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SURFACE PROPERTIES OF A LASER-TREATED BIOPOLYMER

LASTNOSTI POVRŠINE BIOPOLIMERA, OBDELANEGA Z LASERJEM

Iva Michaljaničova[1], Petr Slepička[1], Silvie Rimpelova[2], Petr Sajdl[3], Václav Švorčík[1]

[1]University of Chemistry and Technology, Department of Solid State Engineering, Technická 5, 166 28 Prague, Czech Republic
[2]University of Chemistry and Technology, Department of Biochemistry and Microbiology, Technická 5, 166 28 Prague, Czech Republic
[3]Institute of Chemical Technology, Department of Power Engineering, Technická 5, 166 28 Prague, Czech Republic
iva.michaljanicova@vscht.cz

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Structured surfaces allow the application of commonly used polymers to be extended into specialized fields. This paper describes the construction of surface structures on biopolymer poly(L-lactide) (PLLA), with a method combining krypton fluoride laser (KrF), excimer laser exposure and thermal annealing. PLLA is a commonly used substrate for medical purposes, such as implants and tissue matrices, but it still has a number of limitations, which can be eliminated with its modification. This work is focused on morphological studies and roughness measurements of a structured PLLA substrate using atomic-force microscopy (AFM) and chemical changes investigated with UV-Vis spectroscopy and X-ray photoelectron spectroscopy (XPS). Finally, the biocompatibility of the material was tested using a model cell line of mouse embryonic fibroblasts (NIH 3T3). Using the laser treatment in combination with thermal annealing, we prepared surface layers with various patterns dependent on the chosen input parameters.

Keywords: biopolymer, excimer laser, nanostructuring, thermal annealing, characterization

Strukturirane površine omogočajo razširitev uporabe običajnih polimerov tudi na posebna področja. Članek opisuje pripravo površinske zgradbe biopolimera poly(L-lactide) (PLLA), s kombinirano metodo, z izpostavitvijo excimer laserju (KrF) in toplotno obdelavo. PLLA je običajno uporabljena osnova za medicinske namene kot so vsadki in osnove tkiv, vendar so še številne omejitve, ki se jih da odpraviti z njihovim modificiranjem. Članek je osredotočen na študij morfologije in meritve hrapavosti strukturirane PLLA podlage, z mikroskopijo na atomsko silo (AFFM) in preiskave kemijskih sprememb z UV-Vis spektroskopijo in rentgensko fotoelektronsko spektroskopijo (XPS). Preizkušena je bila tudi biokompatibilnost materiala z uporabo modelne celične linije embrionskih fibroblastov miši (NIH 3T3). Z lasersko obdelavo, v kombinaciji s toplotno obdelavo, so bile pripravljene plasti na površini z različnimi vzorci odvisno od izbranih vhodnih parametrov.

Ključne besede: biopolimer, ekscimer laser, nanostrukturiranje, postopek žarjenja, karakterizacija

1 INTRODUCTION

Poly(L-lactic acid) or poly(lactide) is a biodegradable and bioabsorbable thermoplastic polyester, produced from renewable sources.[1] In medical applications, it is used in the matrices for tissue engineering, stents, sutures or in drug delivery systems, but it still has a lot of limitations, which can be eliminated with its appropriate modification.

Polymer structuring allows a utilization of ordinary materials in highly specialized fields. Nanostructured materials find different applications, e.g., in DNA and protein sequencing[2], in the creation of a suitable synthetic environment for cell growth[3] or in the solar-cell technology.[4] Self-organized structures are prepared with a bottom-up method. A typical example of nanostructuring is a ripple or dot formation caused by laser irradiation. The ripples arise due to the interference pattern formation at a surface and the subsequent response of the surface.[5]

Another example of a self-organizing mechanism is wrinkling instability, which exhibits a variety of surface patterns. Wrinkles are produced by the residual stress, which exceeds the critical value.[6] Wrinkle patterns are included, e.g., in tunable optical devices[7] or flexible electronics.[6–8]

This paper deals with the surface modification of poly(L-lactic acid) using laser treatment in combination with thermal annealing. Treated samples were studied with atomic-force microscopy (AFM), UV-Vis spectroscopy, and ARXPS (angle-resolved photoelectron spectroscopy). Tests of biocompatibility were carried out with mouse embryonic fibroblasts (NIH 3T3). With this modification, we prepared various surface patterns with a wrinkle-like structure.

2 MATERIALS AND METHODS

2.1 Materials and modification

We used biopolymer poly(L-lactic acid) (PLLA, a density of 1.25 g cm^{-3}, T_g = 60 °C, a crystallinity of 60–70 %, 50-μm-thick foils, supplied by Goodfellow Ltd., Cambridge, Great Britain).

For the irradiation of PLLA we used a KrF excimer laser (Coherent Compex Pro 50, a wavelength of 248 nm, a pulse duration of 20–40 ns, a repetition rate of 10

Hz). The beam of the KrF laser was polarized linearly with a cube of UV-grade fused silica 25 mm × 25 mm × 25 mm with an active polarization layer. For a homogeneous illumination of the samples, we used only the central part of the beam profile by means of an aperture 0.5 cm × 1.0 cm. The samples were mounted onto a translation stage, being perpendicular to the laser beam. The chosen pulses were in a range of 100–6000, having laser fluences in an interval of 6–30 mJ cm^{-2}.

The thermal treatment of the polymers was accomplished with a BINDER thermostat. The samples were heated to 60 °C (the glass-transition temperature of PLLA) immediately after the laser treatment. After 30 min of thermal annealing, the samples were cooled down to room temperature (*RT*).

2.2 Measurement techniques

The surface morphology and roughness of the pristine and modified polymer samples were examined with the atomic-force-microscopy (AFM) technique using a VEECO CP II device in the tapping mode. The tapping mode was chosen to minimize the damage to the sample surface. A RTESPA-CP Si probe with a spring constant of 20–80 N m^{-1} was used. The mean roughness value (R_a) represents the arithmetic average of the deviations from the center plane of a sample.

The presence of oxygen and carbon in the PLLA surface layer was determined with X-ray photoelectron spectroscopy (XPS). An Omicron Nanotechnology ESCAProbeP spectrometer was used. The exposed and analyzed area had a dimension of 2 mm × 3 mm. The X-ray source was monochromated at 1486.7 eV. Characteristic O(1s) and C(1s) peaks were searched for. Atomic concentrations of the elements were determined with the CASA XPS program using an integrated area of spectrum lines and relative sensitivity factors, quoted in the database of CASA XPS.

UV-Vis spectra were measured using a Perkin Elmer Lambda 25 spectrometer in a spectral range of 190–1100 nm with a bandwidth of 1 nm (fixed).

2.3 Cytocompatibility tests

For cell-culture experiments, we used an adherent model cell line of mouse embryonic fibroblasts (NIH 3T3) (ATCC, USA). NIH 3T3 cells were cultivated on a regular basis in high-glucose Dulbecco's modified Eagle medium (DMEM, Sigma, USA) supplemented with stable 2 mM L-glutamine, 10 % fetal bovine serum and 1 % MEM vitamin solution (Invitrogen, USA). The cells were maintained at standard conditions (37 °C, a 95 % humidified atmosphere, 5 % CO_2). The cells were maintained in exponential growth.

The bio-response of individual PLLA samples was tested. The polymers were first sterilized in 70 % ethanol for 1 h, air dried, inserted into 12-well plates for cell cultures (VWR, Ø 2.14 cm) and weighted with poly(methyl methacrylate) cavus cylinders. The samples were seeded with the NIH 3T3 cells, with a density of 14,000 cells per cm^{-2} in 800 µL of a complete DMEM. An identical batch of cells growing on a polystyrene Petri dish (PS) was used as a control. The experiments were done in triplicates.

The cells intended for a fluorescence-microscopy analysis were fixed and stained. They were washed twice with phosphate-buffered saline (PBS, pH = 7.4) and fixed with 1 mL of a 4 % formaldehyde (Thermo Scientific, USA) solution in PBS at 37 °C for 20 min. A phalloidin-tetramethylrhodamine B isothiocyanate (Sigma, USA) solution in PBS (0.5 µg·mL^{-1}, 10 min) was used to visualize F-actin; cellular nuclei were stained with a solution of 4',6-diaminido-2-phenylindole dihydrochloride (DAPI, Sigma, USA) in PBS (0.5 µg·mL^{-1}, 5 min). During and after the staining, the cells were rinsed twice with PBS to remove the excess of unbound dyes.

3 RESULTS

Because the laser itself has just a small effect on the surface morphology and the roughness of PLLA, we investigated the influence of the laser treatment in a combination with thermal annealing. We used excimer radiation followed by thermal annealing, which was proven to significantly influence the morphology. For the reverse order of the applied methods, we observed just insignificant morphological deviations on the samples in comparison to the samples treated only by a laser beam. On the contrary, for those samples where the thermal annealing was the second step of the treatment, the sample surfaces were rapidly changed. Moreover, for a low laser fluence and a high number of pulses (6–15 mJ cm^{-2} and 6000 pulses) the surface roughness was also significantly increased.

Figure 1 shows the influence of the applied methods on the sample morphology. The modification of the samples at the top of **Figure 1** shows a structure practically identical with the pristine PLLA and the roughness is also similar to the R_a value of the pristine PLLA, which was determined as 6.9 nm. By applying different laser parameters, we prepared different patterns with various roughness values. An important parameter affecting the sample roughness is the combination of the laser fluence and the number of pulses. If the laser energy is too high, the surface is flattened (30 mJ cm^{-2}, 6000 pulses, R_a = 0.7 nm), but with a lower energy, the surface prepared can be extremely rough, with a spongious structure (15 mJ cm^{-2}, 3000 pulses, R_a = 44.7 nm).

The concentration of the surface oxygen was determined with XPS and the results for the selected samples are listed in **Table 1**. According to these results, it is not possible to conclude that the oxygen concentration decreases or increases after the modification because the differences in the concentration were within the stati-

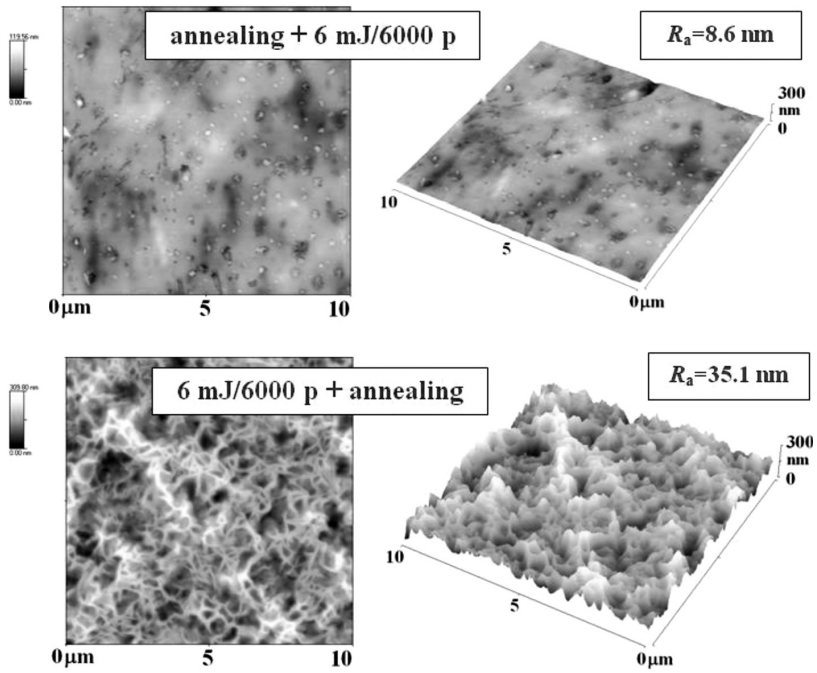

Figure 1: Surface morphology of the samples treated with a combination of an excimer laser (6 mJ cm^{-2}, 6.000 pulses) and thermal annealing (60 °C, 30 min). The samples at the top were first annealed and then modified with the laser; the samples at the bottom were treated in the reverse order. R_a represents the arithmetic mean surface roughness in nm.

Slika 1: Morfologija površine vzorcev obdelanih s kombinacijo ekscimer laserja in žarjenja. Morfologija površine vzorcev, obdelanih s kombinacijo ekscimer laserja (6 mJ cm^{-2}, 6,000 pulzov) in žarjenjem (60 °C, 30 min). Vzorci zgoraj so bili najprej žarjeni in nato modificirani z laserjem, vzorci spodaj so bili obdelani v obratnem vrstnem redu. R_a predstavlja aritmetično sredino hrapavosti površine v nm.

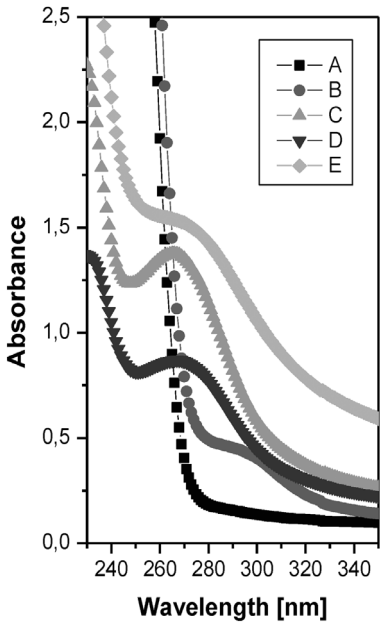

Figure 2: UV-Vis spectra of PLLA. UV-Vis spectra of: A) pristine PLLA and samples exposed to laser fluence 9 mJ cm^{-2} and 30 mJ cm^{-2} (1000 and 6000 pulses) and subsequently treated by thermal annealing (60 °C, 30 min), B) 9 mJ cm^{-2}, 1000 pulses + annealing, C) 9 mJ cm^{-2}, 6000 pulses + annealing, D) 30 mJ cm^{-2}, 1.000 pulses + annealing and E) 30 mJ cm^{-2}, 6.000 pulses + annealing.

Slika 2: UV-Vis spekter PLLA. UV-Vis spekter: A) prvoten PLLA in vzorci izpostavljeni laserju pri 9 mJ cm^{-2} in 30 mJ cm^{-2} (1000 in 6000 pulzov) in nato obdelani z žarjenjem (60 °C, 30 min), B) 9 mJ cm^{-2}, 1000 pulzov + žarjenje, C) 9 mJ cm^{-2}, 6000 pulzov + žarjenje, D) 30 mJ cm^{-2}, 1000 pulzov + žarjenje in E) 30 mJ cm^{-2}, 6000 pulzov + žarjenje.

stical error (about 2 %), which could have taken place during the measurement. It is possible to conclude that the oxygen concentration decreases towards the surface of the spongious structure (the plane-surface oxygen concentration is higher in comparison with the pattern at the very top). This decrease can be contributed to the reorientation of dipoles (oxygen-containing groups) toward the polymer surface.

Table 1: Element concentration of the PLLA surface. The values were determined, with XPS method, for pristine and laser-treated (9 mJ cm^{-2}, 6000 pulses) samples, further for samples treated with laser followed by thermal annealing (9 mJ cm^{-2}, 6000 pulses, 60 °C, 30 min). The samples were measured at angles of 0° and 80°.

Tabela 1: Koncentracija elementov na površini PLLA. Vrednosti so bile določene z metodo XPS na originalnih in z laserjem obdelanih (9 mJ cm^{-2}, 6000 pulzov) vzorcih, nato na vzorcih obdelanih z laserjem, ki mu je sledilo žarjenje (9 mJ cm^{-2}, 6000 pulzov, 60 °C, 30 min). Vzorci so bili merjeni pri kotih 0° in 80°.

Sample	Angle (°)	*C* (amount fractions, *x*/%)	*O* (amount fractions, *x*/%)
Pristine	0	64.4	35.6
	80	67.7	32.3
Laser treatment	0	63.2	36.8
	80	65.4	34.6
Laser treatment + annealing	0	62.6	37.4
	80	67.7	32.3

The UV-Vis absorption spectra of the samples exposed to laser fluences of 9 and 30 mJ cm^{-2} with 1000 and 6000 pulses and subsequently treated with thermal

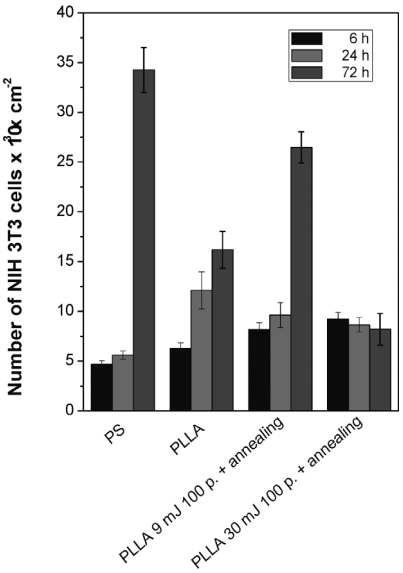

Figure 3: Tests of surface cytocompatibility. Dependence of the number of adhered and proliferated NIH 3T3 cells (6, 24, and 72) h after seeding on pristine PLLA (PLLA) and PLLA modified by laser beam (9 mJ cm^{-2} or 30 mJ cm^{-2}, 100 pulses) and subsequent thermal annealing (60 °C, 30 min). The values for tissue polystyrene (PS) are also shown for comparison.

Slika 3: Preizkus citokompatibilnosti površine. Odvisnost števila oprijetih in razmnoženih NIH 3T3 celic, (6, 24, 72) h po sejanju na prvotni PLLA (PLLA) in na PLLA obdelan z laserskim žarkom (9 mJ cm^{-2} ali 30 mJ cm^{-2}, 100 pulzov), ki mu je sledilo žarjenje (60 °C, 30 min). Za primerjavo so prikazane tudi vrednosti za tkivni polistiren (PS).

annealing (60 °C, 30 min) are shown in **Figure 2**. In the case of the spectra of the modified samples, there are significant peaks at a position of approximately 275 nm. The curve with the most significant peak represents the treatment with a laser fluence of 9 mJ cm^{-2} and 6000 pulses, belonging to a sample with an interesting spongious morphology. The other modified samples also show at least a "small" peak. After the combination of the laser treatment and thermal annealing, a diagonal shift of the absorbance curve was observed.

The cell adhesion represents the first stage of the cell-substrate interaction, thus the quality of adhesion influences cell ability to proliferate and differentiate in the contact with a substrate. After successful adhesion, the adaptation of the cells to the new environment (the lag phase) occurs. For adhesion and proliferation studies NIH 3T3 cells were chosen. The selected samples of the modified PLLA substrate (9 or 30 mJ cm^{-2}, 100 pulses, annealing) were tested and compared with PLLA pristine and control samples of polystyrene used for tissue cultures (PS). Samples with 100 pulses were chosen, because with the increasing number of pulses the material became brittle. From the **Figure 3**, it is apparent that the best results were obtained on PS, which is commonly considered as a model substrate. The PLLA pristine is a material which biocompatibility can be improved by plasma modification. It was shown that modified material with 30 mJ cm^{-2} was slightly less cytocompatible, but with 9 mJ cm^{-2} moderately improved its cytocompatibility. Immediately after seeding cell adhesion was found unaffected. In the **Figure 4**, there are introduced selected pictures of cells growing on treated and pristine PLLA samples.

4 DISCUSSION

By the combination of excimer laser and thermal annealing, it is possible to prepare different surfaces of PLLA with a wide range of surface roughness. The most interesting part of this work is a wrinkle pattern creation. Wrinkles could appear on polymer after annealing as a thin bi-layer film (prepared by treatment and by annealing). In this case, the surface laser treatment and following thermal annealing has created the wrinkle pattern. We suggest that the structure is influenced also during cooling. With less counts of pulses, the staminate

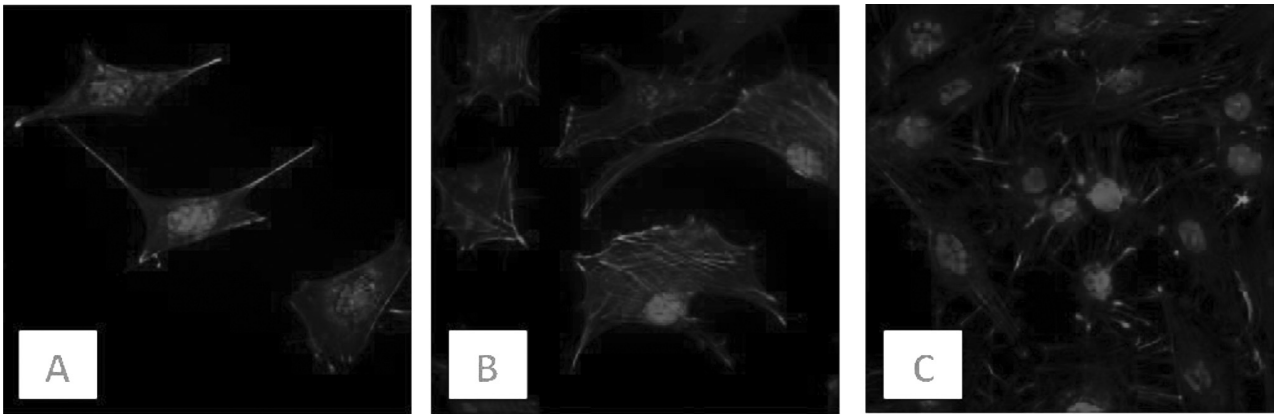

Figure 4: Mouse embryonic fibroblasts (NIH 3T3) growing on different substrates. Proliferated NIH 3T3 cells 72 h after seeding on various substrates: A) modified PLLA (30 mJ cm^{-2}, 100 pulses and subsequent thermal annealing (60 °C, 30 min); B) pristine PLLA, and C) tissue polystyrene for comparison.

Slika 4: Mišji embrionski fibroblasti (NIH 3T3), ki so zrasli na različnih podlagah. Razmnožene NIH 3T3 celice po 72 h po sejanju na različne podlage: A) modificiran PLLA (30 mJ cm^{-2}, 100 pulzov in nato toplotna obdelava (60 °C, 30 min), B) prvotni PLLA in C) tkivni polistiren za primerjavo.

patterns were built up. We propose that difference in the structure was caused by the thickness of laser treated layer, which was insufficient to create wrinkles. Instead of that surface cracking occurred. We suggest that "rods" on the PLLA surface were produced by crystallization of the newly formed material from chopped polymer strings. By reverse order of treatment, when the samples were exposed to annealing and subsequently treated by laser beam, the samples showed the same structure as the samples treated just by laser without annealing. This supports the theory, how the structure was formed.

The peak around area of 275 nm is typical for transitions of n electrons to the π^* excited state, and represents the presence of C=O group. This type of transition needs an unsaturated group in the molecule to provide the π electrons. The diagonal shift could be explained by increasing concentration of double bonds.

Cytocompatibility tests show slight decrease of ability to support cell proliferation for the PLLA treated by high laser fluence, the biocompatibility increases for lower laser fluence. The application of lower energy has therefore similar effect as plasma treatment which we studied in our previous experiments.

5 CONCLUSIONS

Various types of surface structures on modified samples of PLLA were produced by exposure of KrF excimer laser beam and subsequent thermal annealing:

- by UV-Vis spectroscopy we observed new C=O groups and creation of double bonds;
- by choosing optimal input parameters, it is possible to prepare structures from porous and spongeous to flat biopolymer surface with staminate structures;
- the roughness is significantly dependent on laser treatment and annealing input values;
- low laser fluence has a positive effect on cytocompatibility, but high laser fluence loses this effect.

Acknowledgements

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6 REFERENCES

[1] R. M. Rasal, A. V. Janorkar, D. E. Hirt, Progress in Polymer Science, 3 (**2010**) 35, 38–356, doi:10.1016/j.progpolymsci.2009.12.003

[2] B. L. Hancock-Hanser, A. Frey, M. S. Leslie, P. H. Dutton, F. I. Archer, P. A. Morin, Targeted multiplex next-generation sequencing: advances in techniques of mitochondrial and nuclear DNA sequencing for population genomics, Molecular Ecology Resources, 2 (**2013**) 13, 254–268, doi:10.1111/1755-0998.12059

[3] E.-K. Yeong, S.-H. Chen, Y.-B. Tang, The Treatment of Bone Exposure in Burns by Using Artificial Dermis, Annals of Plastic Surgery, 6 (**2012**) 69, 607–610, doi:10.1097/SAP.0b013e318273f845

[4] R. Lecover, N. Williams, N. Markovic, D. H. Reich, D. Q. Naiman, H. E. Katz, Next-Generation Polymer Solar Cell Materials: Designed Control of Interfacial Variables Acs Nano, 4 (**2012**) 6, 2865–2870, doi:10.1021/nn301140w

[5] M. Bolle, S. Lazare, Large scale excimer laser production of submicron periodic structures on polymer surfaces, Applied Surface Science, 1–4 (**1993**) 69, 31–37, doi:10.1016/0169-4332(93)90478-T

[6] C.-M. Chen, S. Yang, Wrinkling instabilities in polymer films and their applications, Polymer International, 7 (**2012**) 61, 1041–1047, doi:10.1002/pi.4223

[7] H. S. Kim, A. J. Crosby, Solvent-Responsive Surface via Wrinkling Instability, Advanced Materials, 36 (**2011**) 23, 4188–4192, doi:10.1002/adma.201101477

[8] D. Y. Khang, H. Q. Jiang, Y. Huang, J. A. Rogers, A Stretchable Form of Single-Crystal Silicon for High-Performance Electronics on Rubber Substrates, Science, 5758 (**2006**) 311, 208–212, doi:10.1126/science.1121401