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Uniform aligned bioconjugation of biomolecule motifs for integration within microfabricated microfluidic devices

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ABSTRACT

Full details and a step-by-step guide suitable for printing proteins aligned to micron-sized sensors and subsequent integration and alignment of microfluidic structures are presented. The precise alignment and grafting of micron-sized biomolecule patterns with an underlying substrate at predefined locations is achieved using a novel semi-automated microcontact printer. Through integration of optical alignment methods in the *x*, *y*, and *z* directions, uniform contact of micron-sized stamps is achieved. Feature compression of the stamp is avoided by fine control of the stamp during contact. This printing method has been developed in combination with robust, compatible bioconjugate chemistry for patterning of a dextran-functionalized silicon oxide substrate with a NeutrAvidin-"inked" stamp and subsequent incubation with a biotin-functionalized protein. The bioconjugate chemistry is such that uniform coverage of the protein (without denaturation) over the printed motif is obtained and reproduction of the initial mask shape and dimensions is achieved. Later integration with a microfluidic structure aligned with the printed motif on the substrate is also described.

Miniaturization of biochemical assays is required for both clinical diagnostic and bioanalytical applications. Lab-on-a-chip formats are advantageous with respect to reduced sample consumption [1] and processing time [2] as well as sometimes providing improved sensitivity [3]. These devices often consist of microfluidic channels for the delivery of analytes to sensors that are usually present in one face of a rectangular microfluidic channel. The bioaffinity sensors are a class of sensor where bioanalytes are detected as a result of a specific molecular binding event on a functionalized sensor surface. Although there has been significant effort invested in the creation of (i) highly sensitive sensors appropriate for bioanalyte detection and quantification and (ii) micron-scale biomolecule patterning methodologies [4–9], approaches to provide both precise alignment and good uniformity of the biomolecule patterns on the underlying microfabricated sensors (and the subsequent integration with microfluidic structures) have been lacking until now.

Alignment of sequential fabrication steps by silicon microelectronic fabrication methods relies on the use of a series of alignment marks [10]. The alignment accuracy depends on a range of factors, including the photolithographic resist as well as the precision of the etch, deposition, and growth process used [10]. These fabrication approaches have been adapted and adopted for the fabrication of microfluidic devices and sensors as well as for patterning of proteins by using microcontact printing (μCP)[1] methods that have been developed [11]. Although the application of μCP methods is well-suited for arrays of one or multiple printed components [12], the alignment of shaped μCP motifs and shaped microfluidic channels with underlying microfabricated structures to micrometer precision is now required. The most relevant approaches that go some way in achieving this include that reported by Chakra and coworkers where antibodies were grafted inside an integrated microfluidic device by means of μCP [13] and that where poly-L-lysine was associated (but not conjugated) with electrode arrays [14]. Although methods for the alignment of biomolecule-patterned motifs with the underlying structures have been reported [13,14], the alignment was assessed only in a qualitative manner and the pattern uniformity was not considered for these methods. Here we report a method that provides both (i) accurate alignment and (ii) high uniformity of the conjugation of the biomolecule, both of which are needed for integrated sensor-microfluidic devices and lab-on-a-chip systems.

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[1] *Abbreviations used:* μCP, microcontact printing; PDMS, polydimethylsiloxane; FITC, fluorescein isothiocyanate; GOPS, glycidyl-3-oxypropyltrimethoxysilane; BSA, bovine serum albumin; TNF-α, tumor necrosis factor alpha; Cy5.5 NHS ester, cyanine 5.5 *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; XPS, X-ray photoelectron spectroscopy; UV, ultraviolet; DMF, dimethylformamide; MWCO, molecular weight cutoff; CCD, charge-coupled device; SNR, signal-to-noise ratio.

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Precise, localized uniform bioconjugation of recognition molecules to only the active region of the sensor and not the surrounding (inactive) regions is critical to achieve the optimal bioaffinity sensor response. The creation of lab-on-a-chip devices for the analysis of very small volumes (10–100 μl) of biological fluids is becoming more important. Firstly in tracking and detecting biomarkers or pharmaceutical agents in microlitre volume blood samples from small animals (i.e. mice) so that blood can be analysed without sacrifice of the animals at every timepoint - thus providing protocols that conform to the principles of the 3Rs (replacement, reduction and refinement). Secondly, clinical studies and diagnostic approaches for use with at risk patients become possible; small blood samples taken with a lancet is less likely to compromise the health of patients as compared to regular intraveneous blood donation (i.e. the elderly). For the sensitive detection and quantification of analytes in integrated sensor-microfluidic devices using very small volumes of analyte, consideration of the channel shape as well as the motif design of the surface-conjugated recognition molecules on the active region of the sensor is required. The reason for this is that consideration of the effective delivery of analytes within the fluid flowing through the microfluidic channel over the sensor is important so that there is uniform association of the analyte with the recognition molecules on the sensor active region. Recently, we investigated an approach for the effective delivery of small volumes (μl) of analytes within microfluidic channels to the active region of a planar optical sensor [15]. Numerical methods were applied to the design of the shape of the channel and also to the shape of the motif on the active region of the sensor. Designs of microfluidic channels and motifs of patterned sensors suitable for nearly complete mass transfer to the recognition molecules on the sensor and uniform association were obtained [15]; this study was the first (as far as we are aware) where the channel and motif shape was considered for the optimized "capture" by association of analyte from low volumes of solution. Other related studies have been focused on the design of integrated microfluidic affinity sensor systems for the evaluation of the binding parameters for biomolecular recognition [16,17].

Here a semi-automated μCP printer for bioconjugation of patterns on surfaces in defined aligned locations is presented. In developing this method, it became clear that the bioconjugate chemistry that is compatible with this μCP method required improvement for the attachment of "sensitive" proteins that are prone to denaturation on the polydimethylsiloxane (PDMS) stamp. The μCP technology, the precision of alignment, and the bioconjugate chemistry, as well as the second alignment of an overlying microfluidic structure, are described in detail. This methodology is likely to have broad applicability to many lab-on-a-chip applications applied to biochemistry.

Materials and methods

Materials and reagents

Materials and reagents were purchased from the following sources: Sylgard 184 PDMS prepolymer and cross-linking catalyst from Dow Corning; photoresists SU-8-2 and SU-8-10 from MicroChem; EC solvent from Microposit; hydrogen peroxide (35%), dextran (500 kDa), anhydrous toluene (99.8%), NeutrAvidin, NeutrAvidin–FITC (fluorescein isothiocyanate), and sodium cyanoborohydride (95%) from Fisher Scientific; glycidyl-3-oxypropyltrimethoxysilane (GOPS, 97%) and bovine serum albumin (BSA, 96%) from Sigma–Aldrich; ammonium hydroxide solution (50%) and sodium metaperiodate (98%) from Alfa Aesar; recombinant murine tumor necrosis factor alpha (TNF-α) and anti-mouse TNF-α antibody from R&D Systems; SureLINK Chromophoric Biotin Labeling Kit from Insight Biotechnologies; cyanine 5.5 *N*-hydroxysuccinimide ester (Cy5.5 NHS ester) from GE Healthcare; and 5-kDa Vivaspin centrifugal filters from Sartorius Stedim. All other reagents used were from commercial sources and of the highest grade (clean room or molecular biology grade). All water used was ultrapure (18.2 M Ω cm). Phosphate-buffered saline (PBS, pH 7.4) was prepared using literature methods [18]. Glass substrates were either 50 × 50 × 1-mm or 76 × 26 × 1-mm Menzel glass. All glass surfaces were cleaned using an RCA clean (immersed in ethanol for 20 min, dried, immersed in 1:1:5 ammonium hydroxide/hydrogen peroxide/water for 30 min, rinsed with water for 10 min, dried under a stream of nitrogen, and then baked at 75 °C for 1 h) before use [19]. Photomasks were designed using the L-Edit mask layout editor (Tanner Research) and fabricated by Compugraphics. A Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies) was used for the optical absorption measurements.

X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) spectra were obtained using a Kratos Axis Ultra-DLD photoelectron spectrometer with monochromatic Al Ka radiation (photon energy = 1486.6 eV) at 120 W power. High-resolution spectra were collected at 40 eV pass energy, whereas surveys are acquired at 160 eV; the analysis area was 700 × 300 μm. All XPS spectra are calibrated to the C(1s) signal, which was assigned a value of 285 eV typically used for polymeric/organic materials [20].

Menzel glass substrates (~1 × 1 cm) were cut, cleaned, and functionalized as for the larger glass substrates described below. Aldehyde-functionalized substrates were coated with NeutrAvidin (1.7 μM) (rather than stamped), incubated under a cover slip in a humid chamber for 1 h, and rinsed extensively with water to remove excess NeutrAvidin. The NeutrAvidin-coated substrates were incubated with sodium cyanoborohydride as described below and then washed.

Contact angle measurements

Droplets (1 μl) of water were placed on the surface and left to stabilize for 15 s before measurement with a Kruss contact measurement tool fitted with a DSA100 goniometer with tilting stage and a Kruss T1C in-built camera. The instrument software was used to calculate the contact angle. Contact angle measurements were obtained from triplicate measurements from three samples.

Image analysis

All images were acquired using an Axio Observer Z1 microscope, AxioCamHR3, and AxioVision software (image size = 1388 × 1040 pixels). Images were obtained using 2.5× and 10× lenses. (Note that the length of the printed taper motif was obtained from an image collected with the 2.5× lens and the width was obtained from an image collected with the 10× lens; thus, different measurement errors are obtained.) MATLAB was used for image analysis.

Fabrication of alignment marks in glass substrates

An ion beam etch process was used to create alignment marks on the glass substrates prior to functionalization. To achieve this, 50 × 50 × 1-mm glass substrates, cleaned with a standard RCA clean process, were baked at 120 °C for 1 h. A photoresist layer (SU-8-2) was deposited by spin coating at 2000 rpm for 30 s, baked at 60 °C for 1 min, and then baked at 90 °C for 3 min. The SU-8 layer was exposed to ultraviolet (UV) light through a photomask with the designed features for 11 s at 10 mW/cm^2 in the mask aligner (MA-6, Suss Microtec), baked for 1 min at 65 °C, and then