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ORIGINAL PAPER

**Antonios M. Douvas · Panagiota S. Petrou
Sotirios E. Kakabakos · Konstantinos Misiakos
Panagiotis Argitis · Evagelia Sarantopoulou
Zoe Kollia · Alkiviadis C. Cefalas**

157-nm Laser ablation of polymeric layers for fabrication of biomolecule microarrays

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Abstract A new methodology for protein microarray fabrication is proposed based on the ablation of polymer film using laser at 157 nm (F_2). The polymer has been selected among others with the criterion of negligible protein adsorption. Improved results have been obtained by pretreatment of the polymer surface with an inert protein. The use of 157-nm laser radiation allowed very good depth control during the polymeric layer ablation process. In addition the importance of laser ablation at 157 nm is based on the fact that irradiated surfaces indicate limited chemical change due to the fact that laser ablation at 157 nm is only photochemical, thus avoiding excessive surface heating and damage. Results of protein microarray fabrication are presented to illustrate the viability of the proposed method.

Keywords Biomolecule micropatterning · Protein microarrays · 157-nm Laser · Polymer ablation · Protein adsorption · Blocking

Introduction

A number of new approaches have been presented during recent years in the area of processing methodologies suitable for microfabrication of devices containing biological systems. New materials have been designed and efficient patterning methods have been proposed for the fabrication of modern multi-analyte devices [1]. Among the variety of patterning methods, one can distinguish micro- and nano-delivery including contact printing and microfluidics-based methods [2–5] and light-guided methods [6–9].

Laser ablation is an efficient direct-write method of the second category that has also been recently proposed for biopatterning [10]. This method has attractive characteristics, since it does not require masks and does not include wet steps that may denature proteins or complicate the process. Herein, we present a new methodology for protein patterning based on laser ablation of polymeric films, which have been selected to resist protein binding. Although a number of protein-adsorption-resistant surfaces have been found based on poly(ethylene glycol) (PEG) [11], in this first investigation we examined polymers from classes broadly used in lithography (i.e., polyacrylic materials or polyphenol derivatives), which are well known for their good film-forming properties. A 157-nm laser (F_2) is used in order to favor photochemical over thermal phenomena and thus improve resolution capabilities. The high absorbance of most organic polymers at the 157-nm spectral region also allows efficient depth control during ablation [12–14]. Results demonstrating the viability of the proposed methodology are presented.

A. M. Douvas (✉) · K. Misiakos · P. Argitis
Institute of Microelectronics, NCSR "Demokritos",
Aghia Paraskevi, Athens, 15310, Greece
E-mail: adouvas@imel.demokritos.gr
Tel.: +30-210-6503114
Fax: +30-210-6511723

P. S. Petrou · S. E. Kakabakos
Institute of Radioisotopes and Radiodiagnostic Products,
NCSR "Demokritos", Aghia Paraskevi, Athens,
15310, Greece

E. Sarantopoulou · Z. Kollia · A. C. Cefalas
Theoretical and Physical Chemistry Institute,
National Hellenic Research Foundation,
NHRF, Athens, 11635, Greece

Experimental

Reagents

Rabbit IgG, bovine serum albumin (BSA, Cohn fraction II, III, RIA grade) and 2,2′-azinobis(3-ethylbenz-thiazoline sulfonic acid) diammonium salt (ABTS) were purchased by Sigma Chemical Co. (St. Louis, MO,

USA). Goat anti-rabbit IgG AF (Alexa Fluor) 488 and streptavidin AF 546 were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Biotinylated BSA (B-BSA) was prepared according to a published method [15]. Microtitration plates were obtained from Nunc A/S (Rockslide, Denmark). AZ5214, a novolac-diazonaphthoquinone-type photoresist, was purchased from Clariant. Poly(methyl methacrylate) (PMMA, $M_w = 350{,}000$) was obtained from Du Pont. Epoxy novolac (EPN), an epoxidized novolac of cresol-formaldehyde, was purchased from Shell with the commercial name Epikote 164; this polymer was fractionated and the medium fraction was used ($M_n = 1{,}277$, $M_w = 2{,}438$, and $I = M_w/M_n = 1.9$). Methyl (3,3,3-trifluoropropyl)methylvinyl siloxane (MTFPMVS) was purchased from United Chemical Technologies, Inc. Poly(2,2,2-trifluoroethyl methacrylate) (PTFEMA, $M_w = 114{,}700$, $M_n = 45{,}900$, $I = 2.5$, and $T_g = 68.5°C$) was synthesized by free radical polymerization of 2,2,2-trifluoroethyl methacrylate (Aldrich) using 2,2′-azobis(2-methylbutyronitrile) (Fluka) as initiator.

Apparatus

The experimental apparatus for laser ablation, which is installed at NHRF, consists of the molecular fluorine laser, which emits at 157 nm, the all stainless steel vacuum chamber, a computer-controlled X-Y-Z translation stage where the polymer substrates were placed, and the focusing optics.

Procedures

Polymer film preparation

The polymeric films were applied by spin coating on a silicon wafer that was covered by a thick film (200–250 nm) of a protein-adsorbing layer (photoresist AZ5214). These films were thermally treated after spin coating at temperatures in the range 90–200°C for a period of 5–30 min. Further treatment of the polymeric films was applied in certain cases, as indicated in Table 1. Film exposures were performed by using a Hg–Xe exposure tool and a 254-nm filter (50-nm bandwidth at half maximum).

Table 1 Processing conditions and protein binding capacity of PTFEMA film (1% w/w PTFEMA in methyl isobutyl ketone)

Sample no.	PAB	Exposure (at 254 nm)	% Adsorption
1	120°C, 30 min	–	0
2	150°C, 30 min	–	0.7
3	200°C, 30 min	–	14.3
4	120°C, 30 min	15 min	7.9
5	150°C, 30 min	15 min	12.2
6	200°C, 30 min	15 min	14.3

Laser ablation of polymeric films

Polymers were efficiently ablated by hitting the target with laser energies from 0.5 to 5 mJ cm^{-2} per pulse at 20-Hz repetition rate at a background pressure of 1×10^{-5} mbar. The laser pulse had pulse duration of 15 ns at FWHM. The laser light was focused on the target with a CaF_2 lens having 5-cm focal length. The CaF_2 lens was protected from the ablation products with a 1-mm-thin CaF_2 window, which had to be replaced after 10 h of operation at 20-Hz repetition rate due to the contamination of the lens from the ablative products of the polymer following its irradiation at 157 nm. Fine patterning of the laser beam on the target was carried out by moving the substrate in the X–Y plane and by fine focusing in the Z-axis with a micrometric translation stage controlled by a PC.

ELISA evaluation of protein binding properties of polymer films

The protein adsorption resistance of the polymeric films was evaluated through a model binding-assay (Fig. 1). For this purpose, the polymeric film surface was first incubated with a 20 μg mL^{-1} B-BSA solution in 0.05 M phosphate buffer, pH 6.5, for 30 min at room temperature (RT). It was then washed with distilled water and blocked by using a 10 μg mL^{-1} BSA solution in 0.05 M

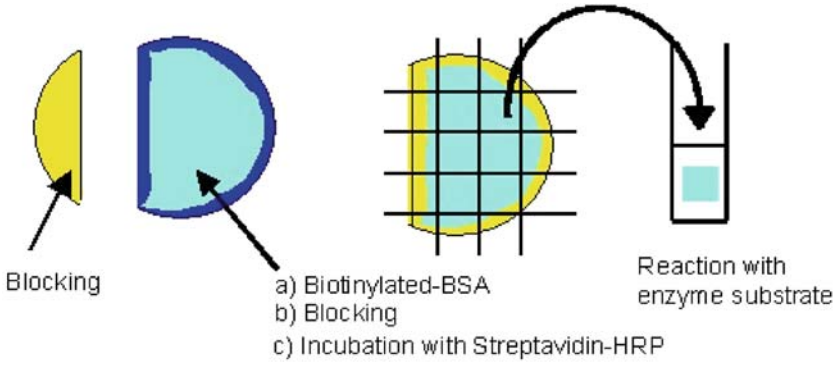

Fig. 1 Methodology for quantitative measurements by ELISA on wafer scale

phosphate buffer, pH 6.5 (blocking solution) for 30 min at RT. After washing with distilled water and drying on a N_2 stream, the wafer was incubated with a 0.25 μg mL^{-1} solution of peroxidase-labeled streptavidin in 0.05 M phosphate buffer, pH 6.5, for 30 min at RT. Finally, the wafer was cut in square pieces (ca. 0.5 cm^2) which where transferred to tubes containing 1 mL of enzyme substrate solution (0.03% H_2O_2, 1.9 mM ABTS in 0.1 M citrate–phosphate buffer, pH 4.5). After 15 min reaction, 100-μL aliquots of this solution were transferred to microtitration plates, and the absorbance at 405 nm was measured by using the Multiscan RC microtitration plate reader (Labsystems, Finland). Non-specific binding was determined by using wafer pieces not coated with B-BSA, whereas standard polystyrene microtitration wells were used as control (100% relative binding capacity).

Fluorometric evaluation of protein binding properties of polymer films

Wafer pieces (3.0×0.5 cm) were immersed in glass tubes containing 1 mL of 20 μg mL^{-1} B-BSA solution in 0.05 M phosphate buffer, pH 6.5 and were incubated for 30 min at RT. They were then washed with distilled water, dried on a N_2 stream, and immersed in tubes containing 2 mL of blocking solution. After 30 min, the pieces were removed, washed with distilled water, and dried as previously. They were then immersed in a solution containing 1 μg mL^{-1} AF 546-labeled streptavidin in 0.05 M phosphate buffer, pH 7.4, for 2 h at RT. Finally, the wafer pieces were washed extensively with 0.05 M phosphate buffer, pH 7.4, containing 0.05% v/v Tween 20 and distilled water and dried on a N_2 stream. Fluorescence images were taken by using the Axioskop 2 plus epifluorescence microscope (Carl Zeiss, Germany) equipped with a Sony Cyber-shot digital camera.

Fluorometric evaluation of protein microarrays

The detection of spots created by laser ablation was based on the immunoreaction of the deposited protein with their fluorolabeled counterparts. Spots of two different proteins, rabbit IgG and B-BSA, were created by successive incubation with the respective protein solutions (20 μg mL^{-1} in 0.05 M phosphate buffer, pH 6.5, 10 min). Visualization was achieved after reaction with a mixture of AlexaFluor 488-labeled anti-rabbit IgG antibody and AlexaFluor 546-labeled streptavidin, respectively.

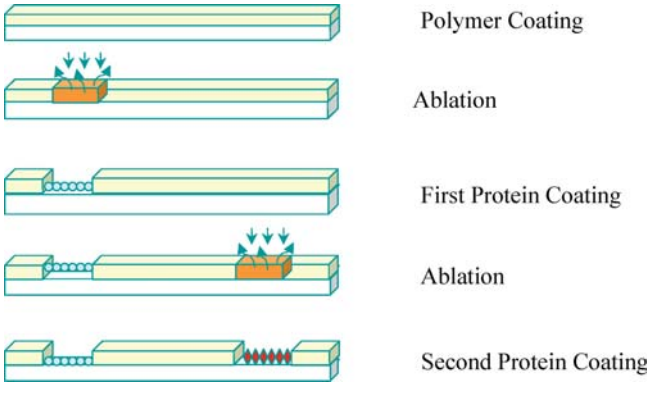

Fig. 2 Schematic representation of laser ablation process for creating spots of different biomolecules on the same substrate

Results and discussion

Process scheme

In our proposed patterning method the polymer must meet the following basic requirements: protein adsorption resistance (negligible protein adsorption), adequate absorbance at 157 nm, and tolerance to protein solutions. Both the second and third requirements are easily met, because most of the polymers have high absorbance at 157 nm and because the protein solutions having almost neutral pH barely affect polymer films. Thus, most effort was devoted to the selection and evaluation of polymer films that could fulfill the first requirement (i.e. films which resist protein adsorption).

The basic scheme proposed for protein patterning using laser ablation of selected polymer films is presented in Fig. 2. In this scheme a spot of a polymer film, which has been selected to resist protein binding as will be discussed below, is ablated with the laser and the first protein is adsorbed onto the substrate. The substrate was either silicon wafer or silicon wafer coated with AZ5214 resist for better adhesion of the upper layer, as explained below. Next, another spot of the polymer film is ablated and a second protein is adsorbed on the ablated region.

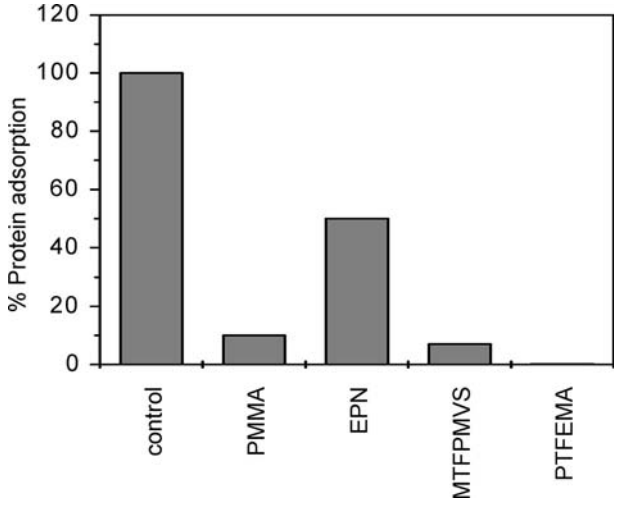

Fig. 3 Results from the evaluation of protein binding capacity of polymeric films studied with the ELISA method. Control corresponds to polystyrene (100% binding capacity)

Polymer selection

A wide variety of polymers were tested in relation to their protein binding capacity: the most important of these were PMMA, EPN, MTFPMVS, and PTFEMA (Fig. 3). The protein binding capacity of these polymers was determined with an ELISA method and was compared to that of standard polystyrene microtitration wells (100% binding capacity). Several formulations of these polymers were tested, containing either the polymer at different concentrations or the polymer with a specific photosensitizer, under similar processing conditions. The lowest adsorptions obtained with the different films are shown in Fig. 2. Protein adsorption with the PTFEMA film was essentially zero.

After the selection of PTFEMA as the polymer that resists protein adsorption the most, we studied further the effect of the formation conditions of the PTFEMA film on its protein binding capacity (Table 1). More specifically, the film formation conditions that were tested were the following: film thickness, post-apply-bake (PAB) temperature, exposure dose with a high-pressure Hg–Xe exposure source (450 W), and sometimes post-exposure bake (PEB). The best results were obtained with films that were not exposed and which were post-apply-baked at relatively low temperature (120°C). Exposure or thermal treatment at high temperature increases the protein binding capacity of the film.

The binding capacity of PTFEMA was also evaluated with the fluorometric method. The protein binding capability of the photoresist AZ5214 was also determined with this method in order to be used as an underlayer that favors protein binding. As was observed, AZ5214 film strongly favors protein binding (Fig. 4a), whereas PTFEMA film obstructs it. In addition, it was found that the PTFEMA film that was deposited on AZ5214 film had better adhesion than onto silicon wafer (Fig. 4b, c). When PTFEMA was directly coated on silicon wafer it was partially removed during incubation in protein solutions giving an inhomogeneous surface coverage. The lowest binding result obtained in Fig. 4c is attributed to the improved PTFEMA film quality over the AZ5214 underlayer compared to the corresponding film over silicon wafer. Furthermore, pretreatment of PTFEMA film with the inert protein BSA strongly impeded protein adsorption (Fig. 4d).

Ablation of PTFEMA films with F_2 laser

The benefit of laser ablation at 157 nm in comparison to the longer laser wavelengths of 193, 248, or 308 nm is based on the fact that at 157 nm ablation is only photochemical with limited thermal effects on the substrate. The laser energy is compensated in a) breaking the chemical bonds of the polymer, b) transforming into kinetic energy of the photofragments that are ejected from the polymer surface with supersonic speed, and c) radiative or non-radiative transitions in the photofragment plume. In this way the substrate heating and its chemical change are limited. As the energy of each photon is compensated in breaking one chemical bond, layers of polymer are removed with atomic precision, and on average 1 mJ cm^{-2} of dose removes 1-nm-thick layers of polymer depending strongly on the background pressure.

Following the experimental procedure a series of experiments were conducted in order to study the process of PTFEMA film ablation. Depth control is necessary to limit laser ablation only to the PTFEMA film without affecting the substrate. The substrates used were

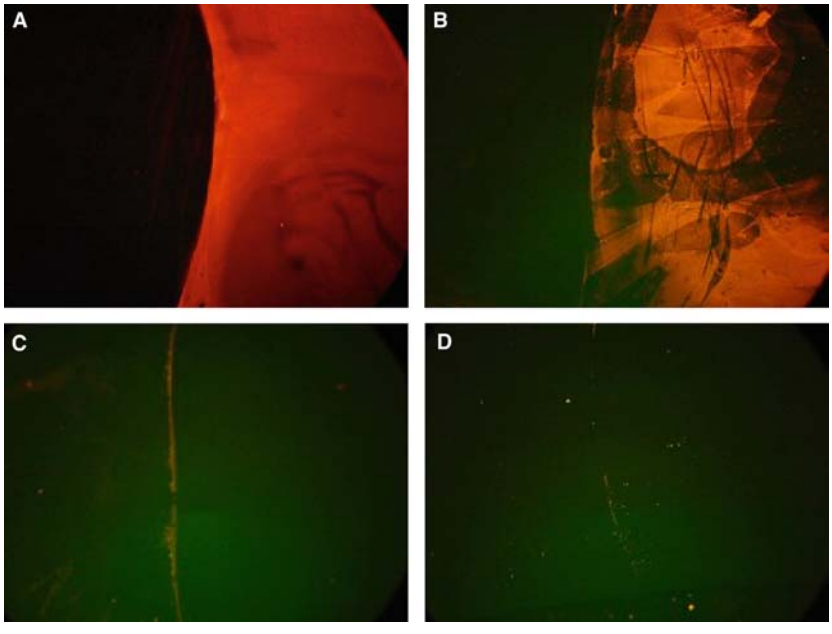

Fig. 4 Evaluation of protein binding capacity of PTFEMA films with epifluorescence microscopy. The *right-hand sides* of the images are the B-BSA-coated area, whereas the *left-hand sides* are the control (no B-BSA coating): **a** AZ5214 film, **b** PTFEMA film, **c** PTFEMA film deposited on AZ5214 film, **d** blocked TFEMA film, which is deposited on AZ5214 film

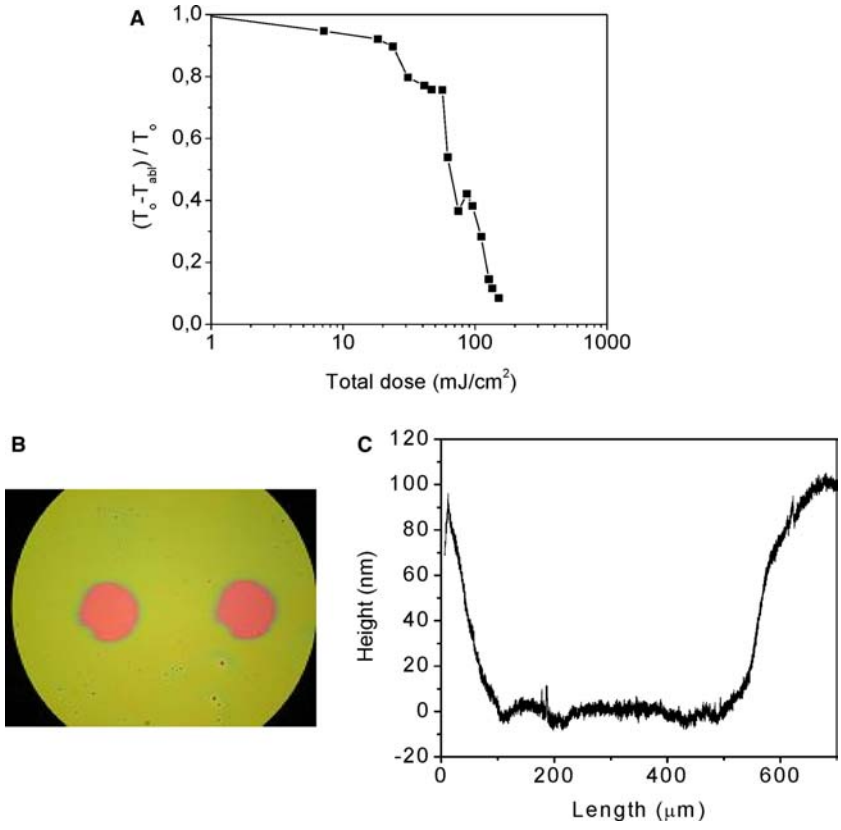

Fig. 5 Laser ablation results of PTFEMA films: **a** remaining thickness of PTFEMA film versus ablation dose, **b** microscope image of two typical spots created by ablation, **c** spot depth profiling obtained by profilometry

bare silicon wafers, silicon wafers coated with AZ5214 film, and PMMA slides. The thickness of the remaining polymeric film versus exposure dose is presented in Fig. 5a. In this case the initial film thickness was approximately 300 nm. A microscope image of two spots that were ablated after 3 min ablation of PTFEMA film (1 mJ cm^{-2} per pulse, 20-Hz repetition rate) is presented in Fig. 5b. In this case the underlayer was AZ5214 resist which appears red in the picture. The profile of one of these spots was observed in a profilometer and it is depicted in Fig. 5c. The diameter of the spot is approximately 400 μm and the film thickness is approximately 100 nm.

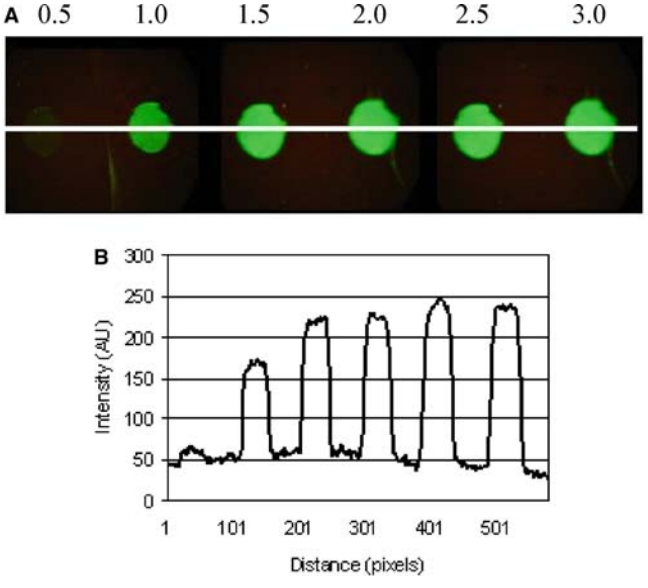

Fig. 6 Fluorescence image (**a**) and fluorescence intensity (**b**) of the spots created on PTFEMA film for ablation times of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 min, respectively. Spot diameter is approximately 400 μm

Protein microarray fabrication

A series of spots created by ablation of PTFEMA film that had been deposited over AZ5214 photoresist underlayer were used in order to study the fabrication of rabbit IgG microarrays. A fluorescently labeled anti-rabbit IgG antibody (AF 488-labeled anti-rabbit IgG) was used to detect rabbit IgG that was adsorbed on the ablated spot areas. Spots created under different ablation times are shown in Fig. 6a. As shown, the fluorescence intensity of spots depends on the ablation dose. A quantitative analysis is presented in Fig. 6b. We notice that 1.5-min ablation time is adequate for complete removal of PTFEMA film and to achieve the maximum fluorescence intensity. Nevertheless, at smaller times, even at 1 min, most of the material has been ablated.

To examine further the capability of the proposed methodology to fabricate microarrays consisting of different proteins, two analytically active proteins, B-BSA and rabbit IgG, were used. The two proteins have been

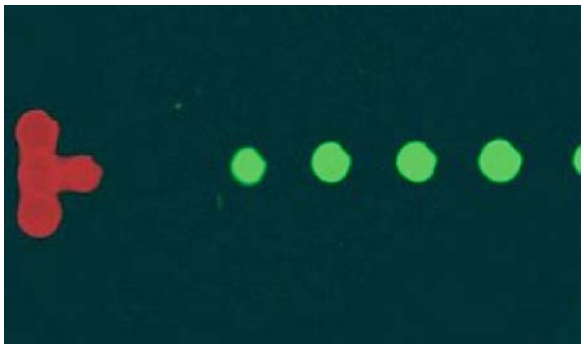

Fig. 7 Microarrays consisted of two proteins, rabbit RIgG (*green*) and B-BSA (*red*), deposited successively after ablation of PTFEMA film and creation of approximately 400-μm spots. Spots were visualized after reaction with anti-rabbit IgG antibody labeled with Alexa Fluor 488 (*green*) and streptavidin labeled with Alexa Fluor 546 (*red*)

deposited in successive steps after successive ablation steps, as shown in the scheme of Fig. 2. To avoid non-specific binding of the two proteins and intermixing of signals, a blocking step with an inert protein (BSA) follows the coating of each analytically active protein. Microarrays of the two different proteins, rabbit IgG and B-BSA, created by successive incubation with the respective protein solutions and visualization after reaction with a mixture of AlexaFluor 488-labeled anti-rabbit IgG antibody and AlexaFluor 546-labeled streptavidin, respectively, are presented in Fig. 7. The high-contrast image is indicative of the negligible non-specific signal and cross-contamination of the spots as a result of the intermediate blocking steps.

Conclusions

A new promising methodology is proposed for the fabrication of multi-protein microarrays in the form of spots. Preliminary results were obtained with spots of dimension between 200 and 500 μm. This approach is based on controlled laser ablation (at 157 nm) of PTFEMA polymeric film. This film has been selected because it prevents protein binding. An underlayer of commercial AZ5214 photoresist has been used owing to its high protein adsorption capability. The whole research is currently under further development focusing on minimizing spots dimensions and on the increase of the number of different proteins deposited.

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