Dry native protein assays on substrates by non-contact Laser-Induced-Forward Transfer LIFT process

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ABSTRACT

Laser-Induced-Forward Transfer (LIFT) is a non-contact method for transferring bioactive substances such as proteins in a microstructured way from a transfer layer on a LIFT target to more or less any surface of a 2D or 3D solid work piece. The quality of the biological transfer layer on the target, i.e. its homogeneity and the bioactivity of the ingredients to be transferred, is crucial for the efficiency of the process and quality of the transferred pattern. Here, aqueous trehalose solutions containing green fluorescent protein (GFP) or the ECM protein laminin type 1 were used in a spin-coating/drying process to render dry thin native protein trehalose films on titanium-coated COP foils. The homogeneity of the layer thicknesses was characterized by spectroscopic ellipsometry and the homogeneity of the GFP distribution within the layer was monitored by fluorescence scan analysis. After application of the LIFT process, the transferred proteins were tested in their bioactivity by fluorescence scan analyses and cell assays.

Keywords: native proteins, trehalose, LIFT, homogenous thin layers, micro array, fibroblast adhesion

1 INTRODUCTION

Micro-structured protein films on solid surfaces with retained protein activity are necessary for a variety of biotechnological and biomedical applications.[1] There are multiple possibilities for generating protein films or patterned protein layers: (electro-)spraying, dip-coating, spin-coating, micro-spotting, micro-contact printing and ink-jet printing. A promising non-contact process to generate micro-scale native protein patterns on 3D and 2D substrates is based on Laser-Induced Forward Transfer (LIFT).[2] This technique uses a laser pulse for jetting a substance from a planar so-called target onto a substrate (Figure 1). The target is coated with a light-absorbing layer and the substances to be transferred. The absence of a dead volume makes this method of printing very efficient and saves valuable resources. LIFT overcome the risk of clogging of nozzles or pins and has recently been adapted for transfer processes of a variety of biomolecules and cells.[3-10]

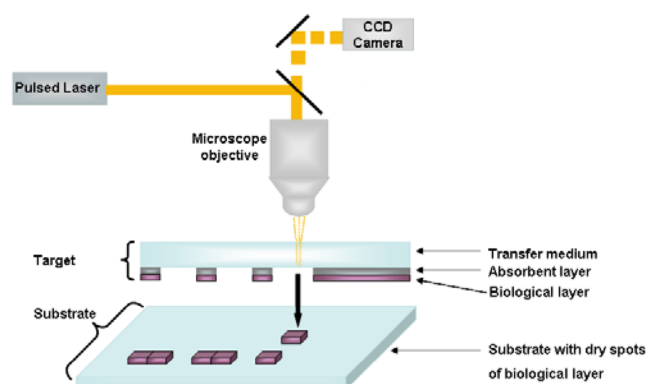

Figure 1: Drawing of the LIFT process in principle according to Barron et al.[11]

Targets comprise a solid transfer medium (glass or polyurethane) which is coated with a light absorbent layer (titanium or silver) and a biological layer. A laser pulse via the transfer medium of the target induces vaporization of the absorbent layer and the local vapor jet propels a small portion of the biological layer from the target onto the substrate. To date, little attention was paid on optimizing the target coating with regard to the homogeneity of the protein distribution and layer-thickness, alternative transfer media or the suitability for storage of ”ready-to-use” targets.

In this study we chose titanium-coated cyclo-olefin polymer (COP) foil as transfer substrate due to its adaquate optical and chemical properties and its possible importance for future scale-up and automation. For the biological layer we prepared protein containing films by spin-coating. film thickness was examined by means of ellipsometric spectroscopy. In order to observe the protein distribution within the biological layer the self-fluorescent green fluorescence protein (GFP) was applied. The quality of the micro-structured patterns transferred by the LIFT process was evaluated by protein activity tests and cell-assays.

2 MATERIALS AND METHODS

2.1 Titanium coated COP foil

For sample preparation cyclo-olefin polymer foils (COP foils, Zeonor®, Zeon Europe GmbH) were coated with a

titanium mirror with a thickness of 100 nm. COP foils were sputtered in a vacuum chamber at 6 µbar with titanium in presence of argon gas (physical vapour deposition).

2.2 Spin-coating

Titanium coated COP foils were incubated in 2% alkaline detergent solution (Hellmanex®, Hellma) for 45 min at 40 °C. Subsequently, the prepared foils were cut in circular pieces with a diameter of 16 mm. The protein-trehalose solution was degassed in a vacuum chamber (Heraeus vacutherm®), for 10 min to a pressure of 100 mbar. To achieve protein-trehalose films a spin-coating device was applied (P6700, Specialty Coating Systems Inc. Indianapolis, USA). 25 µl were deposited on the COP foils and spin-coated for 60 sec at given speeds at 23 °C.

2.3 Evaluation of layer thickness and protein distribution

For layer thickness evaluations 600 mM aequous trehalose solution containing 50 µg mL^{-1} GFP (rTuboGFP, 1 mg mL^{-1}, evrogen, Russia) were spin-coated at 1000 rpm, 3000 rpm and 5000 rpm. Layer thickness was determined by spectroscopic ellipsometry [12, 13] using a SENTECH SE 800 ellipsometer and the analysis software Spectra Ray2 V.5.1, SENTECH Instruments. The determined refractive indices of the organic layers varied from 1.52 to 1.57. The resulting layer thickness was determined to be 171 nm ± 9.9 nm at 5000 rpm, 283 nm ± 11.0 nm at 3000 rpm and 532 nm ± 9.5 nm at 1000 .

2.4 LIFT process

To transfer proteins by LIFT a pulsed laser device (Fraunhofer Institute for Laser Technique ILT, Aachen) was applied. The inserted Q3 laser (CryLas GmbH, Berlin) was used at $\lambda = 355$ nm, pulse width >1 ns, frequency 15 kHz and laser powers 5 mW and 10 mW. The target-substrate distance was adjusted to 70 µm.

2.5 Adhesion of primary fibroblast cells

LIFT generated spots of ECM protein laminin type I (Sigma-Aldrich Chemie Gmbh, Munich Germany) were evaluated by exploiting the adhesion behavior of fibroblast cells. Laminin type 1 was transferred in an arrayed format onto nitrocellulose coated glass substrates. The microarrays were blocked by spray-coating with Stable Guard (SurModics, USA, via Diarect AG, Freiburg, Germany). Fibroblasts were isolated from human foreskin. And precultivated in T-25 culture flasks (Greiner BioOne, Frickenhausen, Germany) in DMEMr (DMEM + 10 % FCS + 1 % Gentamicin (Invitrogen, Karlsruhe, Germany). Incubation of fibroblasts with laminin microarrays was done in DMEM (without FCS) for 2h. In order to avoid unspecific adhesion of the cells to the nitrocellulose, the substrates were shaken every 10 min.

3 RESULTS AND DISCUSSION

3.1 Layer thickness, spin-coating reproducibility and protein distribution

Spectroscopic ellipsometry was applied to evaluate the film thickness homogeneity of spin-coated trehalose-protein films on titanium coated COP foil.

Figure 2: Bar chart of mean layer thicknesses resulting from spin-coating processes at 5000 rpm, 3000 rpm, 1000 rpm. The layers consist of GFP (50 µg mL-1) in dried trehalose (600 mM). Three sample replicates for each speed. shows the coating thicknesses of GFP-trehalose films on COP substrates each with an area of 15 mm x 15 mm. Samples were coated at three speeds 5000 rpm, 3000 rpm and 1000 rpm. The layer thicknesses were measured at nine measuring points per sample. At a speed of 3000 rpm the mean resulting layer thickness was 283 nm which is approximately half of the film thickness of 532 nm resulting at 1000 rpm. At 5000 rpm the layer thickness was 171 nm and therefore about half the thickness of 3000 rpm films. Error bars show the double standard deviation and represent the homogeneity of the layer thickness of each sample. Standard deviations of the film thickness were < 7.1 % of the mean at 5000 rpm < 4.0 % at 3000 rpm and < 2.3 % at 1000 rpm. The reproducibility of the spin-coating technique was evaluated by comparing three independently prepared samples for each speed (samples 1 -3). The overall standard deviations of three samples (27 measuring points) were 5.8 %, 3.9 %, 1.8 % of the mean for 5000 rpm, 3000 rpm and 1000 rpm, respectively. The bulging border areas of the sample pieces where observed to become wider with decreasing speed and were not integrated into analysis.

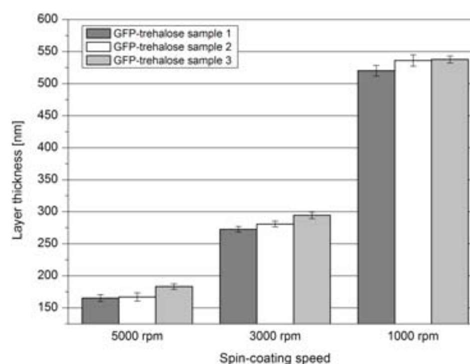

Figure 2: Bar chart of mean layer thicknesses resulting from spin-coating processes at 5000 rpm, 3000 rpm, 1000 rpm. The layers consist of GFP (50 µg mL-1) in dried trehalose (600 mM). Three sample replicates for each speed.

The results demonstrate that spin-coating created protein trehalose films of homogenous layer thickness. The

layer thickness was controlled by spin-coating speed. In order to achieve high protein load per area but minimal width of bulging borders we chose 3000 rpm speed for layer preparation, as far as not stated otherwise.

3.2 Homogeneity of protein distribution

It has been reported that immobilization of proteins and disaccharides in amorphous (i.e. glassy) state protected the proteins from chemical and conformational degradation [14]. During the spin-coating process the solvent (water) evaporates quickly from deposited protein trehalose solutions resulting in amorphous dry coatings [15]. We aimed to investigate whether homogenous protein distribution is achieved within such amorphous trehalose layers. Therefore, self-luminescent protein GFP was incorporated into trehalose layers at 3000 rpm. Protein distribution in dried trehalose films was investigated by means of fluorescence detection: The coated area was divided into rows A-E and columns 1-10. Figure 3 shows the mean fluorescence intensities and standard deviations for each row for two independently generated samples in a bar chart. The overall standard deviation was 17.4 % (sample 1) and 18.5 % (sample 2).

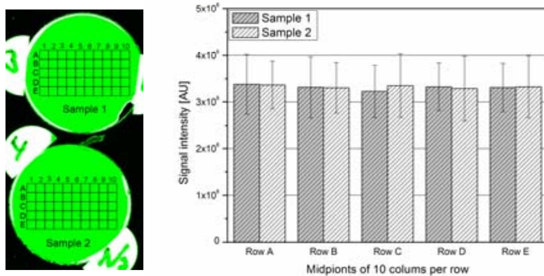

Figure 3: Left: Fluorescence scan image of a d = 16 mm target piece. Right: Midpoint bar chart: Signal intensity of self-luminescent native protein GFP (50 µg mL^{-1}) embedded in dried trehalose (600 mM) film.

3.3 Analyses after LIFT process

LIFT targets were prepared as described using solutions of GFP (1 mg mL^{-1}) and trehalose (600 mM). in order to print protein microarrays via the LIFT process. The biological layers were generated at 1000 rpm spin-coating speed resulting in layers of approximately 550 nm (see section 3.1). GFP was used as indicator protein to visualize native proteins within the transferred spots and to optimize the spot morphology by adaption of the laser parameters. The LIFT process was enforced with two different laser power settings: P = 5 mW and 10 mW. Protein spots were generated applying either three laser shots per deposited spot or one laser shot per deposited spot.

Figure 4 (1a-2b) shows details from the fluorescence scan from 5 x 5 arrays generated by various combinations of the mentioned parameters. As only native GFP molecules emit fluorescence the measured signals proofed that GFP was transferred and deposited in native conformation.

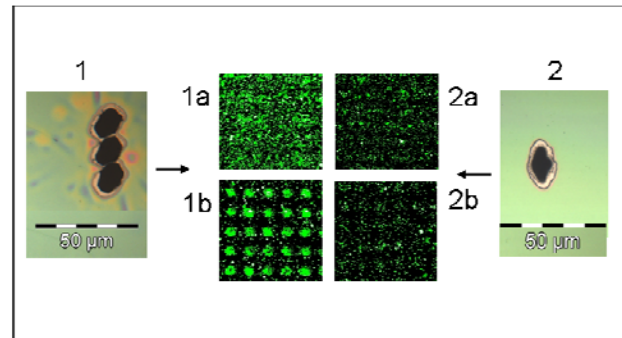

Figure 4: Microscope images of the target and fluorescence scan images of an acceptor glass substrate): Ablation marks from three laser shots at 5 mW (1) and one laser shot at 10 mW (2) and GFP deposited at 10 mW, accumulation of three shots per spot (1a), 5 mW, accumulation of three shots per spot (1b), 10 mW, one shot per spot (2a), 5 mW, one shot per spot (2b).

The presented experiment further revealed that three shots at 5 mW (1b) produced well defined, distinct spots in an arrayed format, while three shots per spot at 10 mW resulted in laminar protein distribution (1a). Printing with one laser shot per spot failed to deliver considerable spots. Atomic force microscopy (AFM) was applied to evaluate the ablation of the biological layer from the COP target during the LIFT process. The AFM image seen in Figure 5 shows well-defined cylindric ablation mark with 40 µm diameter and 600 nm depth caused by the laser induced vapor jet. This results indicates that the biological layer (layer thickness approximately 550 nm, see above) was totally removed from the target.

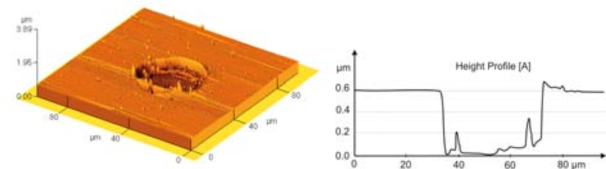

Figure 5: Left: AFM image showing ablation of the protein trehalose layer on COP foil target after one laser shot with 6 mW laser power during the LIFT process. Right: Height profile showing ablation of the protein trehalose layer on target after the LIFT process.

Electron spectroscopy for chemical analysis (ESCA) detected titanium and titanium oxide at the bottom of the laser induced cavity (data not shown), affirming the above stated interpretation

3.4 Cell assay

LIFT targets were prepared as described using solutions of laminin type 1 (50 µg mL^{-1}) and trehalose (600 mM) in order to print protein microarrays for cell adhesion assays. The distance between the deposited protein spots was adjusted to 400 µm.

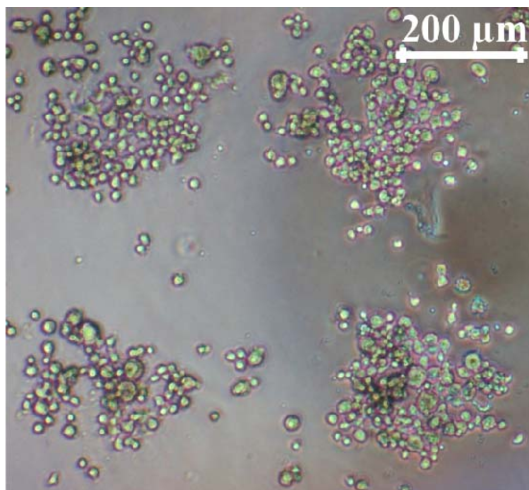

Figure 6: Optical micrograph of fibroblast cells adsorbed to LIFT-transferred laminin type 1 spots on a nitrocellulose coated glass substrate.

The optical micrograph in Figure 6 shows fibroblast cells adhering preferable to four distinct spots on the nitrocellulose covered target. This behaviour is attributed to the micropatterned laminin successfully transferred by the LIFT process.

5. CONCLUSIONS

This study demonstrates that spin-coating is a well suited method for preparation of dry thin films of trehalose and proteins with uniform protein distribution on circular targets. The resulting film thickness was controlled via the spin-coating speed and proofed to be homogeneous all over the coated area (standard deviation per sample < 7.1%). Spin-coated dry thin native protein trehalose films on titanium-coated cyclo-olefin polymer (COP) foil were applied as targets for laser-based protein-transfer by Laser Induced Forward Transfer (LIFT). The titanium coated COP foil proofed to be well suited as transfer medium for the LIFT process. The laser power and the number of pulses were adapted such that arrays of circular spots with diameters of approximately 100 µm were generated on the LIFT substrate. Green fluorescent protein GFP was detected in its native self-luminescent state after being transferred by LIFT and LIFT-generated laminin microarrays mediated microstructured fibroblast adhesion.

Thus, we conclude that dry trehalose protein films are a versatile tool for storing proteins in their native state for subsequent processing, e.g. by means of LIFT. The use of COP foil as transfer medium for the LIFT process enables adaption of the process towards up-scaling and further automation due to the materials mechanical flexibility and processibility.

4 ACKNOWLEDGEMENTS

The authors thank Joachim Mayer, Fraunhofer IGB for AFM images and the Federal German Ministry BMWI (grant ID 16IN0491), the Fraunhofer Gesellschaft and the state Baden-Württemberg for financial support.

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