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Ultrathin Coatings from Isocyanate Terminated Star PEG Prepolymers: Patterning of Proteins on the Layers

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This study presents the easy and fast patterning of low molecular weight molecules that act as binding partners for proteins on Star PEG coatings. These coatings are prepared from isocyanate terminated star shaped prepolymers and form a highly cross-linked network on the substrate in which the stars are connected via urea groups and free amino groups are present. Streptavidin has been patterned on these layers by microcontact printing (μCP) of an amino reactive biotin derivative and consecutive binding of streptavidin to the biotin. Patterns of Ni^{2+}-nitriltriacetic acid (NTA) receptors have been prepared by printing amino functional NTA molecules in freshly prepared Star PEG layers that still contain amino reactive isocyanate groups. Complexation of the NTA groups with Ni(II) ions enabled the binding of His-tag enhanced green fluorescent protein (EGFP) in the desired pattern on the substrates. Since the unmodified Star PEG layers prevent unspecific protein adsorption, His-EGFP could selectively be bound to the sample by immersion into crude, nonpurified His-tag EGFP containing cell lysate.

Introduction

Although the preparation of nonfouling surfaces by the chemisorption of ultrathin layers of linear[1] or star shaped poly(ethylene glycol) (PEG)[2a] is interesting for a number of applications, it is often necessary to generate patterns of biological ligands, proteins, or cells on surfaces. This is obvious for the whole field of microarray technology that originates from DNA arrays but is rapidly evolving over proteins even to cell based arrays.[3−5] While DNA microarrays are produced routinely today,[6−9] the situation differs for protein microarray techniques.[10,11] Although first success is achieved especially with antibody arrays,[12−16] the solid support materials are an important and still problematic aspect.[17−19] On the one side, the proteins have to be immobilized on the surface, and on the other side, they have to remain in their native and biologically active conformation. Since the interacting molecule in most cases is also a protein, the surface between the areas of specifically immobilized proteins has to be inert toward unspecific protein adsorption. Both issues get even more problematic if rather hydrophilic plasma proteins and rather hydrophobic membrane proteins are supposed to be analyzed within the same array.

Protein samples, unlike nucleotide samples for nucleotide arrays, which can be synthesized or amplified easily in large amounts in a high throughput fashion in vitro, typically have to be purified from biological material. Therefore, it is desirable to work with minimal amounts of proteins and, for example, during the workup of recombinant proteins, to minimize process steps that often cause the loss of product such as purification processes. For both reasons, the immobilization of low molecular weight protein ligands and consecutive binding of the desired proteins to these binding partners is advantageous. Such specific protein−ligand interactions are used for the purification of recombinant proteins. Therefore, the proteins are equipped with so-called fusion tags, usually small peptide sequences that do not disturb the structure and function of the protein and allow for specific and reversible binding to purification columns.[20] A widely used system is a polyhistidine tag (His-tag), a series of usually six histidine units attached at the C- or N-terminus of the protein. Purification is performed by immobilized metal chelate chromatography (IMAC), with either Ni^{2+}-nitriltriacetic acid (NTA) or Co^{2+}-carboxylmethylaspartate (CMA) matrixes. Elution of the protein is done by adding imidazole, adjusting the pH, or adding EDTA to complex the metal ion. Nonadhesive surfaces that are equipped with Ni^{2+} loaded NTA receptors on the surface in the desired pattern might therefore be loaded with recombinant His-tag proteins simply by immersing the substrate

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into the crude cell lysate and subsequently washing the sample. So far, embedding of NTA terminated alkanethiols into self-assembled monolayers (SAMs) on gold[21−24] and of NTA terminated lipids into lipid monolayers[25] has been reported. In both cases, the NTA groups were statistically distributed and were interacting with purified His-tag proteins.

Despite spotting small droplets of a solution onto a substrate, the standard method for generating microarrays, there are many other possibilities for generating protein or protein ligand patterns on surfaces such as ink-jet methods,[26] photolithography,[27] or dip pen nanolithography.[28] A novel approach has been developed by Michel et al. that takes advantage of the fact that TiO_2 and SiO_2 surfaces can specifically be coated by adhesive alkane phosphates and nonadhesive poly(L-lysine)-*g*-poly(ethylene glycol), respectively.[29] One of the most commonly used methods is microcontact printing (μCP), a method introduced by the group of Whitesides.[30] In this process, a patterned elastomeric replica (stamp), usually made of poly(dimethylsiloxane) (PDMS), is inked with a solution of the desired molecules and placed in contact with the substrate, leaving behind the desired pattern of ink molecules. This method is easy to apply, is fast, and enables the patterning of square centimeter big areas. Microcontact printing has extensively been used to pattern alkylthiols onto gold[31−33] or alkylsilanes onto silicon.[34−36] Structures down to 50 nm have been created by μCP,[37] but for features smaller than 1 μm, system-specific problems are occurring, that lead to studies about the molecular transport mechanisms involved in the process.[38]

Although the μCP of proteins with untreated PDMS stamps on a single protein level has been reported,[39] PDMS is usually too hydrophobic for stamping of proteins. Therefore, the PDMS is hydrophilized, for example, by plasma treatment, to enable the printing of proteins onto various substrates.[40,41] Other possibilities are to use compounds other than PDMS as stamp materials such as hydrogels[42] or to use the PDMS stamp as mold on the surface that is filled with acrylate functionalized PEG precursors due to capillary forces (capillary force lithography) and polymerized by irradiation.[43] Nevertheless, it is for the reasons mentioned above and also to prevent possible denaturation of the proteins during the stamping process favorable to stamp protein ligands onto a surface and bind the proteins in a second step. Biotin has been stamped on polymer surfaces by microstamping onto an activated polymer surface (MAPS)[44,45] and onto reactive SAMs[46] to yield surfaces where streptavidin was bound in the desired pattern.

This study presents the easy and versatile patterning of biotin and NTA on Star PEG layers by μCP. The possibility to statistically bind proteins to Star PEG layers as well as the negligible interaction of the layers with immobilized proteins has been presented elsewhere.[2b,c] In this study, free amino groups in the layers are used for the covalent binding of amino reactive compounds, whereas amino groups can be covalently attached onto the layers by stamping on freshly prepared and isocyanate containing Star PEG coatings. This way, amino functional NTA has been patterned on Star PEG films. These samples show that the protein repellant properties of the nonmodified areas between the NTA receptors allow specific binding of His-tag EGFP from crude cell lysate. Depending on the procedure for stamping on freshly prepared Star PEG layers, the pattern transfer to the substrate can be modified.

Experimental Section

Reagents and Materials. Silicon (100) wafers were purchased from CrysTec GmbH/Berlin. Glass substrates D 263 T ($50.8 \times 50.8 \times 0.175$ mm^3) were purchased from Schott Desag. Acetone, 2-propanol, and ethanol (Merck, selectipur) were stored in the clean room and used as received. *tert*-Butyl-bromoacetate (Aldrich, 98%), triethylamine (Aldrich, 99%), $CDCl_3$ (Deutero GmbH), *Z*-lysino-*tert*-butylester (Bachem, content 90%, purity >98%), nickel chloride hexahydrate (Aldrich, 99.9998%), NaH_2CO_3, hydrogen (MTI, technical), trifluoroacetic acid (Merck, >98%), anhydrous sodium sulfate (Fluka, >99%), and acetic acid were used as received. Pd/charcoal (20% Pd, Fluka, puriss.) was activated prior to use at 80 °C under vacuum overnight. Tetrahydrofuran (THF) and toluene were dried over $LiAlH_4$, distilled under argon, and transferred into a glovebox. Dimethylformamide (DMF) was distilled from P_2O_5, and chloroform was distilled from CaH_2. *N*-[3-(Trimethoxysilyl)propyl] ethylenediamine (Aldrich, 97%) was stored in the glovebox and filtered before usage. Syringe filters with a pore size of 0.02 μm were purchased from Whatman. Phosphate buffered saline tablets (Sigma) were dissolved in 200 mL of deionized water each to obtain 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4 at 25 °C. Streptavidin Rhodamine Red-X conjugate, Avidin Texas Red conjugate, and Alexa Fluor 532 NHS ester (Molecular Probes) were stored at −20 °C. Streptavidin Rhodamine Red-X conjugate and Avidin Texas Red conjugate solutions were made in phosphate buffered saline with a concentration of 5 μg/mL prior to use. Alexa Fluor 532 NHS ester solutions were made in dry DMF with a concentration of 0.1 mg/mL prior to use. Sylgard 184 (Dow Corning) and (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (ABCR) were used as received. Silicon masters for stamp preparation were generated using lithographic techniques.

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Biotinamido hexanoic acid *N*-hydroxysuccinimide ester (Fluka, purity >97% TLC) was stored at −20 °C.

Methods. ^{1}H and ^{13}C NMR spectra were performed in $CDCl_3$ on a Bruker DRX 400 spectrometer at 400 MHz (^{1}H) or 100 MHz (^{13}C). Chemical shifts are relative to tetramethylsilane. Fourier transform infrared (FT-IR) spectra were recorded on a Bruker IFS 113 V spectrometer. Sonication has been performed using a TK 52H ultrasonic bath. Samples were treated with UV/ozone using a 40 W UV lamp (main emission 185 nm, UV-Technik Speziallampen GmbH) in an oxygen stream of 350 mL/min with a sample distance of 5 mm to the lamp. Films were generated with a CONVAC ST 146 spin-coater. Fluorescene microscopy was performed using an Axioplan2 Imaging microscope (Zeiss) combined with an N XBO 75 lamp (Zeiss) and the Zeiss fluorescence filter set 31 for Streptavidin Rhodamine Red-X conjugate and Alexa Fluor 532. For EGFP, the filter set F41-018 from AHF Analysentechnik AG was used. Pictures were taken with a Princeton Instruments NTE/CCD 512EBFT camera. Intensity for fluorescence measurements is given as counts per second (cps).

Sample Preparation and Star PEG Layer Formation. Cutting, cleaning, and activation of the samples as well as spin-coating of the polymer solution was performed in a class 100 cleanroom. Aminosilylation and preparation of the star prepolymer solutions were done in a unilab glovebox from MBRAUN. Both processes were performed using a Star PEG prepolymer with a molecular weight of 12 000, as described in ref 2a. Briefly, the substrates (glass and silicon) were cleaned by sonication in water, 2-propanol, and acetone for 1 min, activated by UV/ozone treatment for 12 min, and then immediately transformed into a glovebox. After immersion into a solution of 0.3 mL of *N*-[3-(trimethoxysilyl)propyl] ethylenediamine in 50 mL of dry toluene for 2 h, the samples were washed several times with dry toluene and then taken out of the glovebox in dry toluene. For spin-coating, the samples were dried in a stream of nitrogen. Star prepolymers were dissolved in dry THF. Five minutes after the addition of 9-fold excess water with respect to the THF, the solution with a final concentration of 10 mg/mL was used for spin-coating at 2500 rpm for 40 s. The samples were then used for stamping immediately, 1 h after spin-coating, or at least 12 h after spin-coating. Star PEG layers prepared in this way are 30 nm thick and prevent unspecific protein adsorption.[2a]

Stamp Preparation. The silicon masters were cleaned by sonication in acetone, Millipore water, and 2-propanol for 1 min each, then dried in a stream of nitrogen, and then activated by treatment with UV/ozone for 12 min. Then, the masters were fluorinated by gas phase derivatization with (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane under reduced pressure for 2 h. After keeping the fluorinated masters under vacuum for 1 h to remove physisorbed fluorosilane, the masters were used for stamp preparation. Therefore, the two components of Sylgard 184 were mixed extensively in the ratio given by the manufacturer and the masters were covered by a layer of this mixture with the desired stamp thickness in self-made containments of alumina foils. Then, the containments were placed in an exciccator and the pressure was slowly reduced until no more gas bubbles were developing in the PDMS. The degassed stamps were then cured at 120 °C for 2 h, released from the masters, cut into the desired size, usually 1 × 1 cm^2, and used for μCP. The first generation of stamps produced from the fluorinated masters was discharged to make sure that no fluorosilane is incorporated in stamps used for μCP. All microcontact printing was performed under cleanroom conditions. Stamps were cleaned by sonication in ethanol for 3 min and dried in a stream of nitrogen. Then, they were stored under cleanroom conditions overnight to ensure complete evaporation of ethanol. For stamping of Alexa Fluor 532 NHS ester and biotinamido hexanoic acid *N*-hydroxysuccinimide ester, the stamps were hydrophilized by treatment with UV/ozone for 15 min. All stamps were just used once and discharged after use.

Microcontact Printing of Alexa Fluor 532 NHS Ester. A hydrophilized stamp is covered with some drops of a freshly prepared solution of 1 mg/mL Alexa Fluor NHS ester in dry DMF. The solution is homogeneously distributed over the stamp during slow drying of the stamp in a weak stream of nitrogen. After complete drying, the stamp is placed in contact with a completely cross-linked Star PEG layer for 1 min and then peeled off again. The sample is then stored overnight under ambient conditions to allow complete functionalization. After extensive washing with Millipore water, the sample is examined by means of fluorescence microscopy.

Microcontact Printing of Biotinamido Hexanoic Acid *N*-Hydroxysuccinimide Ester. A hydrophilized stamp is covered with some drops of a freshly prepared solution of 10 mg/mL biotinamido hexanoic acid *N*-hydroxysuccinimide ester in dry DMF. The solution is homogeneously distributed over the stamp during slow drying of the stamp in a weak stream of nitrogen. After complete drying, the stamp is placed in contact with a completely cross-linked Star PEG layer for 6 h and then peeled off again. The sample is then extensively washed with Millipore water and immersed into a solution of Streptavidin Rhodamine Red-X conjugate (PBS buffer, pH 7.4, 5 μg/mL) for 20 min. After extensive rinsing with PBS buffer and once with Millipore water, the samples are dried in a stream of nitrogen and examined by means of fluorescence microscopy.

Microcontact Printing of Lysino-nitrilotriacetic acid-tri(*tert*-butylester). A solution of lysino-nitrilotriacetic acid-tri(*tert*-butylester) in toluene with a concentration of 20 mg/mL was then spin-coated on the stamps at 2500 rpm for 40 s. These inked stamps were then placed in contact with freshly prepared Star PEG covered samples and peeled off after varying time intervals.

Deprotection of the NTA Groups. The patterned samples were deprotected using two different methods. One method was heating to 120, 150, or 200 °C for 10 min under reduced pressure to achieved thermal deprotection. The second method was gently shaking in a mixture of 1:5 trifluoroacetic acid (TFA)/chloroform at 50 °C for 2 or 12 h to cleave the esters under acidic conditions. The samples were the rinsed extensively with chloroform and dried in a stream of nitrogen.

Binding of His-tag EGFP. The NTA groups were loaded with Ni^{2+} by immersion of the samples into a 5 mg/mL solution of nickel chloride hexahydrate in Millipore water for 5 min followed by extensive washing of the samples with Millipore water and drying in a stream of nitrogen. Then, the samples were immersed either into a diluted solution of purified His-tag EGFP (PBS buffer, pH 7.4, 30 μg/mL) for 10 min at room temperature or into His-tag containing crude cell lysate with or without the addition of Avidin Texas Red conjugate (PBS buffer, pH 7.4, 5 μg/mL) for 10 min at 4 °C. After all three procedures, the samples were washed extensively with PBS buffer and once with Millipore water, dried in a stream of nitrogen, and examined by means of fluorescence microscopy.

Preparation of His-tag EGFP. *Expression of His-tag EGFP.* His-tag EGFP was expressed using a pET-9d expression vector (Novagen) in which nucleotides 516−848 have been replaced (on the reverse strand) with the EGFP coding sequence (Clontech), flanked 5′ by the nucleotide sequence AACATCACCATCAC-CATCACCCCATGGATCCACCGGTCGCCACC and 3′ by the nucleotide sequence AGCGGCCGCA. The modified vector allows the bacterial expression of EGFP, which is N-terminally tagged with the amino acid sequence MKHHHHHHPMDPPVAT. Bl21-(DE3) bacteria (Novagen), transformed with the His-tag EGFP expression vector, were cultured on LB (10 g/L bactotryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH to 7.5) + 30 mg/L kanamycin plates for 16 h at 37 °C. Highly expressing clones were selected by visual inspection under long wave UV light and grown in LB + 30 mg/L kanamycin for 16 h at 37 °C. The cultures were diluted 1:40 in 0.5 L LB + 30 mg/L kanamycin + 1% glucose + 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for another 2−4 h at 37 °C until OD_{600nm} reached 0.4−0.6. The bacteria were harvested by centrifugation.

Preparation of Crude Cell Lysates. The bacteria were lysed by one freeze−thaw cycle, followed by incubation in 10 volumes 300 mM NaCl, 50 mM $NaPO_4$, pH 7.4 + 1 mg/mL lysozyme + proteinase inhibitor cocktail without EDTA (Roche) for 30 min at 4 °C, and they were then sonicated six times for 10 s on ice. The crude lysate was cleared by centrifugation at 25 000*g* for 30 min at 4 °C and snapped frozen in liquid nitrogen.

Preparation of Affinity Purified His-tag EGFP. The bacteria were resuspended in 1 volume PBS, frozen, and grounded under liquid nitrogen. The baterial powder was thawed in 2 volumes 200 mM NaCl, 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethane-

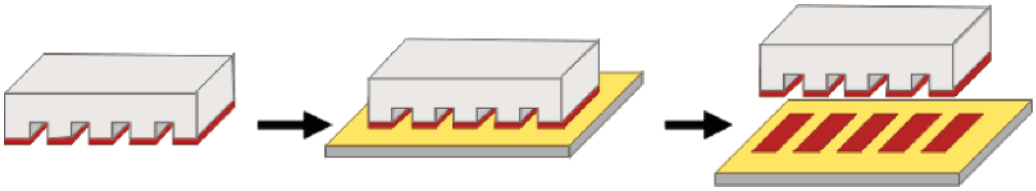

Figure 1. Concept of microcontact printing. A stamp is inked, dried, and then placed in contact with a substrate. After removal of the stamp, the ink stays in the desired pattern on the substrate.

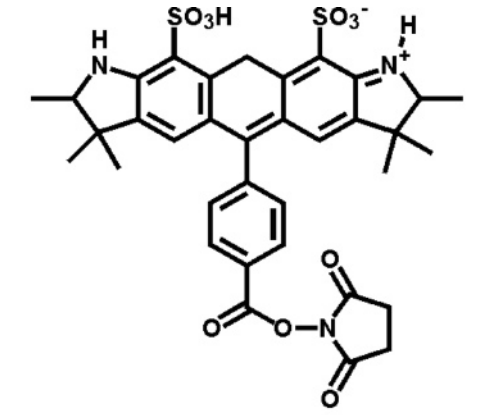

Figure 2. Chemical structure of Alexa Fluor 532 NHS ester (top) and fluorescence microscopy image of a pattern of this dye on a Star PEG layer on a glass substrate (bottom).

sulfonic acid (HEPES), pH 8.0 + proteinase inhibitor cocktail without EDTA (Roche), and sonicated six times for 10 s on ice. The lysate was cleared by centrifugation at 25 000*g* for 30 min at 4 °C, mixed with 1/33 volume of imidazole buffer (300 mM NaCl, 25 mM HEPES, pH 8.0, 300 mM imidazole, and 20% glycerol), and bound to a 5 mL HiTrap chelating column (Amersham Biosciences), which was equilibrated with 300 mM NaCl, 25 mM HEPES, pH 8.0, 10 mM imidazole, and 20% glycerol. His-tag EGFP was eluted with an imidazole gradient of 0−300 mM imidazole over 10 column volumes in 300 mM NaCl, 25 mM HEPES, pH 8.0, and 20% glycerol. His-tag EGFP containing fractions were combined to a total of 15 mL, loaded on a HiPrep 26-10 Sephadex G25 column (Amersham Biosciences), and eluted with 200 mM NaCl, 50 mM HEPES, pH 7.5. The fractions containing protein in the excluded peak were combined and snapped frozen in liquid nitrogen at a final protein concentration of 1 mg/mL.

Synthesis of *Z*-Lysino-nitrilotriacetic acid-tri(*tert*-butylester). A 250 mL three-necked round-bottom flask fitted with a condenser and an argon inlet was charged with a solution of 5 g (13.4 mmol) of *Z*-lysino-*tert*-butylester hydrochloride in 150 mL of dry DMF. After subsequent addition of 7.84 g (10.8 mL, 77.5 mmol) of triethylamine and 26.1 g (134 mmol) of *tert*-butyl-bromoacetate, the reaction mixture was heated to 50 °C and stirred for 5 days at this temperature under an argon atmosphere. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was stirred for 2 days with Millipore water. Then, the water was decanted and the residue was dissolved in THF. The organic phase was dried over anhydrous sodium sulfate and filtrated. The solvent was removed under reduced pressure to yield a brownish, viscous oil that was used without further purification.

Yield: 3.65 g (6.46 mmol, 48%). ^{1}H NMR: 1.40 (s, 18H, (($\mathbf{H}_3$C)$_3$-COCOCH$_2$)$_2$N−); 1.43 (s, 9H, ($\mathbf{H}_3$C)$_3$COCOCH$_2$CH−); 1.49 (m, 4H, *Z*-NHCH$_2$−(C$\mathbf{H}_2$)$_2$−); 1.61 (m, 2H, *Z*-NH(CH$_2$)$_3$−C$\mathbf{H}_2$−); 3.17 (m, 2H, *Z*-NHC$\mathbf{H}_2$−); 3.27 (t, 1H, *Z*-NH(CH$_2$)$_4$C$\mathbf{H}$−); 3.42 (dd, 4H, (($\mathbf{H}_3$C)$_3$COCOC$\mathbf{H}_2$)$_2$N−); 5.06 (s, 2H, C$_6\mathbf{H}_5$C$\mathbf{H}_2$OCONH−); 7.32 (m, 5H, C$_6\mathbf{H}_5$CH$_2$OCONH−). ^{13}C NMR: 22.98/25.58/28.07/29.20/30.09/30.30 (*Z*-NHCH$_2$−(**C**H$_2$)$_3$−, (($\mathbf{C}$H$_3$)$_3$COCO)$_3$); 40.79 (*Z*-NH**C**H$_2$−); 53.89 (−N(**C**H$_2$COOC(CH$_3$)$_3$)$_2$); 65.07 (−**C**HN(CH$_2$-COOC(CH$_3$)$_3$)$_2$); 67.92 (C$_6$H$_5$**C**H$_2$OCONH−); 80.67 (−N(CH$_2$-COO**C**(CH$_3$)$_3$)$_2$); 81.07 (($\mathbf{H}_3$C)$_3$**C**OCOCH−); 127.91−128.40/136.77 (**C**$_6$H$_5$CH$_2$−); 156.46 (C$_6$H$_5$CH$_2$O**C**ONH−); 170.66 (N(CH$_2$-**C**OOC (CH$_3$)$_3$)$_2$); 172.35 (($\mathbf{H}_3$C)$_3$CO**C**OCH−).

Synthesis of Lysino-nitrilotriacetic acid-tri(*tert*-butylester). A 250 mL three-necked round-bottom flask fitted with a gas inlet and a gas outlet was charged with a solution of 3.46 g (6.13 mmol) of *Z*-lysino-nitrilotriacetic acid-tri(*tert*-butylester) in a mixture of ethanol and acetic acid. Then, water was added until the solution was becoming hazy followed by the addition of enough ethanol to obtain a clear solution again. After the addition of a small portion of hydration catalyst (Pd/C, 20% Pd), the solution was stirred for 6 h while hydrogen was bubbled through the solution. After filtration, the reaction mixture was washed with an aqueous NaH_2CO_3 solution until the aqueous phase kept a basic pH value. Then, the organic phase was dried over anhydrous sodium sulfate and filtrated. The solvent was removed under reduced pressure to yield a yellowish, viscous oil that was used without further purification.

Yield: 2.73 g (6.34 mmol, 98%). ^{1}H NMR: 1.38 (s, 18H, (($\mathbf{H}_3$C)$_3$-COCOCH$_2$)$_2$N−); 1.39 (s, 9H, ($\mathbf{H}_3$C)$_3$COCOCH$_2$CH−); 1.59 (m, 4H, H$_2$NCH$_2$−(C$\mathbf{H}_2$)$_3$−); 2.01 (s, 2H, $\mathbf{H}_2$NCH$_2$−); 2.62 (t, 2H, H$_2$-NC$\mathbf{H}_2$−); 3.24 (t, 1H, H$_2$N(CH$_2$)$_4$C$\mathbf{H}$−); 3.40 (dd, 4H, (($\mathbf{H}_3$C)$_3$-COCO C$\mathbf{H}_2$)$_2$N). ^{13}C NMR: 23.14/28.01/28.10/30.44/33.31 (H$_2$-NCH$_2$−(**C**H$_2$)$_3$−, (($\mathbf{C}$H$_3$)$_3$COCO)$_3$); 41.85 (H$_2$N**C**H$_2$−); 53.64 (−N(**C**H$_2$COOC(CH$_3$)$_3$)$_2$); 65.24 (−**C**HN(CH$_2$COOC(CH$_3$)$_3$)$_2$); 80.47 (−N(CH$_2$COO**C**(CH$_3$)$_3$)$_2$); 80.85 (($\mathbf{H}_3$C)$_3$**C**OCOCH−); 170.56 (N(CH$_2$**C**OOC(CH$_3$)$_3$)$_2$); 172.30 (($\mathbf{H}_3$C)$_3$CO**C**OCH−). IR (ν, cm^{-1}): no signal for aromatic CH; 1593.5 ((N−H) deformation oscillation).

Results and Discussion

The general concept of microcontact printing is displayed in Figure 1. An elastomeric stamp, in this study prepared from PDMS with either 5 μm broad lines in 5 μm distances or dots with a radius of 5 μm in distances of 10 μm, is inked with a solution of the molecule that is intended to be patterned on a substrate. After drying, the inked stamp is placed in contact with the substrate. The contact times are system dependent and vary in this study between 1 min and 6 h due to different reactivities between the ink molecules and the corresponding functional groups in the layers. After peeling off the stamp, the desired molecules stay on the substrate in a negative image of the pattern on the stamp either physisorbed or chemisorbed. In this study, the patterned molecules are always covalently bound to the samples which are always Star PEG layers on silicon or glass substrates.

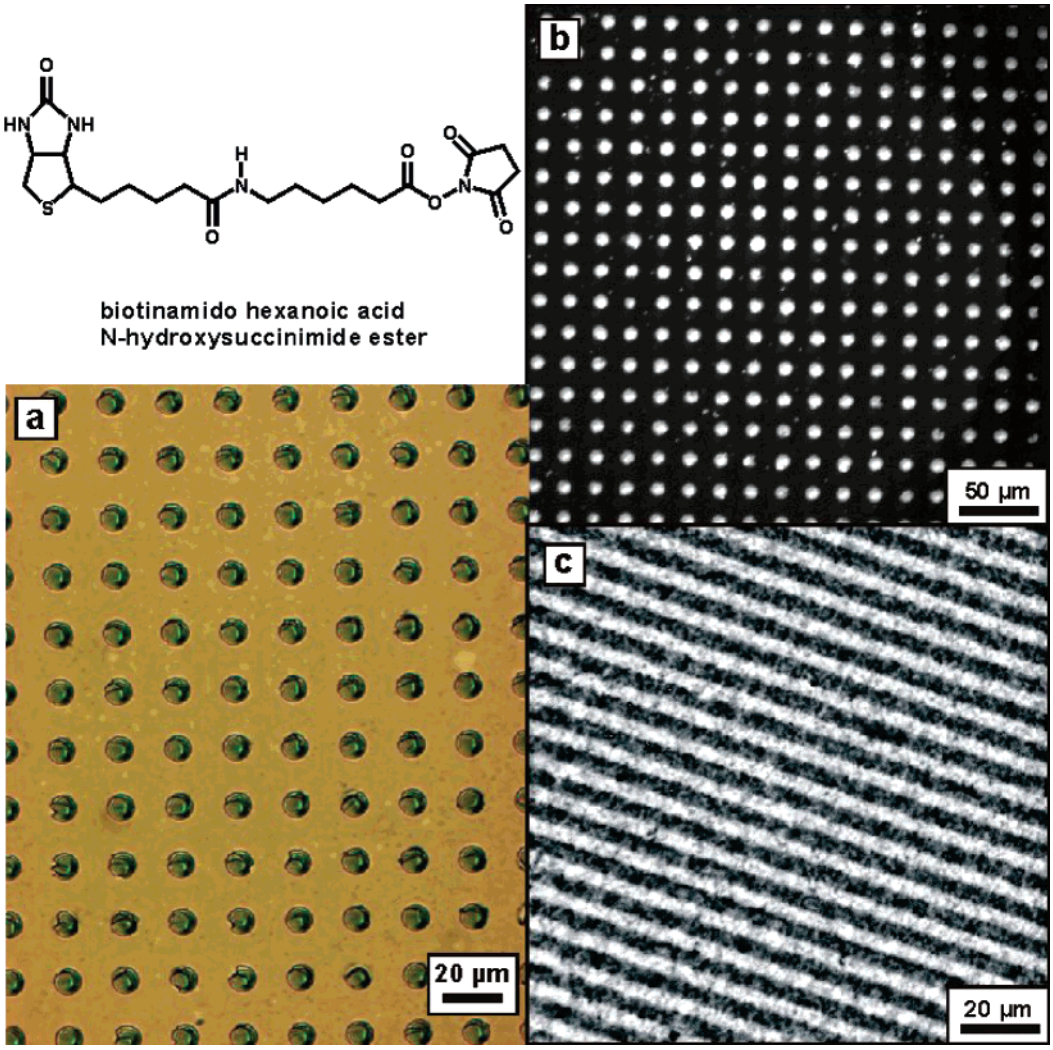

Figure 3. Chemical structure of biotinamido hexanoic acid *N*-hydroxysuccinimide ester, light microscopy image (DIC) of this molecule stamped onto a Star PEG covered silicon wafer (a), and fluorescence microscopy images of labeled streptavidin specifically bound to this dot pattern (b). Part c shows a fluorescence microscopy image of labeled streptavidin bound to a line pattern of the biotin derivative on a Star PEG covered glass substrate.

The chemical composition of the Star PEG system allows chemical modification via two general routes. One possibility is to pattern fully cross-linked, mechanically stable layers that contain accessible amino groups. Therefore, the ink has to be amino reactive, which often means water sensitive. The other possibility is to use the fact that freshly deposited layers still contain many isocyanate groups. These easily react with ink molecules that contain alcohol or amino groups and are easy to handle. Nevertheless, in this case, it has to be considered that the layers are not fully cross-linked and therefore mechanically not stable, more like a very viscous liquid than like a solid film. Both strategies have been pursued in this study, and they are discussed in the same order as they are listed in this paragraph.

The first attempts to achieve structures on cross-linked Star PEG layers have been performed with Alexa Fluor 532 NHS ester. The use of this commercially available, amino reactive fluorescent dye has the advantage that the visualization of the pattern transfer can directly be done after stamping. The chemical structure of the dye as well as a fluorescence microscopy image of a line pattern is displayed in Figure 2. It can be seen that the lines are thinner than the structure on the stamp. This is explained by the very hydrophilic character of the dye with ionic groups and a free sulfonic acid group. Although the stamp has been hydrophilized, the dye still does not wet the stamp homogeneously and forms thin lines in the middle of the bars on the stamp. These lines are then transferred to the substrate after drying.

The next step was to pattern a cross-linked Star PEG layer with a biological target molecule that can in a second step be specifically recognized by a protein that is fluorescently labeled for visualization. This has been accomplished by stamping biotinamido hexanoic acid *N*-hydroxysuccinimide ester onto Star PEG layers and consequent immersion of these samples into a solution of fluorescently labeled streptavidin. Figure 3 presents optical (DIC) and fluorescence microscopy images of patterns generated in this way on glass and silicon. Since the biotin derivative used as ink here is not as hydrophilic as the fluorescence dye discussed above, the stamp is wetted homogeneously and the pattern transfer is nice and regular. The necessity of the protein resistant properties of the unmodified Star PEG is obvious in these experiments, since surfaces that allow unspecific protein adsorption on unmodified surface areas would result in a very bad signal-to-noise ratio or even in no pattern formation at all.

Also, patterning of biologically relevant molecules on freshly prepared Star PEG coatings that still contain a lot of reactive isocyanate groups has been performed. The

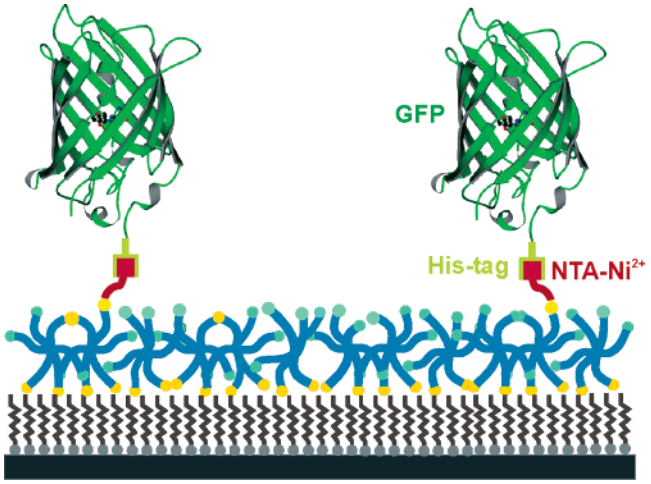

Figure 4. Schematic of the specific binding of His-tag EGFP to NTA modified Star PEG layers by complex formation of the imidazole units in the His-tag and the Ni^{2+} loaded NTA groups.

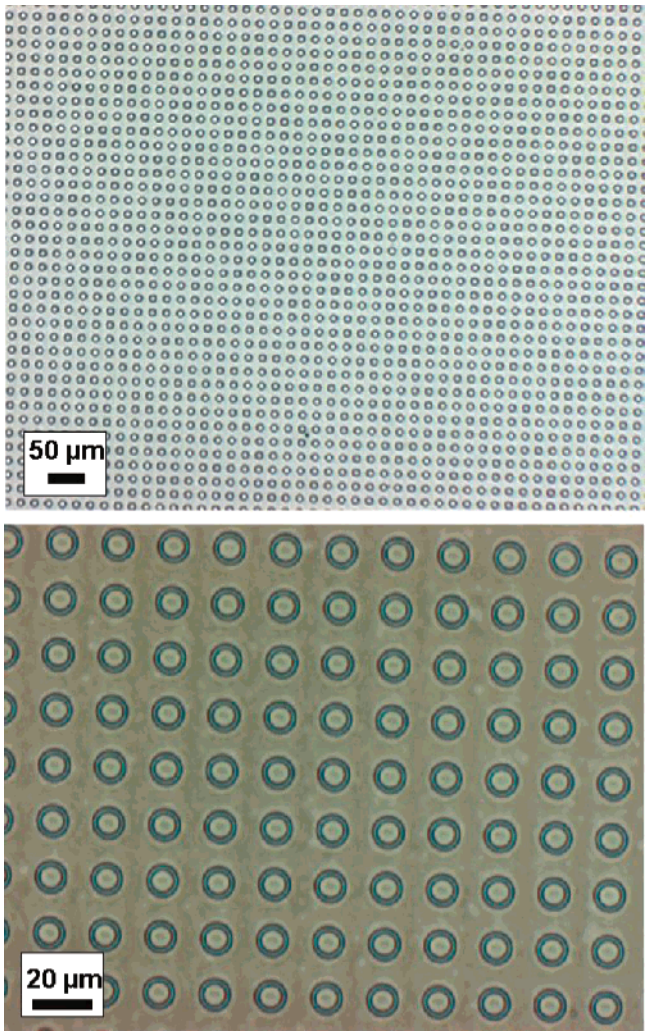

Figure 5. Optical microscopy images of lysino-nitrilotriacetic acid-tri(*tert*-butylester) stamped into a freshly prepared Star PEG layer on silicon.

most interesting and versatile ligand used for patterning of the Star PEG surfaces is the NTA receptor. As discussed in the Introduction, this molecule is widely used for the purification of recombinant proteins, what results in the fact that complete libraries of His-tag proteins exist that can readily be stamped or spotted onto suited samples for

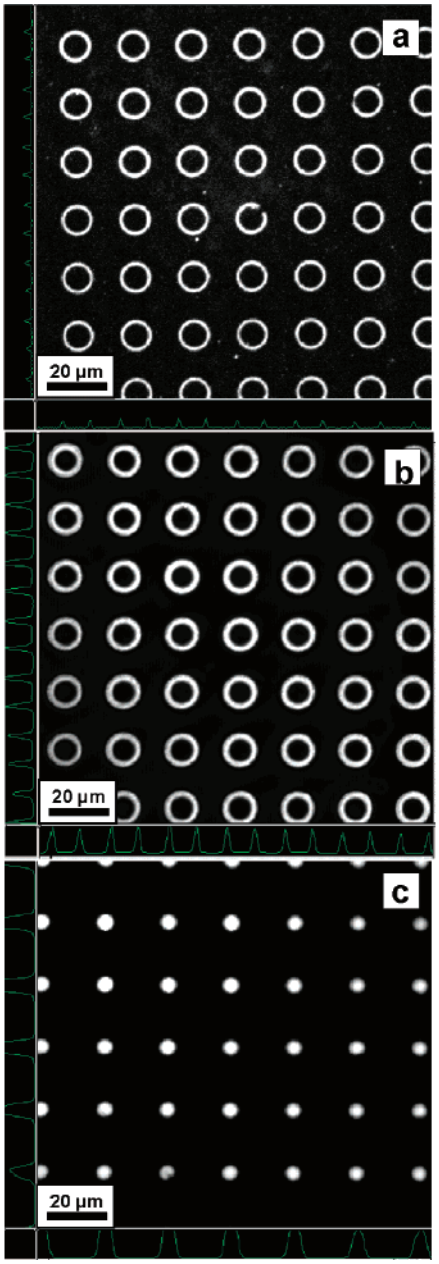

Figure 6. Fluorescence microscopy images of EGFP on NTA patterns that have been thermally deprotected at 120 °C (a) and 200 °C (b) and wet chemically deprotected with TFA at 50 °C (c). As indicated by the fluorescence intensity profile on the left side and at the bottom of each picture (the scale is the same for all pictures), more NTA is deprotected with increasing temperature but complete deprotection is only reached by wet chemical deprotection. All films have been prepared on silicon substrates.

various applications. In this study, His-tag EGFP has been used to enable easy and direct visualization of the NTA−His-tag recognition. When expressed in cells, GFP undergoes a posttranslational autocatalytic chromophore formation and matures into a stable fluorescent protein[47,48] that can be detected in vivo. Therefore, it is widely used as a fluorescent biological marker to visualize biological processes in living cells and organisms. Its fluorescent properties are well-known, and examinations are performed on the single molecule level.[49] A scheme of the

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(49) Zumbusch, A.; Jung, G. *Single Mol.* **2000**, *1*, 261.

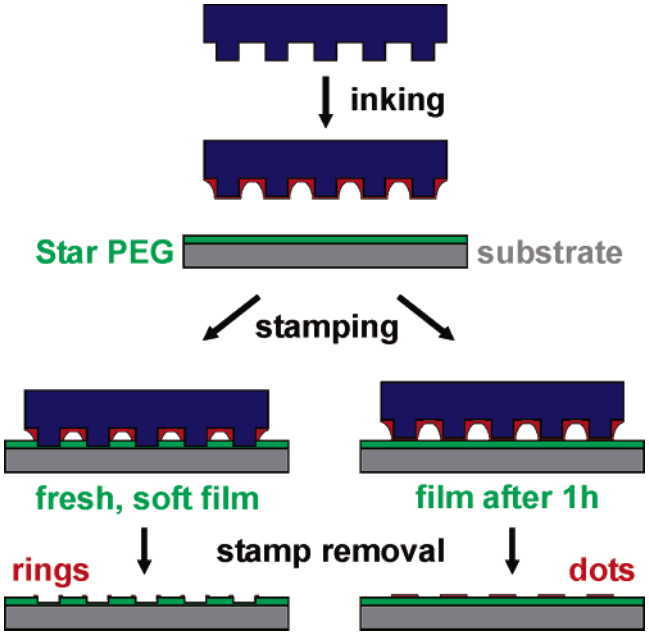

Figure 7. Scheme of the stamping procedures that result in different surface patterns. Placing the stamp on freshly prepared layers generates rings (left side), whereas stamping on films 1 h after spin-coating results in dot patterns (right side).

immobilization of His-tag EGFP on NTA modified Star PEG coatings is shown in Figure 4. The NTA group forms a complex with the Ni(II) ion, but since Ni(II) complexes have octahedral geometry, two coordination sites are filled with slightly coordinated water. The nitrogen atoms of the imidazole rings in the His-tags show a strong affinity toward these free coordination sites and complete the complex. This binding is reversible, since the complex can be destroyed by adding imodazole, adjusting the pH or complexing the Ni(II) with EDTA, which releases the protein from the surface.

Since the NTA group is very polar and wetting problems already disturbed proper pattern transfer for the Alexa Fluor dye, lysino-nitrilotriacetic acid-tri(*tert*-butylester) was chosen as the ink for μCP. This ink is rather hydrophobic, is quite soluble in organic solvents, and is wetting nonactivated PDMS stamps homogeneously. Figure 5 shows light microscopy images of lysino-nitrilotriacetic acid-tri(*tert*-butylester) stamped onto a freshly prepared Star PEG film. The homogeneity of the pattern transfer is almost perfect. The ringlike structures will be addressed later. The disadvantage is that, after patterning of the surface, a deprotection step is necessary to generate free NTA groups. The first strategy was thermal deprotection at different temperatures. Fluorescence microscopy images of His-tag EGFP bound to Ni(II) loaded NTA patterns on Star PEG samples after deprotection at 120, 150, and 200 °C. The fluorescence intensity profiles are

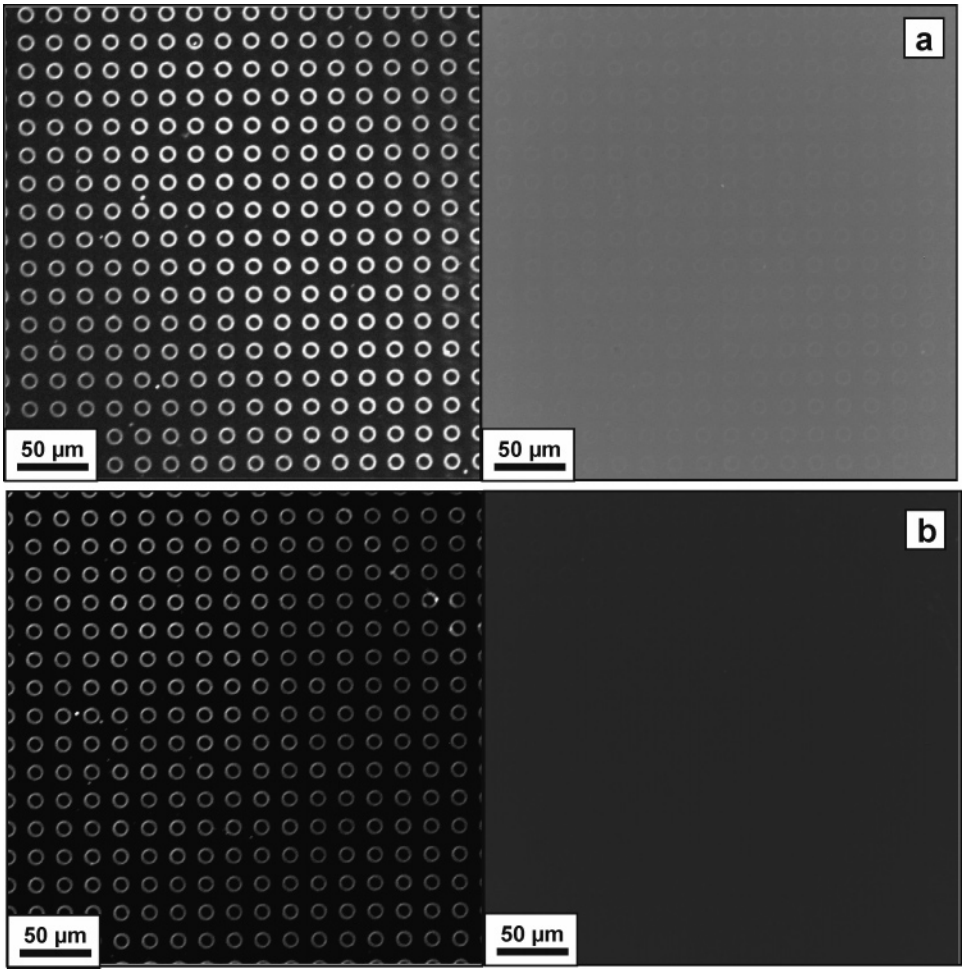

Figure 8. Fluorescence microscopy images of Ni(II) loaded NTA patterned Star PEG films on glass substrates after immersion into crude His-tag EGFP containing cell lysate without (a) and with (b) the addition of Avidin Texas Red conjugate to the cell lysate. The left image shows the fluorescence intensity in the green channel, and the right picture displays the fluorescence intensity in the red channel.

given so that it is more clear that, with rising temperature during the deprotection, more NTA groups are fully deprotected and thereby functional in the following steps for protein immobilization. However, the Star PEG layers are unaffected only at 120 °C and start to partially decompose at 150 °C, while after deprotection at 200 °C big areas of the sample are decomposed. Therefore, wet chemical deprotection of the NTA groups with trifluoroacetic acid (TFA) was performed as an alternative way. A comparison of the thermally deprotected sample at 120 and 200 °C and wet chemically deprotected sample by treatment with TFA in chloroform at 50 °C for 2 h is displayed in Figure 6. The fluorescence intensity profile given indicates that, by wet chemical treatment, all NTA groups are deprotected and the highest fluorescence intensity results. In addition to this, the Star PEG layers were absolutely unaffected by the treatment with TFA for 2 h.

The three pictures displayed in Figure 6 and thereby the two different patterns have all been produced using the same stamp. This originates from the fact that, as already mentioned above, freshly prepared Star PEG films are almost like a very viscous liquid. If the stamp is placed in contact directly after spin-coating of the layer, the stamp sinks in the coating, as displayed in Figure 7. If the stamp is put on the film 1 h after layer deposition, the film is already cross-linked enough to prevent sinking of the stamp into the layer. To describe the mechanical properties of the layers more quantitatively, Star PEG gels from 10 wt % solutions in water have been prepared. These gels show a gel point 450 s after the addition of water to the Star PEG. At the gel point, the G' value does not exceed a few pascals, demonstrating the low mechanical stability of these gels. After 60 min, the G' value of such gels is still increasing but is already larger than 1800 Pa, which shows the mechanical stability of such gels. These numbers can now be correlated to the Star PEG layers presented in this study and underline the viscous character of freshly prepared layers and the mechanical stability of the layers 1 h after layer preparation. Sinking of the stamp into the layer results in ringlike structures as patterns on the surface, whereas placing the stamp on partially cross-linked layers generates dot patterns. Therefore, the pattern generated with one stamp can reproducibly be varied by changing the stamping procedure. The effect of smaller stamp feature sizes on the pattern formation is not yet clear and is being examined at the moment.

The most challenging application is to specifically bind His-tag EGFP by the immersion of a NTA modified Star PEG substrate into the crude, His-tag EGFP containing cell lysate. Figure 8 shows fluorescence microscopy images of NTA patterned Star PEG samples after loading with Ni(II) and immersion into crude, His-tag EGFP containing cell lysate. The upper two pictures show an image taken with the fluorescence filter system for GFP on the left side and a picture taken with the filter system for red fluorescent dyes on the right side. While the pattern of specifically attached His-tag EGFP is clearly displayed in the green channel, the red channel shows negligible fluorescence intensity due to the marginal fluorescence of the EGFP. The lower two pictures show the same experiment with Avidin Texas Red conjugate added to the cell lysate. Since no increased fluorescence intensity is detectable compared to the cell lysate without the addition of the fluorescently labeled avidin, the selectivity of the NTA−His-tag bonding and the compatibility of the Star PEG surfaces with the system was demonstrated.

Conclusions

In this study, we present the possibility to covalently bind molecules to Star PEG layers in patterns by microcontact printing. Stamping on fully cross-linked layers requires amino reactive inks such as NHS esters. An amino reactive biotin derivative has this way been patterned onto Star PEG layers, and streptavidin was selectively attached to the surface in the desired pattern. Microcontact printing on freshly prepared Star PEG films allows the covalent binding of amino functional molecules on the layer. With this method, amino functional NTA molecules were patterned on freshly prepared Star PEG coatings. Due to the hydrophilic character of NTA, the acid groups had to be protected by ester groups for the stamping, and deprotection after the transfer onto the samples was performed both thermally and wet chemically. His-tag EGFP was selectively bound to such NTA patterns, even when the sample was immersed into His-tag EGFP containing, crude cell lysate. This offers the possibility to bind His-tag proteins directly from the crude cell lysate onto Star PEG surfaces. If this is done by spotting, many different proteins can be tethered to such a substrate, sparing one expensive and product consuming purification step per protein. Since the Star PEG coatings do not negatively influence the native conformation of specifically bound proteins, this system has a big potential for protein microarray applications.

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