In solution, only collisions between molecules of sufficient energy to overcome the activation barrier lead to the formation of reaction products. The rate of the reaction is influenced by the temperature, concentration of the reactants, solvent, orientation of the reactants, and structure of the molecules. Catalysts or enzymes lower the activation energy by making available alternative reaction pathways and preorganize the reactant molecules in close proximity, which leads to an increase in the effective concentration of the reactants. Herein, we report the first example in which a reaction (amide-bond formation) is made possible because the reactant molecules are physically pushed together. An elastomeric stamp (as used in microcontact printing) comes into conformal (van der Waals) contact with a functionalized surface, thus forcing “ink” molecules very close to the surface. We speculate that the nanoscale confinement of the ink at the interface between the stamp and the self-assembled monolayer (SAM), in combination with the preorganization of the reactants in the monolayer, facilitates the formation of covalent bonds.

The on-chip synthesis of single-strand DNA molecules has revolutionized genotyping research. Fodor and co-workers developed photolithographic techniques in combination with wet surface chemistry to attain >99% yield in coupling steps for the preparation of libraries of peptides or oligonucleotides. Usually, a range of catalysts and activated substrates are required for efficient covalent-bond formation on surfaces. In our research on patterned self-assembled monolayers, we were intrigued by the rapid and efficient formation of SAMs on gold or Si/SiO₂ through the use of microcontact printing (μCP). When an elastomeric stamp inked with trichloroalkyl silanes is placed on a clean Si/SiO₂ surface, the formation of the polysiloxane network is essentially complete within minutes, whereas this process could take hours in solution. Microcontact printing has previously been used to couple molecules to a reactive surface, but in all cases a catalyst or activated substrate was used to induce covalent-bond formation. Herein we report the formation of new bonds solely as a result of the nanoscale confinement of molecules between stamp and surface.

Amide-bond formation is a suitably challenging test case, as such reactions require catalysts (4-(dimethylamino)pyridine (DMAP), N-hydroxybenzotriazole (HOBT), or dicyclohexylcarbodiimide (DCC)) or elevated temperatures and long reaction times when performed in solution. In a typical reaction (Figure 1), we prepared a clean amine-terminated SAM surface on gold. To ensure all amines were deprotonated, we washed the surface with a saturated solution of K₂CO₃. A flat hydrophilic stamp (treated for 30 s with an oxygen plasma and stored under millipore water) was inked with a solution of an appropriate Boc-protected amino acid (1 mM) in ethanol, dried under a stream of nitrogen, and placed on this surface. If necessary the sample was heated with the stamp on a hotplate (to approximately 40 °C). After a certain reaction time, the stamp was lifted off and the substrate washed vigorously with ethanol and water to remove all noncovalently bound molecules. The surfaces were then characterized by ellipsometry, FTIR spectroscopy, and contact angle goniometry.

In Figure 2a typical results of the ellipsometry measurements are shown for three different amino acids. We found that Boc-protected proline (Boc-proline) was the most reactive amino acid with a maximum thickness increase observed after only 10 s. Fmoc-protected alanine (Fmoc-alanine) was also reactive at room temperature but a reaction
time of 30 mins was required for a maximum thickness to be reached. However, Boc-protected tyrosine (Boc-tyrosine) required a reaction temperature of 40°C and a printing time of 30 min. The different reactivities of the various amino acids indicate that a chemical reaction takes place. After removal of the Boc protecting group under acidic conditions (25% trifluoroacetic acid in dichloromethane, 10 min), we measured the FTIR spectrum of the proline bound to the amineterminated SAM (Figure 2b). The bands at $\tilde{v} = 1651$ and 1533 cm$^{-1}$ are characteristic for amide I and amide II absorptions. The only source for these absorptions is the formation of amide bonds between printed amino acids and amine-terminated SAMs. We also observed changes in the advancing contact angles from 61° for the free amine to 74° for Boc-tyrosine, 82° for Boc-proline, and 83° for Fmoc-alanine, which changed to 76° after deprotection.

As a control, a flat oxidized PDMS stamp was immersed in ethanol with no reagent; the stamp was dried as before and printed onto an amine monolayer for 30 min at room temperature. Ellipsometry showed an increase in thickness of 0.2 nm, but this is within the error of ellipsometry measurements on gold and consistent with the findings of Glasmastar et al.[10] The experiment was repeated with a 10 mM solution of Boc-proline in ethanol and a non-oxidized stamp. The ethanolic solution did not ink the hydrophobic PDMS stamp in a homogeneous way, and only a patchy increase of <0.3 nm was observed. The silica layer of the oxidized PDMS can act as an acid catalyst,[11] and to show that this did not influence our reactions under the stamp, we prepared permanently hydrophilic PEG-coated stamps according to Delamarche et al.[12] When we repeated the chemistry with the modified stamp, our result was consistent with the results with our nonmodified stamps, thus indicating that there is no acid effect on the PDMS. Finally we attempted the reaction without the use of a stamp. A drop of the solution of Boc-proline was placed on the surface of an amine SAM, and the solvent was allowed to evaporate. The surface was left overnight, and ellipsometry showed no evidence of a reaction having taken place. Again the thickness increase was 0.2 nm, which is within the error limits of the instrument.

The tripeptide arginine–glycine–aspartate (RGD) is involved in the interactions between extracellular matrix proteins, such as fibronectin, and the integrin proteins located in the cell membranes of most human cells. The immobilization of RGD peptides on a surface enhances the adhesion of tissue-forming cells.[13–15] To further support our hypothesis we decided to synthesize the tripeptide RGD on the surface through multiple stamping steps. The first step involved printing a 2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (Fmoc-AEEA-OH) linker onto an aminopropyltrimethoxysilane (APTS) SAM on silicon. Subsequent removal of the Fmoc group was followed by printing Boc-aspartate (D), Boc-glycine (G), and Boc-arginine (R) (to allow for the correct N–C coupling direction). 3T3 mouse cells were seeded in cell-culture medium without fetal bovine serum (FBS) on top of treated surfaces; under these conditions no nonspecific cell spreading was observed. Cell attachment was evident, however, on the printed surfaces, thus indicating that the RGD was present on the surface, and that a multistep sequence can be carried out by repetitive stamping (Figure 3).

To demonstrate the viability of this approach, a 20-mer peptide nucleic acid (PNA) strand was synthesized step by step on the surface. First the Fmoc-AEEA-OH spacer was printed onto the APTS monolayer. Unreacted amines were acylated with a capping solution (acetic anhydride/2,6-lutidine/N,N-dimethylformamide (DMF)) to prevent further unwanted reactions. The Fmoc protecting groups were
subsequently removed with DMF/piperidine. By using the same printing activation cycle and commercially available nucleotides the sequence CACT-GAACCTGCTACCTAA-Fmoc was printed in 20 steps. Ellipsometry measurements showed a steady increase in thickness with each base that was added, and a slight decrease for each Fmoc group removed (Figure 4). The ellipsometry data show a fairly linear increase in thickness for the first five bases (Figure 4c), but deviation from the initial slope seems to indicate a decrease in “yield”, that is, thickness increase per base. The final thickness of the PNA 20-mer is roughly half that which could be expected from extrapolation of the initial increases. However, per printing step, this would still mean an average of well over 90% efficiency.

The PNA chip was also analyzed by FTIR spectroscopy (Figure 4d). The spectrum shows clear evidence of amide bonds in the PNA chain at $\tilde{\nu} = 1650$ cm$^{-1}$. There is also evidence for phenyl-ring stretching vibrations at $\tilde{\nu} = 1550$ cm$^{-1}$. The peak at $\tilde{\nu} = 1745$ cm$^{-1}$ is due to the urethane groups of the benzhydryloxy carbonyl (Bhoc) protecting groups. A band of peaks at around 2950 cm$^{-1}$ (not shown) result from the different types of CH stretching. The peak at 1465 cm$^{-1}$ might arise from the conjugated C–N double bonds in the purine and pyrimidine molecules.

Prior to hybridization, the terminal Fmoc group was removed, followed by the Bhoc groups along the PNA backbone (50% trifluoroacetic acid, 2 h). The PNA on the surface was hybridized with a DNA 16-mer that was complementary to the 16 top-most bases with a hexaethylene glycol–Cy3 fluorophore attached. The hybridization was visualized by using a laser fluorescence scanner (Figure 4b). There was a clear increase in fluorescence on the PNA-modified surfaces after removal of the Bhoc protecting groups. On another section of the silicon chip two other non-complementary PNA sequences were prepared, one with a 6-base and the other with a 20-base mismatch. The DNA probe described earlier did not hybridize with these sequences, thus demonstrating a lack of nonspecific binding and that all fluorescence intensity is due to the presence of the PNA 20-mer.

We have demonstrated that a covalent amide bond is formed in the absence of catalysts or activated substrates in the nanoscale confinement between stamp and surface. The interaction of DNA molecules and cells with these surfaces provides further proof of the viability of our approach. This rapid method of forming covalent bonds is now being investigated for its ability to promote reactions that normally require high temperatures, pressures, or catalysts. We are also carrying out kinetic studies to determine the actual rate enhancement in this system. We believe this printing method has enormous potential for applications in the field of peptide arrays for protein studies. The formation of new covalent bonds can be combined with nanocontact printing as a means to synthesize molecules on the sub-50-nm scale.

**Experimental Section**

All Boc-protected amino acids were purchased from Novabiochem with $>98\%$ purity and checked in-house by mass spectrometry for Fmoc acid and Boc residues. PNA monomers, the AEEA linker, and deblocking reagents were purchased from Applied Biosystems. 11-
sulfanylundecylammonium chloride was synthesized according to a procedure described elsewhere.[17] The Cy3 fluorescence DNA probe was obtained from Amersham Biosciences. All other materials were used as received from Aldrich. Ethanol was obtained as AR grade 100% and all water used was obtained from a Milli-Q water purification system at 18.2 MΩ. All substrates were stored under nitrogen gas. Ellipsometry measurements were carried out on a DRE ELX-02C ellipsometer with a 632.8-nm laser at 70° from the normal. A refractive index of 1.5 was assumed for all monolayers used. FTIR transmittance FTIR spectra were recorded on a BioRad FTS 6000 spectrometer. The gold was evaporated by using a BOC Edwards Auto500 Thermal Evaporator & RF Sputter Coater (NXE-141-000) in thermal deposition mode. Contact angle goniometry was performed by using a homemade stage comprised of a computer-controlled micro syringe and digital camera. The fluorescent probe was made visible on an Axon instruments Genepix personal 4100A scanner. Poly(dimethylsiloxane) (PDMS) elastomeric stamps were made as described in the literature. All PDMS stamps were freshly oxidized by using an Emitech K1050X plasma asher for 30 s.

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