solution was dropped (i) (Figure 4(b)/1) and (ii) on a ODT terminated glass substrate with physisorbed BG. The sample shown in Figure 4(b)/3 was prepared as the sample in Figure 4(a)/4 and shows the fluorescence of the His-SNAP-GFP protein bound via the covalently to an amino-functionalized glass substrate coupled BG. The intensity difference between the fluorescence of the line and the fluorescence of the areas beside the line is identical to the difference in fluorescence intensity between sample b/3 and b/1. Thus it can be concluded that there is a significant unspecific adsorption of the protein to the OTS terminated surface, however, the specific BG-based binding of the His-SNAP-GFP is preferred.

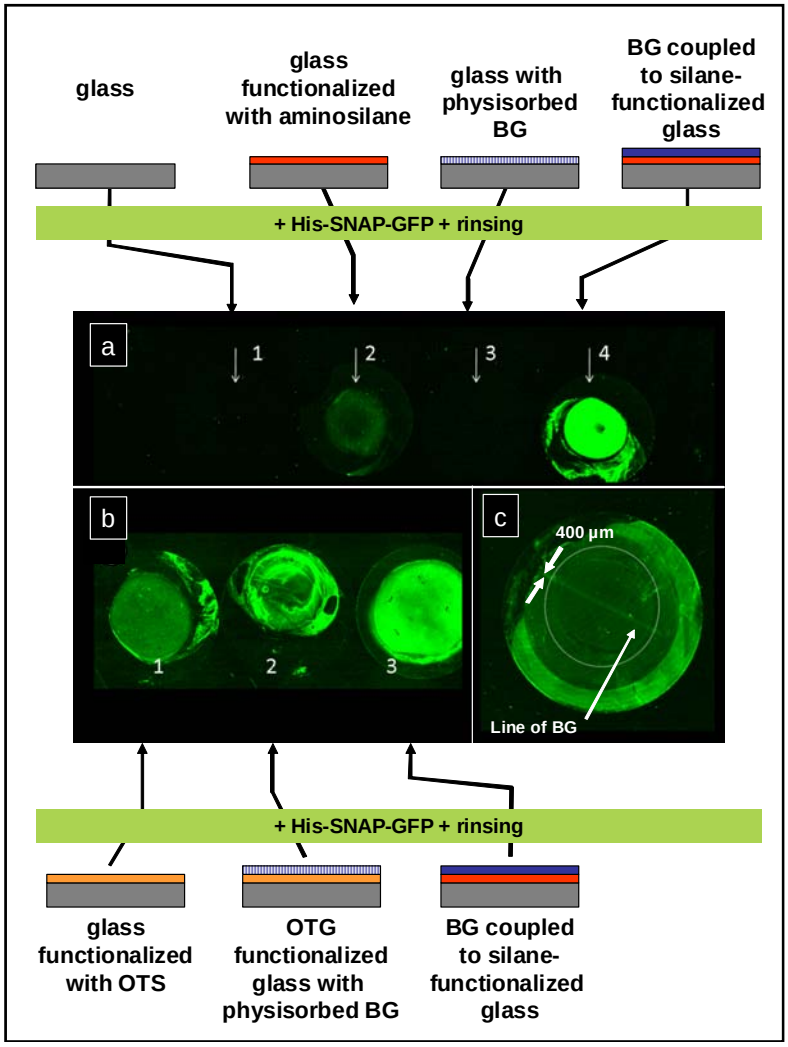


Figure 4
Phosphorimager scans of the GFP fluorescence. His-SNAP-GFP bound to a glass substrate, functionalized with benzylguanine (BG) in (a)/4 and (b)/3. For negative control the His-SNAP-GFP solution was dropped on glass in (a)/3, on aminosilane functionalized glass in (a)/2, on physisorbed BG in (a)/1, on OTS in (b)/1 and on BG physisorbed on a OTS functionalized glass substrate in (b)/2. (c) shows the BG-functionalized line prepared by the magneto lithography process.

Results of His-SNAP-GFP coupling on Quantumdots (QD)

After the immobilizing the His-SNAP-GFP on flat substrates has been successful experiments for the immobilization of the His-SNAP-GFP fusion protein on particulate nanoparticles, so called qantumdots (QDs) were carried out. The particles consist of a CdSe/ZnS core which is coated with a amino-functionalised PEG shell. The NH_2-groups of the particle surface were coupled to a benzylguanin-n-hydroxy-succinimide (BG-NHS). The benzylguanine (BG) group is the used for the coupling of the His-SNAP-GFP protein (see Figure 5). This work is done collaboration with Dr. Ljiliana Fruk and Prof. Ulrich Nienhaus.

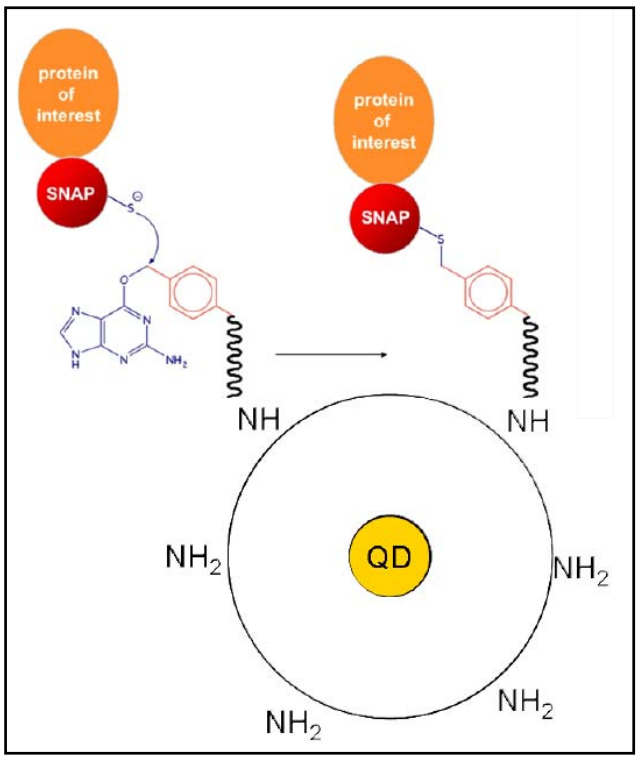


Figure 5:
Coupling scheme for a SNAP fusion protein to the benzylguanine (BG) -terminated surface of quantumdots (QD)

2. Coupling techniques for cadherins

We decided to develop a method to couple Cadherin-ectodomains, which mediate homophilic binding and thereby intercellular adhesion, via thiols to gold surfaces. Supervised by Robert Tampé (University of Frankfurt) the PhD student Sinem Engin learned to generate self-assembled monolayers (SAMs) consisting of matrix thiols and thiols with nitrilo triacetic acid (NTA) head groups to which His-tagged E-cadherin (EC1-5His) was coupled in presence of Ni^{2+} . Binding of the EC1-5His was confirmed by Surface Plasmon Resonance (SPR) measurements, immunostaining and cell adhesion assays (Fig. 5). Unfortunately, the signal to background ratio was too low to continue with the cell experiments.

To establish an alternative method we have chosen the SNAP-tag system, which allows covalent binding of the EC1-5SNAP to SAMs that contain benzylguanine-thiols (BG-thiols). The SNAP is an enzyme that removes the guanine from the thiol while the fusionprotein is covalently attached to the benzyl group of the thiol (Fig. 6).

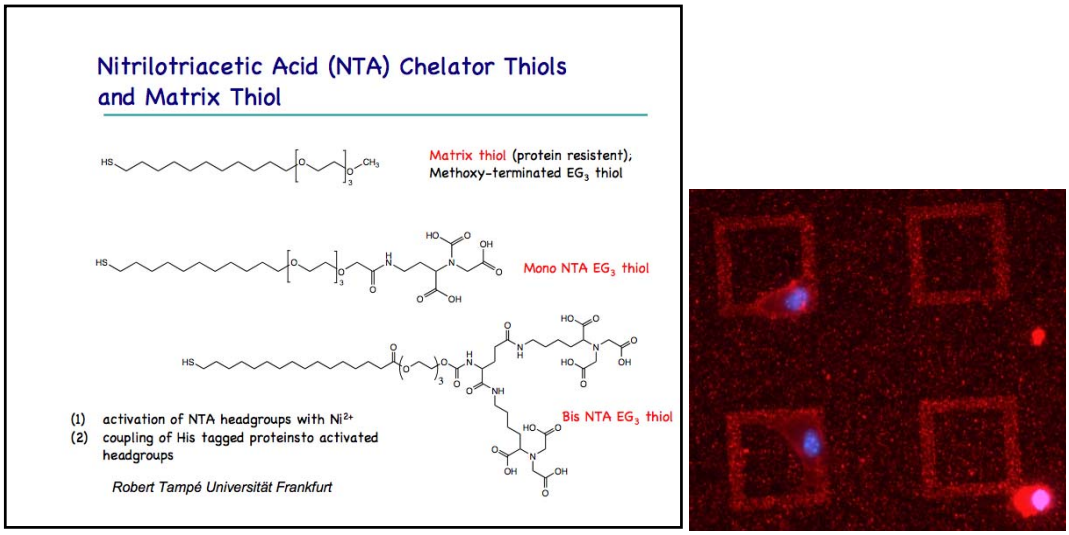


Fig. 5: Mono- and Bis-NTA thiols used for the coupling of His-tagged E-cadherin (EC1-5His), (left); E-cadherin expressing cells on µCP rectangles of Bis-NTA thiols to which E-cadherin ectodomains (EC1-5His) are immobilized. E-cadherin (red) was visualized by immunstaining with the DECMA antibody, the cell nuclei by DAPI binding (blue). Side length of rectangles 40µm, (right).

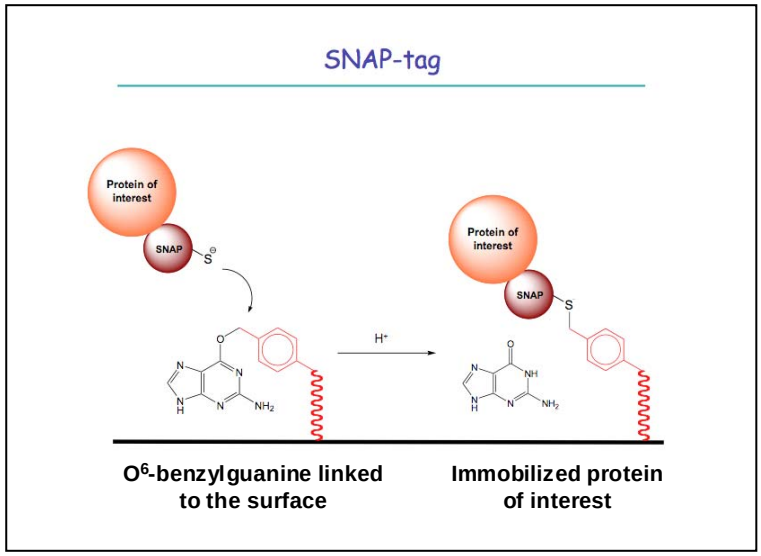


Fig. 6: SNAP / benzylguanine reaction

Matrix thiols of different chain length and different ratios of matrix to headgroup thiols were tested. Correct SAM formation was proved by XPS, ellipsometry, TOF-SIMS and AFM.We used a His-SNAP-GFP fusionprotein to test the functionality of the BG-thiols in SNAP-tag protein binding (Fig. 7). The density in protein immobilization was quantified by QCM and AFM measurements (for detail see publication [28]).

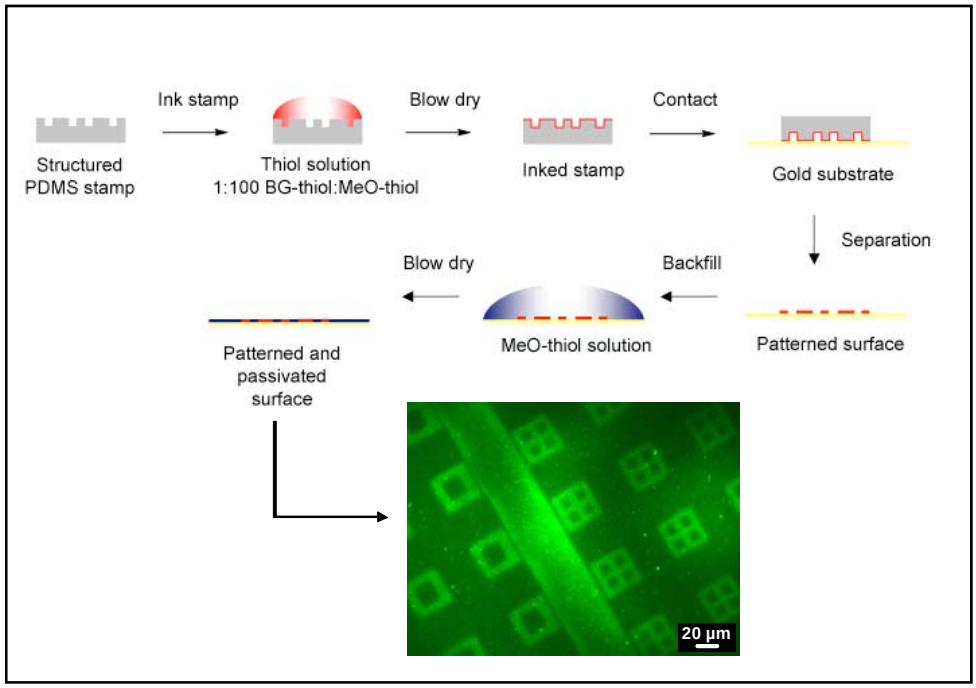


Fig. 7: µCP of benzylguanine-thiol and passivation by backfill with matrix-thiol (scheme), His-GFP-SNAP indicates the pattern of the stamp (fluorescence microscopy image).

Apart from µCP we also design patterns by dip-pen lithography. However, in writing thiols we were not able to create stable patterns. We plan to improve the method by replacing matrix thiols by BG-thiols using dip-pen lithography. But when we used lipids with BG-headgroups synthesized by Dr. Alexander Welle, we could couple Snap-tagged proteins to the patterns (Fig. 8).

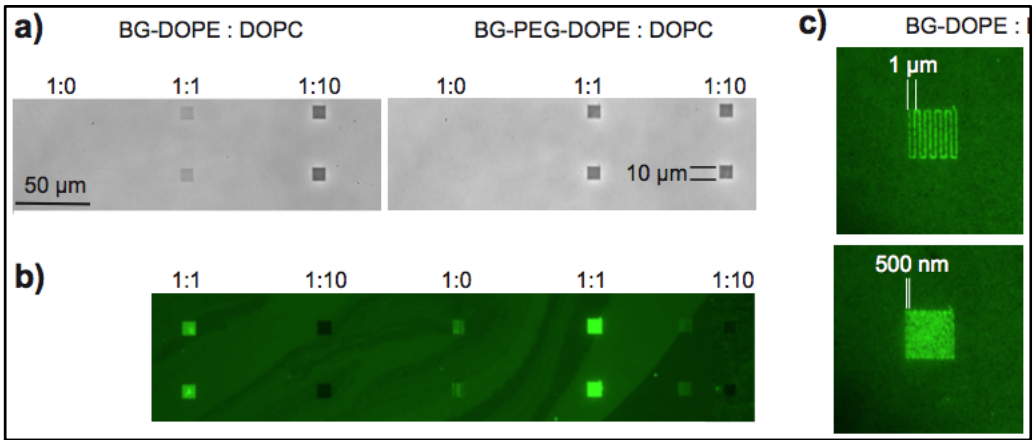
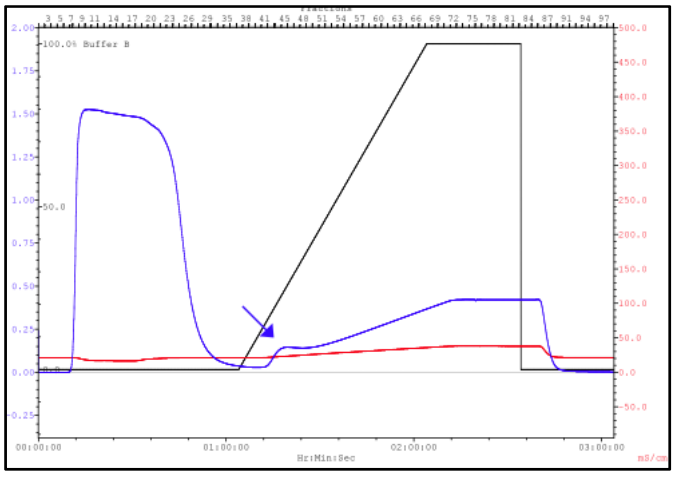


Fig. 8: Dip-pen lithography of lipids with different ratios of BG-DOPE coupled with His-SNAP-GFP. (a) phase contrast, (b) corresponding fluorescence image, (c) higher magnification of 1:1 mixture BG-DOPE:DOPE.

3. Peptide and protein production

In the last funding period we developed in collaboration with Horst Kessler's group (TU Munich) a technique to bind isothiocyanate-terminated peptides with different RGD-motifs covalently to amino-groups [18]. The RGD-motif is present in a subset of extracellular matrix molecules and recognized by integrin receptors at cell surfaces. The efficiency and reproducibility of our one-step peptide coupling method prompted us to apply it in future for other peptides specific for other extracellular matrix proteins, e.g. laminin or collagen. Although substrate adhesion was not in the direct focus of the previous grant application we found it helpful to work with peptides because the cloning, production and purification of cadherins and the development of suitable immobilization techniques took more time.

The extracellular domains (EC1-5 or EC1-3) of different cadherin subtypes (E-cadherin, Cadherin-11, N-cadherin) were cloned as fusion proteins with 12 histidine residues and the SNAP-tag at the C-terminus. The combination of two tags was essential to enrich these fusionproteins via NTA/Ni^{2+} affinity chromatography (IMAC: Immobilized Metal Affinity Chromatography). The highest efficiency of protein production was achieved with HEK 293 cells. We also synthesized a His-GFP-SNAP fusionprotein (GFP = green fluorescent protein) to establish a fast read-out system in controlling the coupling reaction of SNAP-tag proteins to benzylguanine thiols. Cadherin Ectodomains (EC1-5, EC1-3) production and purification has been established (Fig. 9). E-cadherin and N-cadherin ectodomains are in use (see below).



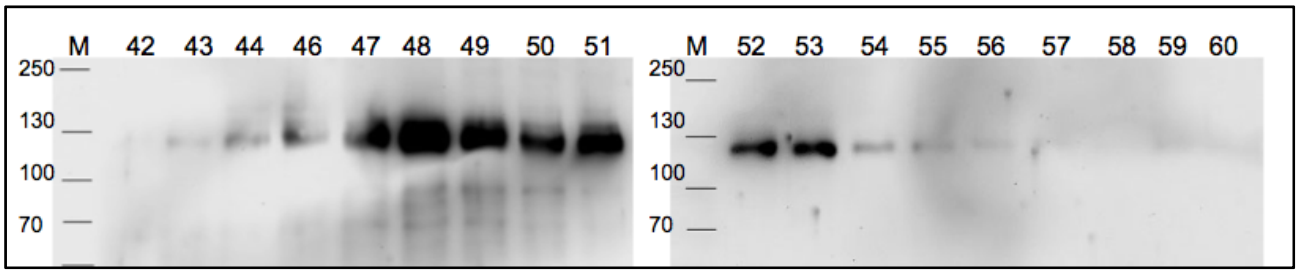


Fig. 9: IMAC chromotography profile showing the elution of N-cadherin EC1-5 (arrow, left side). Immunoblot of eluate fractions, showing N-cadherin EC1-5 of correct size in fractions 43-54 (below).

5. Biocompatibility and Cell Differentiation Assays

Patterning of surfaces by µCP and peptide coupling as shown in scheme 1 was highly reproducible. We proved the functionality and the biocompatibility by cultivating mouse fibroblasts (Ltk$^-$-cells) on these surfaces. Cells recognized RGD but not beta-Alanine containing peptides or ODT passivated gold stripes confirming the specificity of the created pattern. Cells also preferred to grow on the RGD-stripes in long-term cultivation (Fig. 10, [20]).

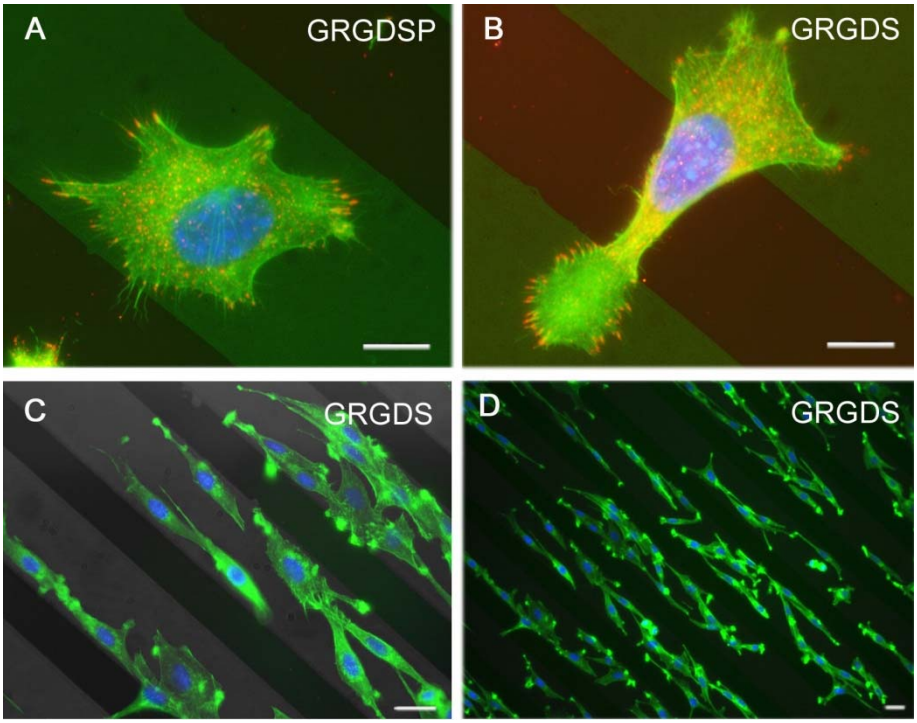


Fig. 10: Mouse fibroblasts (Ltk$^-$-cells) spread on µCP/peptide surface structures. (A) and (B) 45 min after seeding on surfaces in serum free medium. (C) After 4 h and (D) after 48 hours in serum containing medium (scale bar in A, B, C, and D 20 µm).

Structuring surfaces by µCP according Fig. 7 and immobilization of E-cadherin and N-cadherin via the BG-SNAP reaction was successful and also proved for biocompatibility. Specificity in recognition of the immobilized cadherin was investigated by cells transfected either with E- or N-Cadherin-GFP. Only when E-cadherin was presented on surfaces cells expressing the E-cadherin-GFP cells spread and formed maximal contact zones (Fig. 11).

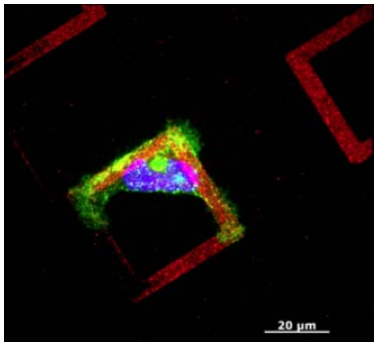
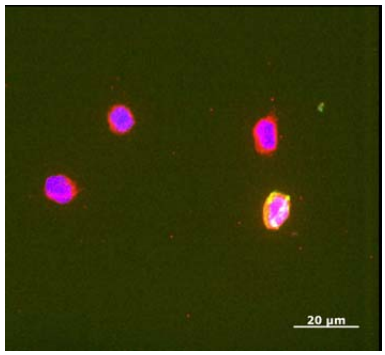


Fig. 11: Hela-cell expressing E-cadherin-GFP (green) adhering to E-cadherinEC1-5 (red rectangle) bound to µCP BG-thiol (left). Transfected HeLa cells do not adhere to thiol SAM surfaces without E-cadherinEC1-5 (right).



Murine embryonic stem cells express E-cadherin. When seeded onto E-cadherinEC1-5 immobilized surfaces they adhered to the functionalized areas. Long-term cultivation confirmed the biocompatibility of these surfaces (Fig. 12).

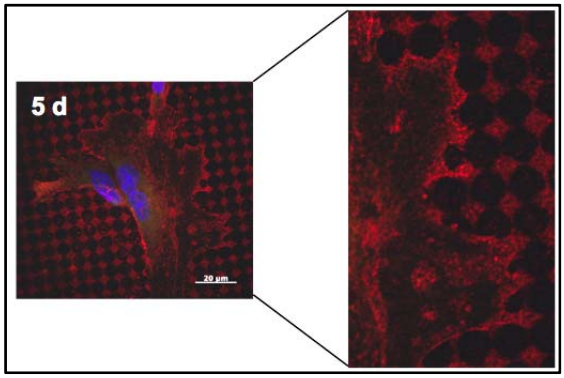


Fig. 12: Murine embryonic stem cell cultivated for 5 d on E-cadherinEC1-5 immobilized surfaces. Immunstaining for E-cadherin (red), DAPI staining for nuclei (blue).

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