Towards protein pattern transfer for biosensor applications

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Abstract

This paper presents a very simple, industrially-scalable method for transferring a high-resolution, biologically-active protein pattern from one substrate to another. We demonstrate the transfer of a protein pattern formed initially by microcontact printing from a silicon surface (to which this form of printing is applicable) onto a glass or polymer substrate, almost independently of the surface/bulk properties of the second substrate. A very thin, spin-coated layer of a sugar is used to preserve the structure and organization of proteins during the subsequent plasma deposition of a siloxane polymer, after which the protein pattern could simply be peeled off the silicon substrate and glued onto any other desired substrate.

Keywords: biosensors - protein pattern transfer - atomic force microscopy - microcontact printing - fluorescence

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1. Introduction

The immobilization of antibodies, and more generally proteins, is a crucial factor affecting their recognition by antigens. It has therefore drawn particular attention in the past decade, mainly due to its importance in many biotechnological applications. Biological interactions such as biotin-avidin complexes (Vareiro et al., 2008), or protein A or G (Briand et al. 2006; Ha et al., 2007), chemical interactions, like glutaraldehyde or PDITC (1,-4 phenylene diisothiocyanate) linkage (Lee et al., 2006; Raj et al., 2009; Rao et al., 1998; Weiping et al., 1999) and physical interactions, such as simple adsorption (Dreesen et al., 2004), have been studied for antibody immobilization on surfaces. It appears that protein immobilization is strongly dependent on surface properties such as composition, reactivity, wettability, roughness etc (Sethuraman et al., 2004; Xu and Siedlecki, 2007). For example, protein adsorption has proven to be different on gold, silicon and stainless steel substrates (Nakanishi et al., 2001), to depend on surface charge and hydrophobicity, and to vary strongly with the nature of the protein (Lu et al., 2007). Thus, it is not surprising that immobilization strategies developed up to now are not applicable to every substrate. The most characterized and understood substrates are mainly not appropriate for biotechnological applications. On the other hand, polymer substrates, usually used in bio-medical or -technological devices because of their interesting optical properties, low time-consuming and production costs, are more difficult to manipulate or modify. Characterizing the adsorbed state on these substrates can also be more difficult.

These problems can be overcome using the transfer strategy outlined in Scheme 1 and described as follows. During the first step a selected protein is deposited on a selected substrate (Scheme 1.1) (Chen et al., 2003; Shi et al., 1999; Wang et al., 2004). Particularly, some metallic or semiconducting materials are used as substrates for the immobilization of a

protein pattern, made of antibodies (a 5-6 nm thick layer). Such substrates also allow easy characterisation using standard surface analysis techniques. We used microcontact printing (Crivilliers et al., 2007; Mrksich and Whitesides, 1995) onto a silicon surface, to which this form of printing is applicable, to prepare the initial high-resolution pattern. The second step consists of transferring the antibodies onto a second substrate, which may have unfavourable surface characteristics. This transfer is performed by first spin-coating the adsorbed protein with a thin protective layer of a sugar (a disaccharide, such as trehalose) (Scheme 1.2). This coating (< 5nm thick) functions as both a protecting layer and fixing agent, allowing the structure, organization and orientation of proteins to be maintained during the transfer process. This step is followed by the deposition of a layer that acts as the physical interface and mechanical support: a plasma deposited polymer-like layer (around 100 nm thickness) (Scheme 1.3) (Gandhiraman et al., 2005). This sandwich can then simply be glued to a second substrate – glass or polymer for example (Scheme 1.4 and 1.5). The only limitation of this process is therefore the glue used to transfer proteins to the second substrate. Indeed, it should adhere to the second substrate but not disadvantageously affect the specific properties for which the second substrate has been chosen – optical clarity, for example. Finally, the substrates are separated by simply pulling them apart, the deposited proteins transferring to the second substrate (Scheme 1.6). We here demonstrate this process and show that the resultant surface retained the protein pattern, and its biological activity, almost independently of the nature of the surface to which the pattern had been glued.

2. Materials and Methods

2.1. Chemicals.

D-(+)-Trehalose dihydrate, hydrogen peroxide, sulfuric acid, hexamethyldisiloxane from Sigma-Aldrich and millipore purified water (18MΩ.cm) were used without further treatment. Albumin from bovine serum was diluted in phosphate buffer solution (PBS) (0.01 mol/L phosphate buffered saline (NaCl 0.138 mol/L; KCl - 0.0027 mol/L); pH 7.4, at 25 °C, Sigma-Aldrich) at a concentration of 30 mg/ml. Hyperpure silicon polished (100) n-doped silicon substrates were provided by Wacker Chemitronic GmbH. Glass substrates were microscope slides (Corning Inc.). The silicon and glass substrates were first washed by dipping in piranha solution (1:3 concentrated H_2O_2:H_2SO_4 solution) for 10 min and thoroughly rinsed with deionized water (DI water). Cycloolefin polymer, Zeonor® (Zeonor 1060R), was provided by Åmic AB (Sweden). The slides were cleaned using UV-ozone treatment (Novascan PSD-UV) for 15 min at room temperature.

2.2. Microcontact printing of proteins.

Patterned or flat poly(dimethylsiloxane) (PDMS) stamps were fabricated by pouring a 10:1 (v/v) mixture of Sylgard 184 elastomer and curing agent over a patterned silicon master (stripes of 15 μm, separated by 15 μm). The mixture was cured for one hour in an oven at 60ºC, then carefully peeled away from the master and left in the oven for another 18 h at 60ºC to ensure complete curing. Prior to inking of the stamps, all the stamps were oxidized by exposure to UV/ozone (Novascan PSD-UV) for 15 min. This process favored the hydrophilicity of the stamp and the homogeneous spreading of the ink. Immediately after ozone treatment over a period 10 minutes several droplets of a protein solution (the ink), were

deposited on the stamps. Subsequently the stamps were blown dry in a stream of nitrogen, and were brought into contact with the substrate without the use of additional pressure. Substrate and stamp were kept in contact for 10 min before careful separation. Each stamp was used to print 5 samples, then discarded and a new one used. Different proteins in a PBS solution were used: 10 μg/mL human immunoglobulin G (hu-IgG) and 10 μg/mL goat-anti human immunoglobulin G (α-hu IgG) (from Biomeda Corp, Foster City, California, USA). Hu-IgG and α-hu IgG were also labelled with DY-636 using the following procedure: 1 mg of DY-636-NHS ester was dissolved in 1 mL of deionized water, from which 267 μL were added directly into an eppendorf tube (1.5 mL) containing a PBS solution (2 mg, 12.5 nmol, pH = 8.0) of polyclonal IgG and allowed to gently shake at room temperature for 4 hrs. After that, the reaction mixture was placed in a refrigerator overnight to let the unreacted dye to hydrolyze and then the reaction was worked up by size-exclusion chromatography (NAP-5 or NAP-10 columns). The amount of molecules attached to IgG was subsequently determined by the UV-vis spectroscopy from all available fractions

2.3. Spin coating of a sugar layer.

A 5 10^{-5} mol/L trehalose / deionized water solution was spin coated at 5000 rounds per minute (rpm) for 30 s. The use of such a dilute sugar solution ensured that the protective sugar layer was very thin. This layer was approximately 3-5 nm thick (as determined by atomic force microscopy scratching experiment).

2.4. Plasma enhanced chemical vapour deposition (PECVD).

The PECVD deposition of a polymer-like layer was performed using a computer controlled Europlasma CD 300 PECVD system. The deposition was performed using base pressure ~100 mTorr, hexamethyldisiloxane (HMDSO) (Sigma-Aldrich) 100 standard cubic centimeters per

minute (sccm), O_2 100 sccm, plasma power 20W for 15min. The thickness of the layer was approximately 100 nm (as measured by spectroscopic ellipsometry assuming a refractive index identical to silicon dioxide).

2.5. Glue.

The glue used was a cyanoacrylate based glue (Henkel Consumer Adhesives) for glass as a substrate 2 and a two-part epoxy glue for cycloolefin polymer as substrate 2.

2.6. Transfer process.

A selected protein was microcontact printed onto a silicon surface (5-6 nm thick layer), a 5 10^{-5} mol/L trehalose / deionized water solution was then spin coated on top of the patterned surface (< 5 nm thick). This was followed by the deposition of a polymer-like layer (~ 100 nm thick) using a 13.56 MHz RF, capacitively coupled plasma in a plasma enhanced chemical vapour deposition (PECVD) reactor with hexamethyldisiloxane (HMDSO) as a precursor. This upper polymer-like layer was then glued with any suitable substrate-specific adhesive to a second substrate (cyanoacrylate contact adhesive and epoxy were both successfully used). Finally the substrates were separated by simply pulling them apart. In this way the microprinted pattern of proteins is transferred to the second substrate.

2.7. Immunoassay.

For immunoassay experiments, the surfaces of transferred antibody microprinted lines were immersed in a bovine serum albumin solution (BSA) (30 mg/ml) for one hour in order to prevent further protein adsorption on non-covered surface areas. A subsequent immersion in a Cy5-labelled α-hu IgG solution was performed for one hour. The Cy5-labelled α-hu IgG was

diluted in a 1:10 w/w solution of powdered milk in PBS to reach a final concentration of 10μg/ml.

2.8. Characterizations.

The contact angle of bidistilled water on treated solid surfaces was measured in air at room temperature using a video-based optical contact angle measuring apparatus (FTA200, First Ten Ångstroms) with an electronic syringe unit. Uniform drops were deposited on the surface and after the drops had reached a quasi-stable configuration, (15 seconds); digital images of the drops were recorded. Using FTA32 Video 2.0 software, left and right contact angles were calculated from the images. All measurements were performed in triplicate.

The film thickness was measured using J.A. Woollam Co., Inc EC-400, M-2000UI Spectroscopic Ellipsometer. The spot size of the incident light on the substrate was 5 by 1.5 mm^2. $SiO_xC_yH_z$ and protein layers were modeled as a simple silicon dioxide dispersion layer to extract an effective thickness.

XPS was used to evaluate the elemental composition of the surface of samples. The spectra were recorded at a 35° take-off angle with an SSX-100 (Surface Science Instrument) spectrometer using a monochromatized Al-Kα radiation (1486.6 eV) as the X-ray source. Binding energy (BE) survey scans were carried out from 0 to 1100 eV, while narrow scans of photoelectrons peaks for C1s, O1s and N1s core-levels were taken in the recommended range of BE. The pressure in the analysis chamber was maintained at about 4×10^{-9} Torr during analysis. The analyzed core-level lines (C1s, O1s and N1s) were calibrated with respect to the C1s characteristic binding energy of the aliphatic carbons, fixed at 285.0 eV. Peaks of C1s, O1s and N1s are deconvoluted using a mixed Gaussian-Lorentzian fit program (Winspec) with a Gaussian character which was free to vary between 60% and 100%.

Atomic force microscopy (AFM) examinations were performed with a commercial instrument (Dimension 3100 AFM using a Nanoscope IIIa controller equipped with a phase imaging extender, Digital Instruments) operating in Tapping-Mode™ (TM-AFM), using standard silicon tips (Tap300Al, BudgetSensors,) with 42 N/m nominal spring constant and 300 kHz nominal resonance frequency. All images were recorded in air at room temperature, at a scan speed of 1 Hz. The background slope was resolved using first or second order polynomial functions. No further filtering was performed.

Fluorescence microscopy images were taken using an array scanner (ScanArray Gx, Perkin Elmer).

3. Results and discussion

The physico-chemical properties of all surfaces used during the process were investigated by water contact angle (WCA) and X-ray photoelectron spectroscopy (XPS). The silicon wafer was cleaned using *piranha solution* and UV/ozone treatment. This procedure is known to yield a thin hydrated silicon oxide layer (presenting hydroxyl groups at the surface) with a low contact angle (<10°) (Arvidsson et al., 2007). After adsorption of a complete protein layer, human immunoglobulin G (hu-IgG), the water contact angle increased from < 10° to ~ 68°. The relatively hydrophobic nature of the protein layer (as previously shown (McClellan and Franses, 2005)) is confirmed by this WCA increase. The XPS high resolution spectrum of the hu-IgG layer (Fig. 1A) displayed a peak at 401.2 eV (FWHM = 1.37 eV), assigned to nitrogen atoms in uncharged amines and amides (Briand et al., 2006; Kim et al., 2008). This provides another good evidence for the adsorption of hu-IgG on the surface. After spin coating with trehalose, the WCA decreased slightly to 53°, which is indeed in the range observed for polysaccharides (Assis and Hotchkiss, 2007) and confirming the presence of a sugar layer at the outer surface. However, a peak (Fig. 1B) at ~ 401.2 eV (with FWHM = 1.31 eV) in the XPS spectrum, identical to that observed before sugar deposition, is still visible. The AFM-measured thickness of the sugar layer (< 5 nm) was less than the photoelectron escape depth so a nitrogen peak from hu-IgG should be observed in the spectrum. Moreover, as trehalose molecules (nitrogen free) are only physisorbed on the protein surface, the N1s peak should be similar to one observed before sugar spin coating. After the next step, the preparation by PECVD of a continuous thick polymer-like layer (~100 nm as deduced from ellipsometry) was confirmed by the disappearance of the N1s peak in the XPS spectrum (see Supplementary Informations). O1s peaks (Fig. 1C) were observed at 532.0 and 533.4 eV. The

first peak indicates the presence of an oxidised silicon coating on the surface (Si-*O*-Si) (Nema et al., 2004; Gengenbach and Griesser, 1999) while the second peak at 533.4 eV corresponds to C-*O* and C-*O*-O^- bonding, indicative of a siloxane network ($-(Si(CH_3)_2-O)_n-$) (Nema et al., 2004). The C1s (Fig. 1D) peak could be fitted by 3 contributions at 284.5, 285.8 and 286.9 eV, attributed to *C*-Si, *C*-H and *C*-O bonds, respectively (Gengenbach and Griesser, 1999; Tlili et al., 2008). The preparation of $SiO_xC_yH_z$ layer was thus confirmed. The contact angle increased to ~ 87° after the PECVD deposition, highlighting a hydrophobic surface, in agreement with previous studies (Azioune et al, 2007; Yeo et al., 2006).

We wanted to demonstrate both the accurate transfer of an imprinted pattern from one substrate to another, and that the transferred pattern retained biological activity. We measured the pattern using Tapping-mode™ atomic force microscopy and confirmed biological activity by measuring specific capture of antigen by a transferred pattern of antibody. It was easy to identify by atomic force microscopy the pattern before and after the transfer process. Figure 2 shows three-dimensional representations of the surface morphology of a microcontact printed hu-IgG pattern on the silicon substrate before (Fig. 2A) and after (Fig. 2B) transfer of this pattern to a glass substrate. Both exhibit lines of ~12 μm width, separated by ~12 μm. The measured height of the printed protein lines on the silicon substrate was 5-6 nm. This height corresponds to the thickness of a monolayer of adsorbed antibody layer as deduced previously by neutron reflectometry (Xu et al., 2006a). After transfer to the glass substrate and washing with water, the measured height of the lines was about 6-7 nm. These images indicate the transfer of structures (microcontact printed lines) from one substrate to another. A slight increase in thickness can however be observed. The sugar layer can be responsible of it. Indeed, after transfer to substrate 2, the antibody pattern is present on the surface but also the sugar layer (or a part of it) located between the protein lines. This sugar layer (or a part of it)

between lines is assumed to be washed away during the washing step performed just after separation of both substrates. Oppositely, sugar molecules directly interacting with antibodies in the lines, through van der Waals or hydrogen bounding should not be washed away, therefore increasing protein lines thickness after transfer. This observation also implies that the sugar coating was not destroyed by the subsequent PECVD process.

Fluorescence microscopy was used to quantify the efficiency of the protein transfer and to confirm that biological activity had been retained. Two experiments were performed (Figs 3). The first experiment was to transfer a printed pattern of anti-human immunoglobulin G labelled with DY-636 (DY-636 α-hu-IgG). Fluorescence imaging on these samples verified the presence of the protein on the first substrate before transfer (Fig. 3A), as well as on the second substrate after transfer process (Fig. 3B and 3C), structured in lines according to the original pattern stamp. The lateral resolution of the fluorescence scanner used induced pixelisation of the image but did not influence the fluorescence intensities measured. The graph in figure 3 (top right) highlights fluorescence intensity profiles measured perpendicularly to the printed lines. By comparing in this graph the fluorescence intensities on the silicon substrate before transfer (black line) and on the second substrate after transfer onto glass (red line) and Zeonor® (green line) as second substrates, we were able to quantify the transfer efficiency as 43 ± 4 %. The missing material has probably been removed by the washing step before the measurement or some of the proteins have stayed on the first substrate. Although this transfer was not as efficient as expected, it seemed to be almost independent of second substrate surface properties, as expected. In this case glass and a hydrophobic cycloolefin polymer, Zeonor®, that is widely used as platform in biosensors technology (Dudek et al., 2009; Jonsson et al., 2008; Raj et al., 2009) because it is easy to mould accurately and has low background fluorescence under UV excitation, were used as

second substrates and provided similar results. This also demonstrates the extension of our process to different substrates, almost independently of their surface properties.

Although a fluorescent signal was measured, it did not allow us to postulate that antibodies transferred using this process are still capable of recognising antigens. A second experiment was performed to prove it. The second substrate carrying a transferred pattern of ✔-hu IgG was dipped in a bovine serum albumin solution (BSA) for one hour in order to prevent further protein adsorption on non-covered surface areas. A subsequent immersion in a solution containing a fluorescent antigen, DY-636-labelled human IgG solution, demonstrated the recognition of the surface-patterned antibody by the antigen. Fluorescence microscopy highlighted that the adsorbed fluorescent antigen reproduced the imprinted surface pattern of the antibody (Fig. 3D and 3E). The graph in Fig. 3 (dark blue and light blue lines) shows fluorescence intensity profile measured perpendicularly to the printed lines after biomolecular specific recognition of antibodies by antigens after transfer onto glass (Fig. 3D) and Zeonor® (Fig. 3E) as second substrates (respectively). Assuming similar printing and transfer properties for labelled and non-labelled antibodies, we can conclude that about 43 ± 2 % of the transferred antibodies were recognized by antigens. The proportion was similar after transfer onto glass and onto Zeonor® surfaces. This ratio is very acceptable however revealing some inhomogeneities. These inhomogeneities were already observed in Fig. 2B and can originate from different factors but one of the most predominant appears to be the sugar layer. Indeed, during experiments, transfer efficiency degradation when thicker sugar layers are used was noticed. Thiner layers were therefore used as proposed in this paper. Unfortunately, this sometimes lead to inhomogeneous deposition of sugar and therefore inhomogeneities in the transferred pattern. Microcontact printing procedure can also be responsible of it. Finally, strong interactions between proteins and substrate 1 can also lead to this undesired characteristic of the transfer pattern. In this latter case, a possible solution could be to

minimize protein pattern-substrate 1 interaction through use of self-assembled monolayers presenting methylene functionalities for example, well known to induce low interaction with proteins. It is well known that antigen binding capacity of adsorbed antibody varies strongly with parameters that were uncontrolled parameters in this experiment, such as surface coverage and the nature of the antibody (Xu et al., 2006b). Indeed, these results highlight the need of optimizing the process by carefully controlling each step in order to get low standard deviation and homogeneity of transferred patterns. However, we demonstrated here the concept and its application to a range of different substrates presenting different surface/bulk properties.

4. Conclusions

In conclusion, we have demonstrated a generic process that allows a protein pattern to be transferred, with retention of biological activity and of micrometer-scale pattern features, to almost any surface, regardless of its protein adsorption characteristics. In the developped procedure, a very thin (3-5nm), spin-cast sugar layer sufficiently protects an adsorbed protein layer to allow plasma-polymerisation of a protective and mechanically supportive layer on top. This assembly could then simply be glued to any other substrate and the original deposition substrate pulled apart to leave the protein attached to the second substrate, with pattern details preserved on the micrometer scale and biological activity intact. The procedure has been demonstrated by stamp-printing a high-resolution antibody pattern onto a silicon substrate and then transferring this pattern to a glass or plastic surface. Antigen recognition by the transferred pattern confirmed retention of biological activity. Moreover, orientation of antibodies on the first substrate can easily be performed using self-assembled monolayer, following the technique developed by Wang et al. (Wang et al., 2004). The combination of both techniques (pre-orientation and transfer process) opens the possibility to transfer to a second substrate patterns made of appropriately previously oriented antibodies, which is a major advantage compared to alternative methods like dip-pen nanolithography, affinity printing, in-mold patterning or other inkjet spotting techniques (Biancardo et al., 2008; Renault et al., 2002; Salaita et al., 2007).

5. Acknowlegments

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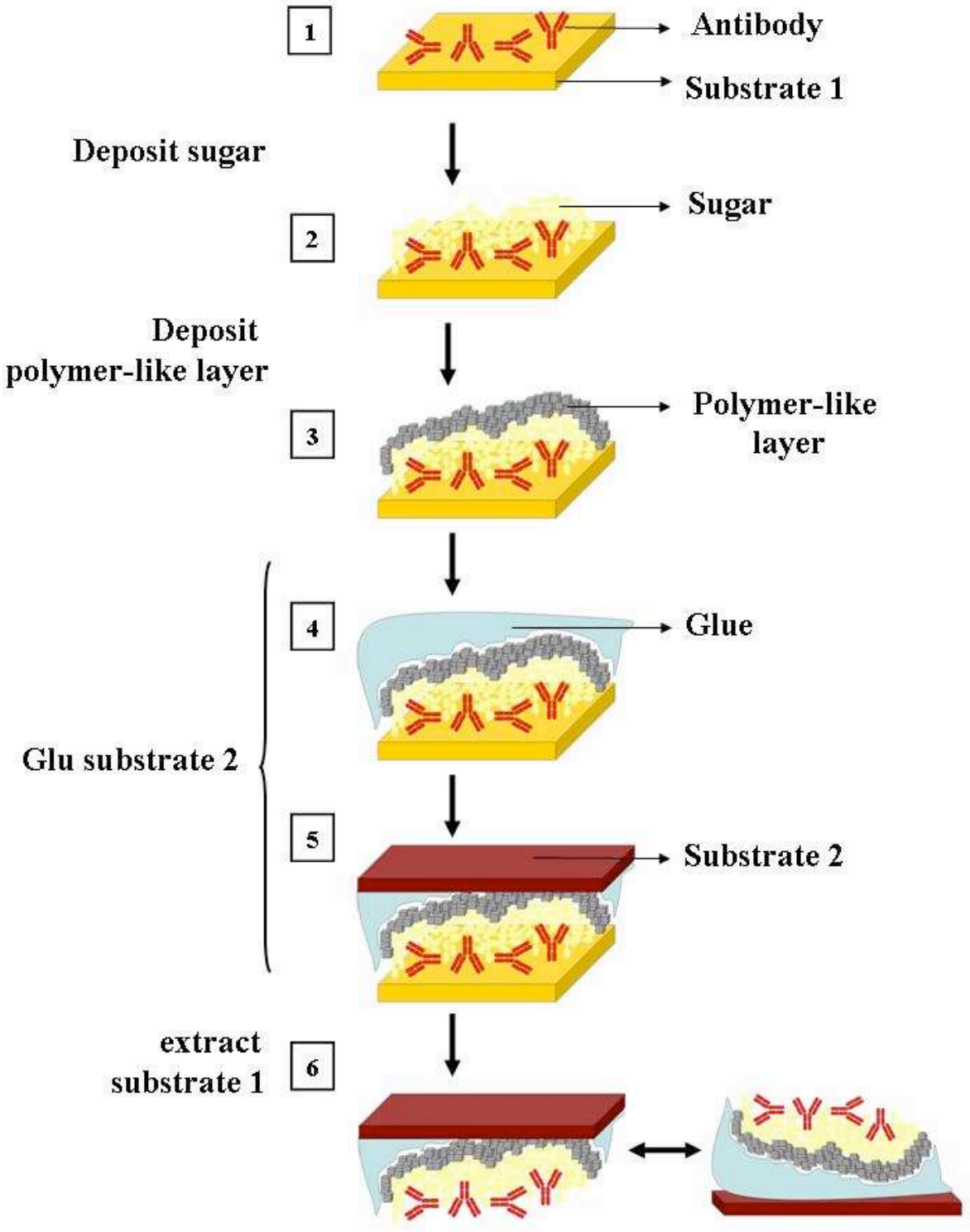

1
Antibody
Substrate 1
Deposit sugar
2
Sugar
Deposit
polymer-like layer
3
Polymer-like
layer
4
Glue
Glu substrate 2
5
Substrate 2
extract
substrate 1
6

Scheme 1

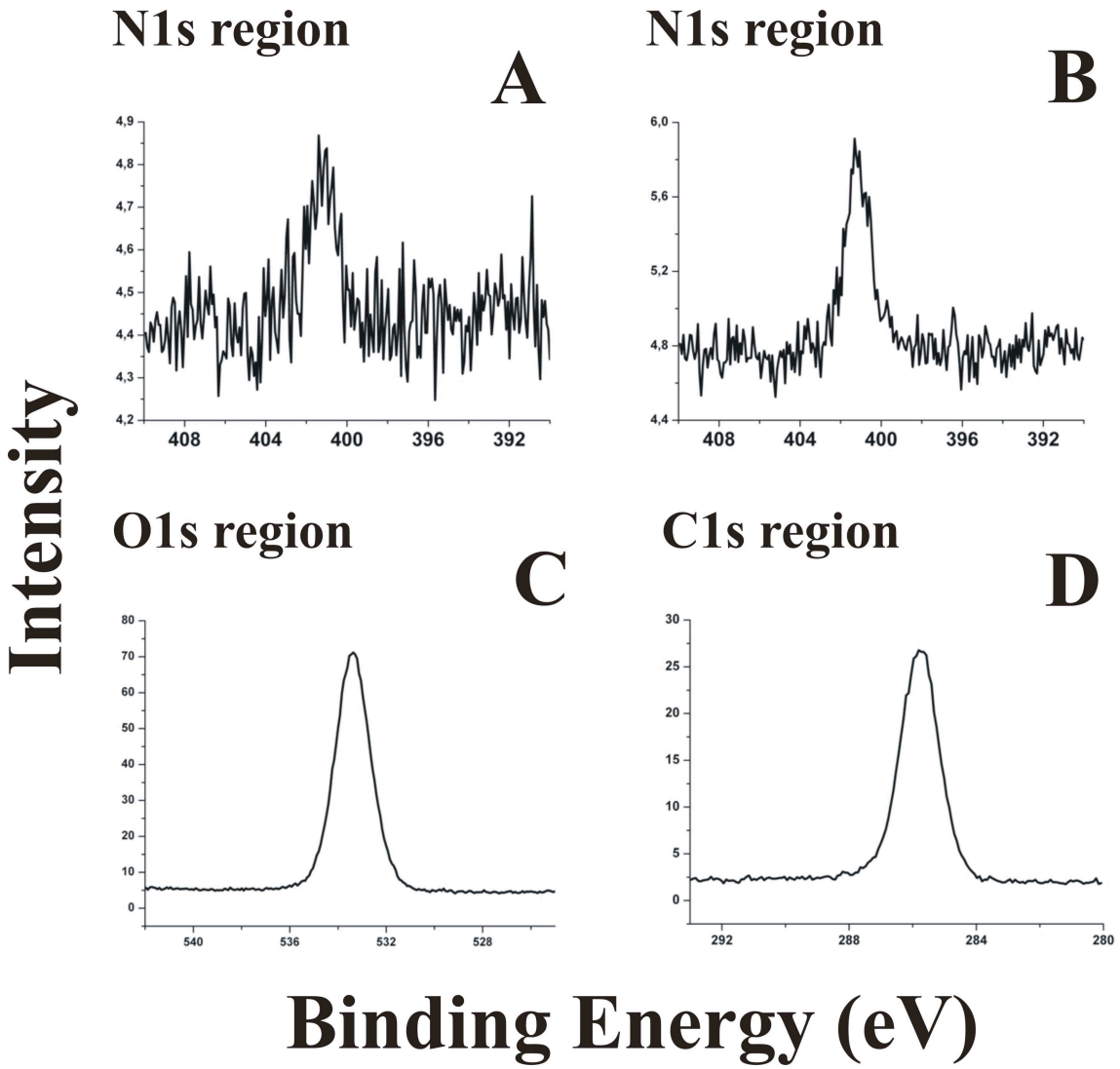

N1s region
A
N1s region
B
O1s region
C
C1s region
D
Intensity
Binding Energy (eV)

Figure 1

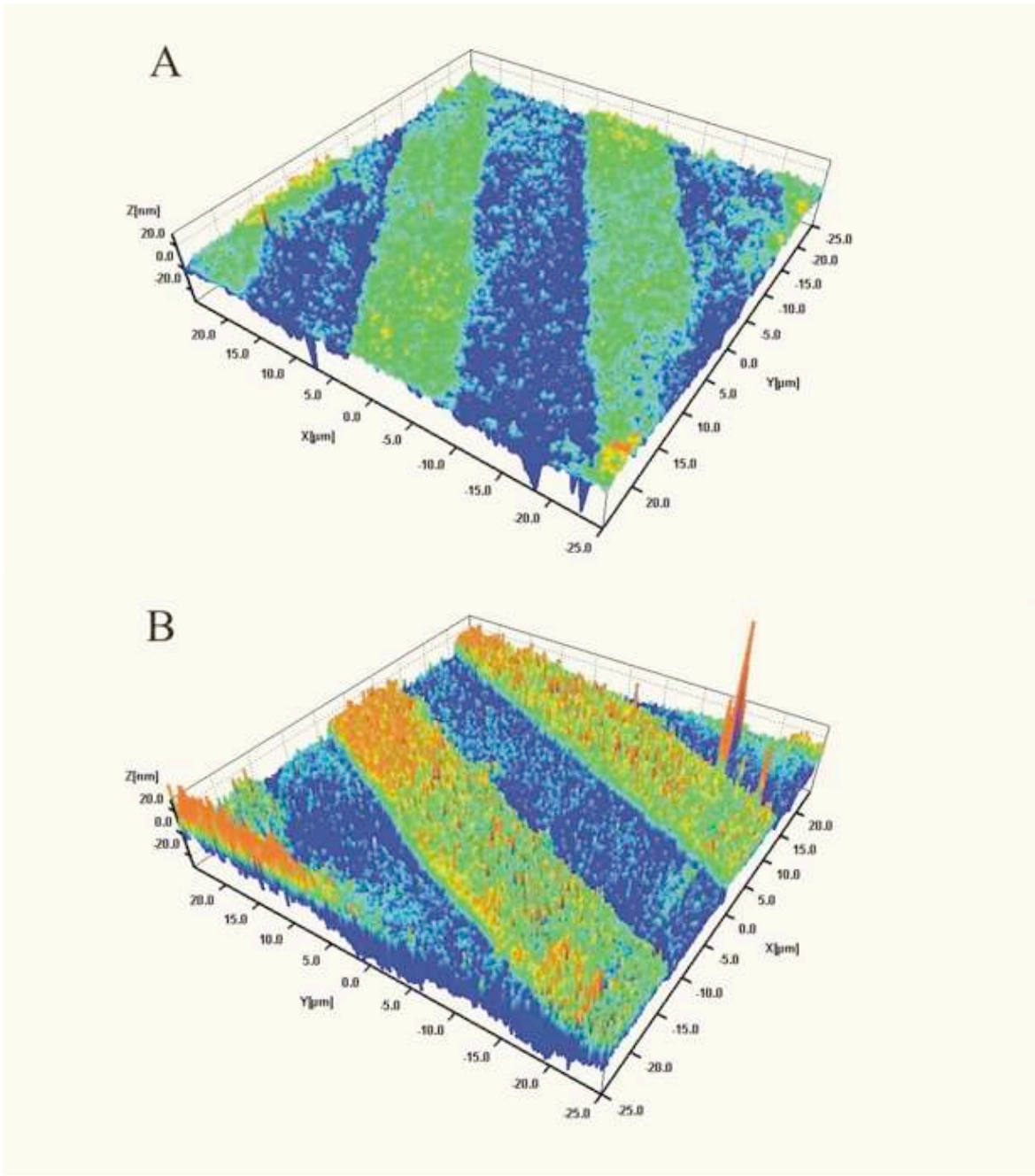
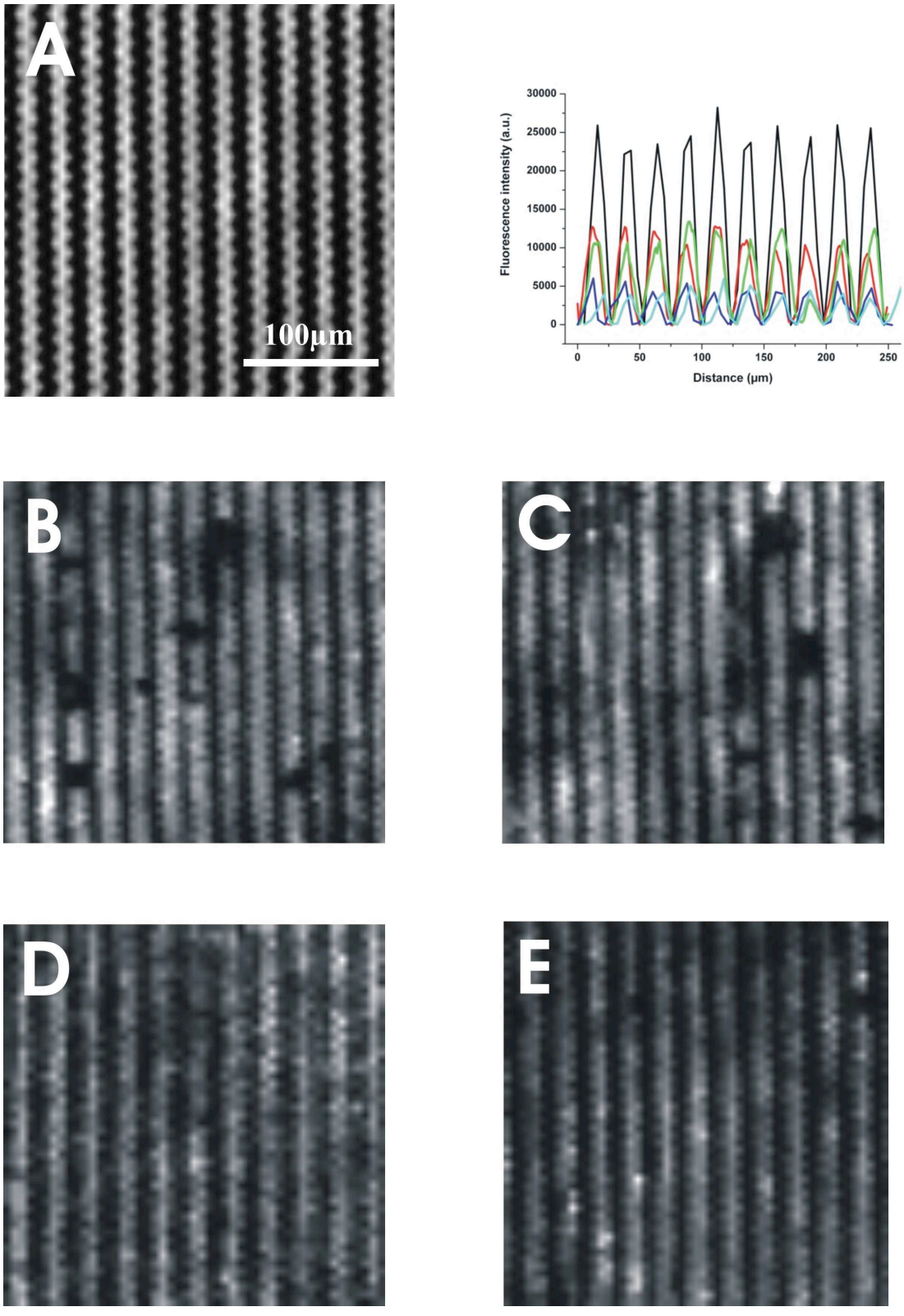


Figure 2


A
100µm
Fluorescence intensity (a.u.)
30000
25000
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Distance (µm)
B
C
D
E

Figure 3