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Porous/Gold surface Preparation for Protein Microarray Applications

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Abstract. Surface chemistry is extremely important in microarray technology, since it determines the successful attachment of the molecules, spot morphology or signal intensity. This paper describes development of functionalised gold layer surfaces deposited onto porous silicon as substrates for microarray experiments. Two gold textures were studied in order to find the best ratio between signal intensity and spot morphology. The experiments revealed: (i) that porous silicon substrate ensures spots uniformity and good response of the signal intensity with respect to concentration; (ii) saturation bands for high concentrations for both types of substrates.

Key-words: protein microarray, porous silicon, gold layer.

1. Introduction

In a typical protein microarray experiment, proteins are arrayed on a functionalised surface as spots (capture molecule); the array is probed with a sample and the interaction with the capture molecule is recorded using different detection methods. [1] Each array can contain hundreds of capture molecules (e.g., cell lysate extracts, proteins) and the interaction of these with a single protein can be analysed simultaneously. Thus, protein microarray technique seems to become a powerful analytical

tool for clinical applications. In order to enable rapid clinical validation, a protein microarray platform should be able to perform high throughput and high sensitivity detection of markers in minute amounts of samples. [2]

One of the most challenging tasks of protein biochip technology is the immobilization of proteins onto surfaces so that they fully retain biological activity [7]. Therefore, immobilization of proteins onto solid surfaces is the key step of a microarray experiment. The most common mechanisms for protein attachment to functionalized surfaces are electrostatic or hydrophobic interaction, physical entrapment, covalent binding and biorecognition. [1] The covalent mechanism requires the presence of reactive groups on the surface (usually electrophilic groups such as epoxides, aldehydes, succinimidyl esters/ isothiocyanate functionalities) able to react with nucleophilic groups (amino, thiol, hydroxy, carboxy) on the biomolecules. [8, 9, 10] The functional surfaces are performed by modification of the solid support (glass, porous silicon, gold) with chemically functionalized organosilanes, self-assembled monolayer (SAM) or polymers. [11]

Albumin is the most abundant blood plasma protein and contributes in 80% to colloid osmotic blood pressure. [3] The most outstanding property of albumin is its ability to bind reversibly an incredible variety of ligands [4]. Bovine serum albumin (BSA) is widely used in proof-of principle protein studies due to its good stability in various media and reproducibility between experiments.

This paper describes the development of functionalised gold layer surfaces deposited onto porous silicon and use of them in microarray applications to find the best ratio between signal intensity and spot morphology. Two gold textures were used to prepare the functionalised surfaces onto which fluorescently labelled proteins (BSA-Cy3) were attached.

BSA (bovine serum albumin) is the principal carrier of fatty acids that are otherwise insoluble in blood plasma. It also performs many other functions such as, sequestering oxygen free radicals and inactivating various toxic lipophilic metabolites such as bilirubin. [5] Albumin has a high affinity for fatty acids, hematin, bilirubin and a broad affinity for small negatively charged aromatic compounds. It forms covalent adducts with pyridoxyl phosphate, cysteine, glutathione, and various metals, such as Cu (II), Ni (II), Hg (II), Ag (II), and Au (I). As a multifunctional transport protein, albumin is the key carrier or reservoir of nitric oxide, which has been implicated in a number of important physiological processes, including neurotransmission. [6]

2. Experimental

2.1. Substrate preparation and surface functionalization

The silicon slides were cleaned in Piranha solution (H_2SO_4: H_2O_2, 3:1) at 80°C for 30 minutes in order to remove organic impurities and then washed for 20 minutes in deionised water (18 MΩ). The slides were then dried under nitrogen flow.

Porous silicon was generated by anodization of *p*-type silicon wafers with (111) crystal orientation and resistivity of 10–20 Ωcm without backside illumination. A

current density of 10 mA/cm^{2}was applied for 20 min and the electrolyte solution was 4% HF in dimethyl formamide (DMF). Before gold deposition, the porous silicon (PS) was boiled in HCl: HNO_3 (3:1) for 10 min in order to remove any organic and inorganic impurity.

The substrates were coated with gold of 100 nm thickness, deposited by vacuum thermal evaporation; two types of surfaces were investigated: (i) PS/gold and (ii) the PS/gold subjected to an additional annealing process at 700°C for ½ h in forming gas for rearrangement of Au surface atoms in (111) crystalline orientation. The (111) gold layers are of great interest to generate self assembled monolayers (SAMs) for the attachment of a new material layer, for protein, enzyme and oligonucleotide bio-immobilization, or in molecular electronics. The spontaneous adsorption of organothiol compounds onto gold leads to a highly ordered and densely packed layer, improving the availability of active surface groups. In order to obtain such monolayers, all slides were immersed in a 0.05 mM 11-mercaptoundecanoic acid solution for 12 hours, under argon atmosphere. After this step, the slides were washed with ethanol, dry under vacuum and functionalized with EDC/NHS (for amide bond coupling), for the protein immobilization on microarray slides. The slides were kept under vacuum until further use.

3. Microarray production

3.1. Protein Labeling

Bovine serum albumin (BSA) was labeled with Cy3 NHS ester using the following protocol: 1 mg/ml in PBS pH = 7.4 protein solution was mixed with 1 mg Cy3 NHS ester and stirred in dark for 2 h at room temperature. The reaction was quenched by addition of one-tenth volume of phosphate buffered saline (PBS). The labelled protein was purified using NAP columns.

3.2. Protein Microarray Fabrication

After substrate preparation, the slides were printed in triplicate, 6×6 spots per subarray, 48 arrays per slide, using a contact printer OmniGrid Micro printer (Genomic Solutions). The stamp time for all the samples was 5 000 ms; the humidity in the printer chamber was 80% and temperature, 20°C. In order to control the pin contact force and duration, the following motion parameters of the instrument set-up were established: acceleration 2 000 mm/s^2; velocity between 10 and 170 mm/s.

The protein samples were prepared using BSA in 10× PBS (pH = 7.4) in 10 different exponential dilutions. Slides were washed in 1× PBS (pH 7.4) using a wash station with 400 rpm. Several analyte concentrations (0.08, 0.16, 0.24, 1 mg/mL) were evaluated. All experiments were repeated three times. Results are expressed as the mean standard error of the signal to noise ratio averaged across each experiment for the replicate spots of interest.

4. Results

The samples were characterized after each technological process. The PS surface structure containing pores of 1 μm diameter after porosification process. The PS samples with gold layer before and after annealing are presented in Figs. 1 and 2.

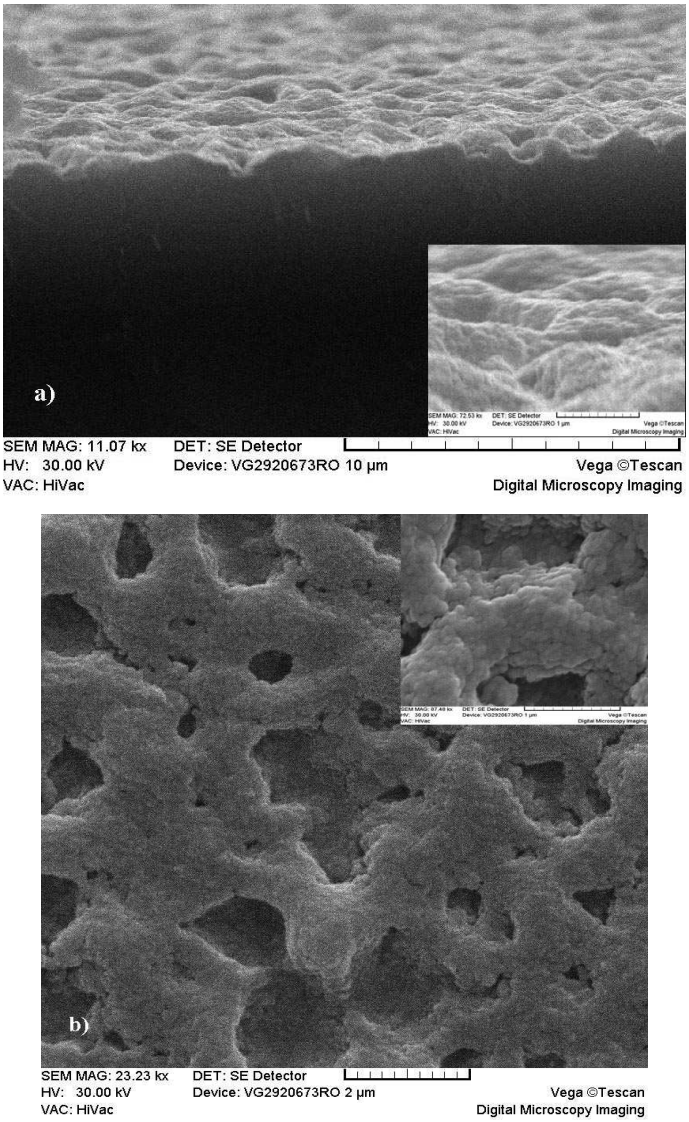

Fig. 1. SEM image of PS/Au substrate before annealing; a) cross section, b) surface image.

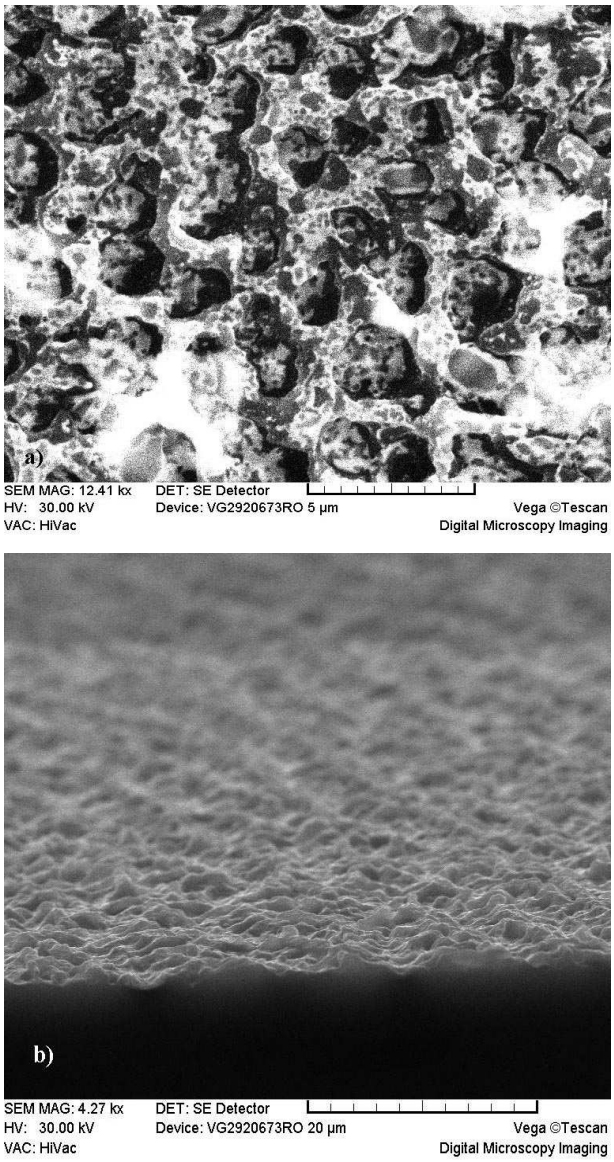

Fig. 2. SEM image of PS/Au substrate after annealing: a) surface image and b) cross section image.

Microarrays were scanned at 635 nm (CY5 channel) and 532 nm (Cy3 channel) at 5-µm resolution using a scanning laser confocal fluorescence microscope (UC4 Microarray Scaner, Genomic Solutions). For all microarray experiments, the laser power was 80% and the PMT gain was 30-40%. The images were analyzed by quantifying the pixel density (intensity) of each spot using Gene TAC Analyzer (Genomic Solutions). The mean signal intensity of each spot was used for data analysis. All

fluorescent intensities in the scanned images were processed as background-corrected mean fluorescence intensities of the pixels within the spot. Statistical analysis was performed with Origin Pro 7.5.

The image files on all of the slides were collected prior to set background fluorescence, after printing and after 30 min washing. In Fig. 3a there are represented the microarrays slides performed by contact printing of labelled BSA.

BSA attachment onto the prepared surface was evaluated after several washing steps. The fluorescence images of all slides were collected after printing and after 30 min washing and are shown in Fig. 3b. The images indicate a stable attachment of the protein on the Au/PS substrate.

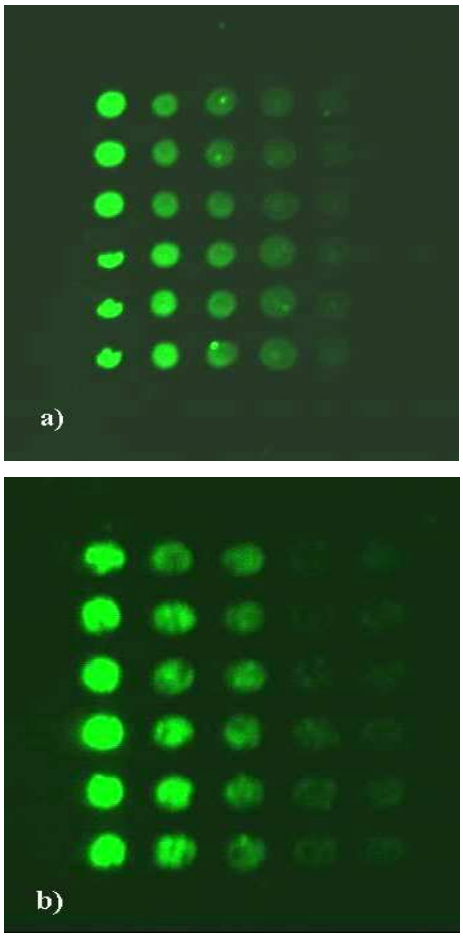

Fig. 3. Image slide with PS/ gold
a) after printing, b) after 30 min washing.

SEM images of BSA printed on porous silicon with both types of gold layers, before and after thermal treatment are shown in Fig. 4.

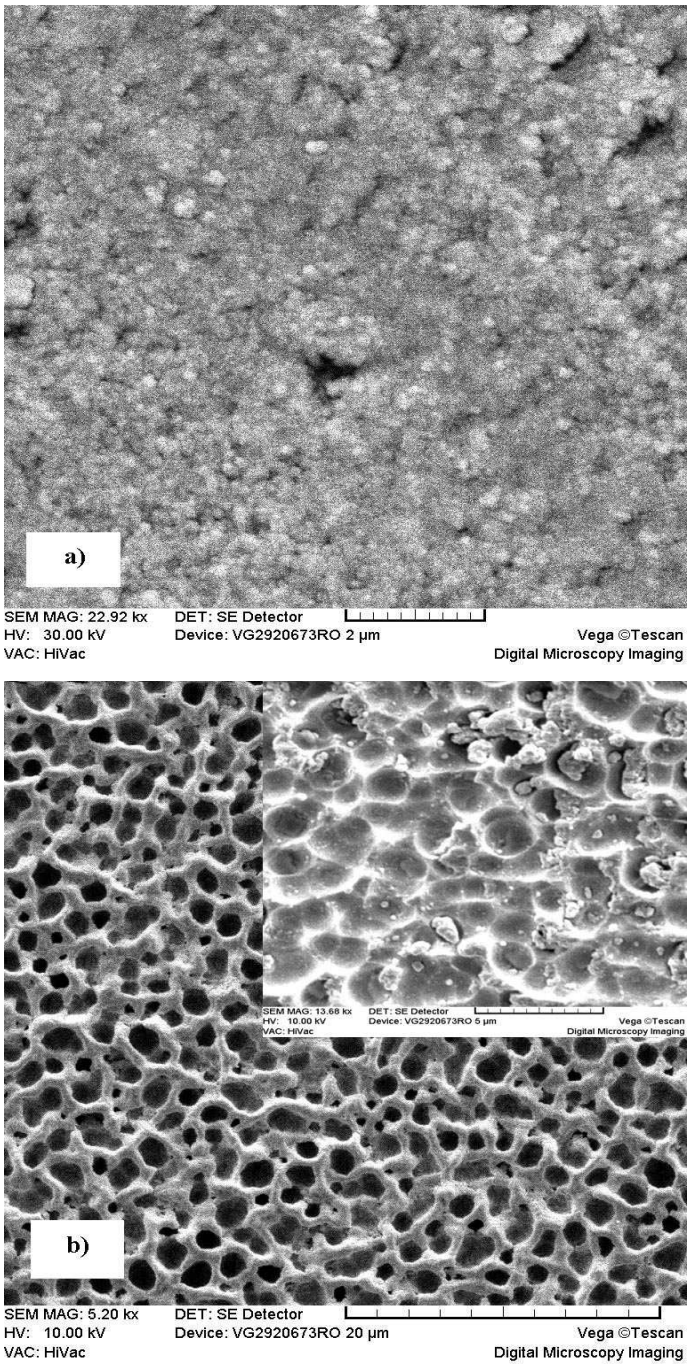

Fig. 4. SEM image of PS/Au substrate with BSA,
a) before annealing, b) after annealing.

Statistical analysis results are plotted in Fig. 5, representing the fluorescence spot intensity versus concentration. The intensity of spots signal was analysed in order to investigate the protein attachment to the surface.

On several slides, especially for high protein concentration printed a tail was observed after washing. We assume that this appears due to surface saturation in protein.

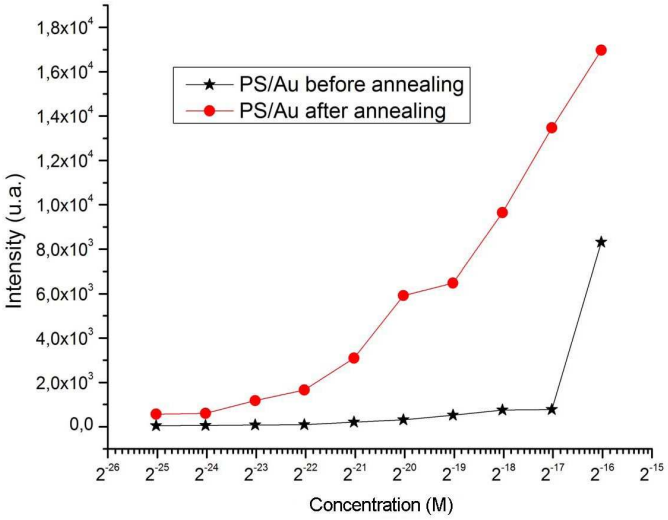

Fig. 5. Fluorescence intensity versus protein target concentration on PS/Au and PS/Au after annealing, scanned at 27.5 % PMT gain.

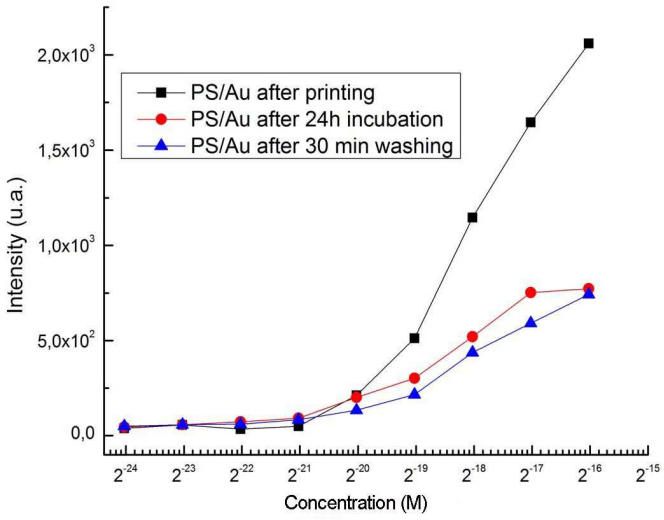

Fig. 6. Fluorescence intensity versus protein target concentration on PS/Au after printing, 24 h incubation and after 30 min washing, scanned at 27.5 % PMT gain.

5. Conclusion

The experiments revealed that: (i) *that porous silicon substrate* covered by PVD gold thin layer *ensures spots uniformity and good response of the signal intensity concentration*; (ii) both types of Au/PS samples (annealed and non annealed) exhibit saturation bands for high protein concentrations.

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