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Laser-triggered writing and biofunctionalization of thiol-ene networks

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Abstract

In the present work the light responsivity of ortho-nitrobenzyl esters (*o*-NBE) is exploited to inscribe μ-scale 2.5D patterns in thiol-ene networks by direct laser writing. For this purpose, a multi-functional thiol and a photosensitive alkene with an *o*-NBE chromophore

are cured upon visible light exposure without inducing a premature photocleavage of the *o*-NBE links. Once the network is formed, a laser beam source with a wavelength of 375 nm is used for selectively inducing the photocleavage reaction of the *o*-NBE groups. Positive tone patterns are directly inscribed onto the sample surface without the requirement of a subsequent development step (removing soluble species in an appropriate organic solvent). Along with the realization of dry-developable micropatterns, the chemical surface composition of the exposed areas can be conveniently adjusted since different domains with a tailored content of carboxylic groups are obtained simply by modulating the laser energy dose. In a following step, those are activated and exploited as anchor points for attaching an Alexa-546 conjugated Protein A. Thus, the laser writable thiol-ene networks do not only provide a convenient method for the fabrication of positive tone patterns but also open future prospective for a wide range of biosensing applications.

1. Introduction

Stimuli-responsive or “smart” (co)polymers refer to macromolecules that undergo physical changes or chemical reactions upon certain stimuli such as temperature, magnetic field, pH or light.[1] In recent years, numerous stimuli responsive (co)polymers have been synthesized and employed in a multitude of applications.[2] Among them, photo-responsive ones are of great interest, due to the spatial and temporal control of photoreactions (i.e. modification on demand). This is related to the precise control of light sources compared to other physical or chemical stimuli.[3] Moreover, most of the photochemical processes do not need complex technological

steps, and the irradiation parameters, such as light intensity, wavelength and irradiation time, can be easily modulated to adequately comply with the system.[3] For these reasons, in the last years, several photosensitive moieties were introduced in various polymer networks in order to achieve switchable properties.[4]

The mechanism triggered by light as external stimulus relies on either reversible or to irreversible photoreactions, both involving chemical transformation of the molecules. Prominent candidates of the former are azobenzene or coumarin derivatives, whilst the photocleavage of ortho-nitrobenzyl ester (o-NBE) moieties belongs to the latter type of photoreactions.[4–6] Among those two classes of photosensitive chromophores, o-NBE chemistry was widely explored as a common route in the design of polymer networks with photo-responsive properties.[7,8] Thus, the use of o-NBE-based binding motifs has become a common strategy in polymer chemistry for producing photolabile networks.[9–12] The key advantage of o-NBE chemistry relies on the photo-controlled degradation of the polymer or polymer network under UV light.[11–14] Upon UV irradiation, o-NBE groups undergo a cleavage reaction yielding carboxylic acids and o-nitrosobenzaldehyde as primary photoproducts.[13,14]

Johnson and coworkers were the first in designing a crosslinked network based on o-nitrobenzyl alcohol derivatives.[9] Later, Hanxu et al. introduced o-NBE groups in hydrogels, showing a fine control of the cellular microenvironments through the UV-induced de-crosslinking of the hydrogels.[10] Other works explored the possibility to fabricate 3D hydrogels containing o-NBE links by applying two photons absorption techniques.[11,12] In a very recent work, networks based on o-NBE chemistry were successfully employed for 3D print and then erase 3D structures by employing a two wavelengths two photon equipment.[15] In previous studies, we explored the possibility to introduce o-NBE moieties in a wide range of different networks such as epoxy and

methacrylic ones as well as thiol-ene, thiol-epoxy and thiol-yne polymer networks fabricated by click chemistry.[16–20] In this context, thiol-ene networks containing o-NBE moieties were used as positive/negative tone photoresist with good resolution.[21] In addition to the use of o-NBE for producing photoresists, we also showed the possibility to modify surface properties of the network through UV–induced photocleavage, by exploiting the formation of polar species such as carboxylic acids.[16] The high control obtained by light-irradiation was further employed to move water droplets on gradually irradiated photopolymer surfaces.[17]

In this work we further explore the capabilities of these networks. First, photocured networks were obtained by curing thiols with alkenes containing o-NBE moieties upon visible light exposure. Then, we investigated the formation of 2.5D positive tone micropatterns (i.e. the possibility to reproduce xy patterns with controllable depth) in thiol-ene photopolymers with o-NBE binding motifs by direct laser writing technique, avoiding conventional photolithographic techniques.[22] Here we also evidences that the use of a laser source for activating the cleavage reaction is of particular interest since it allows not only high spatial control, but also enables dry-development (i.e. removal of cleaved molecules without the use of a solvent) while typically solvents are required to remove the soluble species formed in the exposed areas.[15–20,23,24]

More importantly, we exploited the photo-isomerization of o-NBE groups to change the chemical surface composition of the thiol-ene photopolymers. In particular, free carboxylic acid groups were formed by the cleavage reaction in different laser-drawn domains, whose concentration could be conveniently adjusted by tuning the laser beam intensity. Spatially controlled surfaces with a tailored content of carboxylic groups were obtained and employed to graft proteins on the patterned polymer surface with a high selectivity and control of the functionalization density.

2. Results and discussion

The photosensitive thiol-ene network formed by the visible light induced click reaction between Trimethylolpropane tris(3mercaptopropionate) (TMPMP) and vinyl-NBE ((2-nitro-1,4 phenylene) bis(methylene) acetate) was prepared according to literature (Figure S1).[21] The formation of the network was confirmed by means of FT-IR spectra, which were taken before and after the curing reaction. As described in detail in Supporting Information File, the thiol-ene reaction occurs simultaneously by step-growth reaction between the thiol moieties and vinyl double bonds. The final monomer conversions amount to 88% for thiol and C=C bonds, respectively (Figure S2). The photocured material results in a transparent soft film, easy to handle, with a glass transition temperature (T_g) about – 10 °C (Figure S3).

The insoluble 3D network was then degraded by a UV laser working at 375 nm, which induced the cleavage reaction of the o-NBE links. Compared to classic photolithographic processes, the direct laser writing is characterized by several advantages such as a high time-space control of the reaction, a uniform energy irradiation and the possibility to control the 2.5D profile, simply by modulating the energy dose of the laser beam. The comparison between the two approaches is displayed in Figure 1.

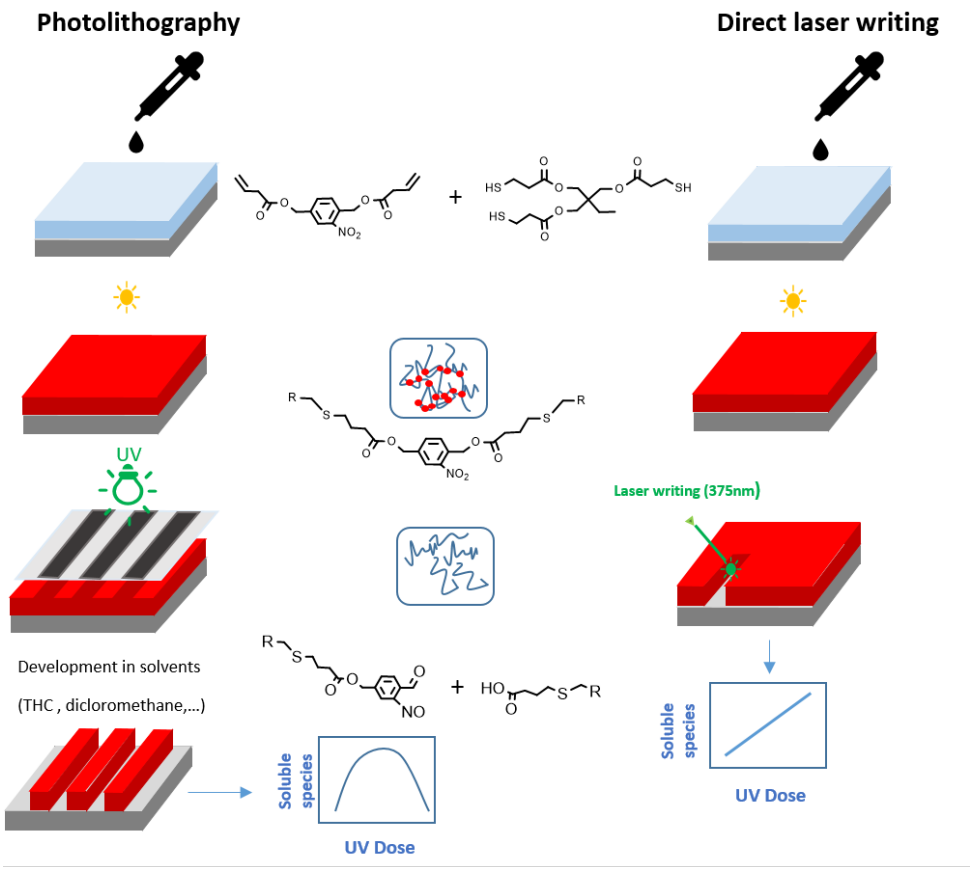


Figure 1: Comparison between photolithography and direct laser writing for the introduction of micropatterns in photo-sensitive thiol-ene networks.

Compared to standard lithography, direct laser writing shortens the process steps and allows a higher degree of freedom in patterning by the use of an electronic control of the light source. It facilitated a direct patterning by following a STL file (standard tessellation language, a file format used for reproducing computer-aided design- CAD), without the use of expensive and fix-design quartz masks.[25] Moreover, we observed that in the laser-induced patterning process, the cleaved network is directly abstracted by evaporation: this avoids the use of solvents in the developing step, differently from classical photolithography processes (Figure 1).

This last point involves a further advantage, related to the maximum yield of soluble cleavage products formed by the photolysis. In previous works, it was shown that the amount of soluble species in thiol-ene networks with o-NBE groups reaches a maximum at a certain dose if the irradiation is carried out with an UV-lamp. At prolonged UV exposure, a re-crosslinking of the cleaved polymer chains is observed through secondary reactions (e.g. formation of azobenzene groups by dimerization of nitroso-benzaldehyde moieties) that leads to an increase in the gel content.[16–21]

In contrast to the irradiation with the UV-lamp, the cleaved off polymer chains are immediately removed by evaporation during the laser writing process, without the possibility for the degraded network to go through secondary photoreactions. In the laser writing process, we observed that the amount of removed material has a linear dependence to the energy dose of the laser beam if this exceeds 20 % (Figure 2b). The results suggest that a minimum of energy is required in order to sufficiently de-crosslink the network and to remove the cleaved off species. By varying the energy dose of the laser beam, the z-depth of the patterns was conveniently adjusted over a broad range. In particular, the z-depth goes from 130 nm at 20 % of energy dose to almost 10 microns at 100 % laser energy dose (Figure 2 a,b). Moreover, the sharp profiles obtained (Figure 2c) indicate that the material was removed by evaporation and not by shape modification (i.e. plastic deformation) suggesting that a precise 3D patterning of the film could be obtained by laser irradiation (Figure 2d). As reference, similar experiments were performed on a classic thiol-ene network: TMPMP was cured with tri(ethylene glycol) divinyl ether (without o-NBE links) upon visible light exposure, reaching comparable final monomer conversions. In this network, without photolabile compounds, we did not observe any degradation or change in z-depth, which confirms the active role of the o-NBE groups in the photo-controlled patterning process.

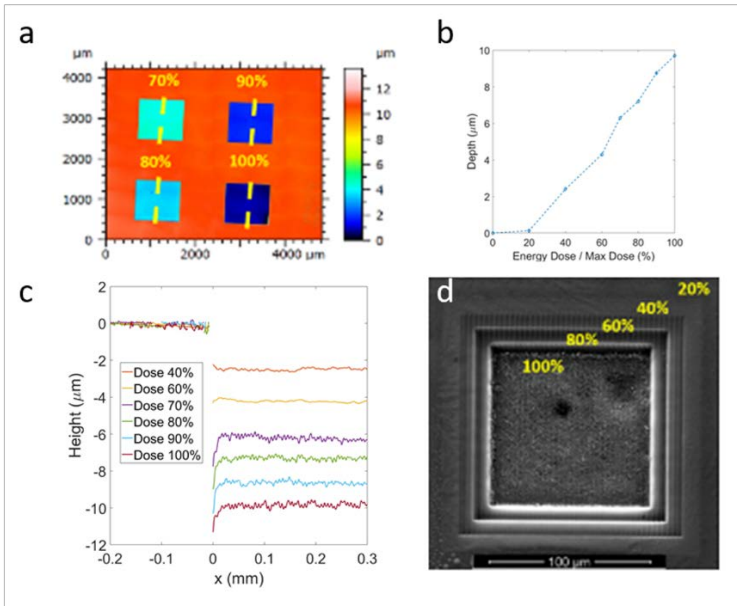
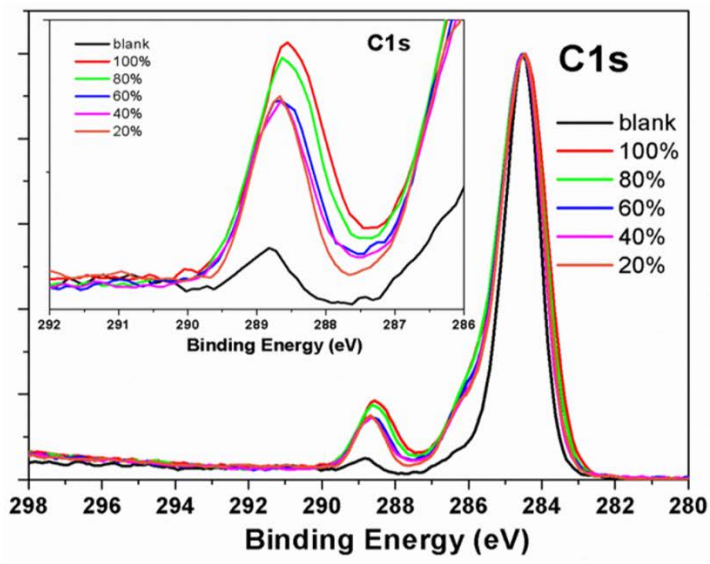


Figure 2: a) Profilometer of visible light cured TMPMP/vinyl-NBE samples, cleaved at different energy doses (70%, 80%, 90% and 100%). b) Depth profile/laser energy dose (in %) of visible light cured TMPMP/vinyl-NBE samples, cleaved at different energy doses. c) profilometry of patterns obtained at different laser doses. d) SEM of a 2.5D pattern generated with different energy doses.

In addition, the photo-responsive nature of the o-NBE links in thiol-ene click networks was used for altering the surface properties in a spatially controlled way. In previous works, we used the presence of polar species such as carboxylic acids, that are formed upon photocleavage, in order to switch the surface polarity of thin polymer films,[16] showing also the possibility to move a water droplet on the irradiated surface.[17]

Advancing from a selected change in surface wettability, in the present study we exploited the photo-generated carboxylic acid moieties as reactive anchor groups for the subsequent attachment of proteins. The better time and space control of the photocleavage reaction and the formed cleavage products realized upon laser beam irradiation enabled a tailoring of the carboxylic acid content in the exposed areas of the surface. Selected sample domains were irradiated with a

different laser beam intensity and the presence of carboxylic groups on the surface was characterized by XPS as a function of the energy exposure dose (Figure 3).



Laser Energy dose	$\frac{[-COOH]}{[C_{TOT}]}$
Blank	1.99%
20%	6.92%
40%	8.12%
60%	8.50%
80%	9.90%
100%	10.73%

Figure 3: C1s HR XPS spectra of visible-light cured TMPMP/vinyl-NBE samples, which were then cleaved upon exposure to a laser beam with varying exposure dose. The inset graph shows a magnification of the carboxylic chemical shift, with the peaks located between 288.6 and 288.8 eV. In the Table, UV laser energy dose vs –COOH/C_{TOT} peak area, calculated from HR XPS spectra by a deconvolution procedure applied to C1s peaks, are listed.

As shown in Figure S5 in Supporting Information File, the C1s region of the cured thiol-ene sample has been deconvoluted with four components: one for the C-C/H peak at 284.5 eV (peak I), the second one (peak II) at +0.7 eV from peak I, related to C-NO_2 and C-SH bonds;[26,27] the third one (peak III) at +1.6 eV due to *C-O-C=O and the last one (peak IV) at +4 eV for the C-O-*C=O. The irradiated (i.e. cleaved) samples, instead, need five components, in order to overlap completely with the raw signal: the first three already mentioned, peak I, II and III, and two new ones arise for the C=O bond at +2.8 eV and the latter one at +4.2 eV for the -COOH group. The

appearance of these two new components is in good agreement with the photo-cleavage mechanism of o-NBE group (see Figure S4). The peak related to carboxylic groups overlaps with the ester at +4.0 eV, which will represents a sort of offset in the calculation of the peak area ration for the -COOH group towards the total C1s peak area. Since the aim of this work is related to use the formed carboxylic groups for a further modification step, the content of -COOH groups was determined as a function of the UV laser energy. In the table reported in Figure 3, for each sample the -COOH peak area over the total C1s peak area is calculated versus the laser energy dose. It is evident that the -COOH content increases with rising laser irradiation. In order to be more rigorous, due to the energy overlapping between ester and carboxylic C, we reported a value (1.99 %) even for the blank sample; however we expect that this should be only related to C-O-*C=O/C_{TOT}, so we should not find available carboxylic groups in the next investigations. On the other hand, at maximum laser energy the content of -COOH groups amounts to 10.73%, indicating an extensive photoisomerization reaction. In order to show clearly the trend, all the C1s graphs were plotted after normalization in the inset of Figure 3. This shows the -COOH group region in more details: the maximum of each peak located between 288.6 and 288.8 eV is clearly visible, with an increasing relative intensity. While it is obvious that we obtained a higher degree of formation of carboxylic groups by increasing the laser irradiation dose, this trend doesn't seem linear as, for instance, for cleavage profiles. One of the possible explanations related to this not linear behavior could be hydrophobic recovery.[28]

So, in order to have a better characterization of the correlation between laser irradiation dose and surface modification, the -COOH groups formed by the photo-cleavage reaction were used as anchor points for attaching a fluorescent protein in a spatially controlled manner. In particular, an Alexa-546 conjugated Protein A was used. This allowed us, not only to evaluate the available

surface active moieties, but also to demonstrate that these functional groups can be exploited for the immobilization of biomolecules such as proteins, which is of primary interest in several applications (e.g. biosensors, microarrays, Lab-On-a-Chip).[29,30]

For this purpose, an array of three aligned squares (50x50 µm) for each different laser energy dose (20%, 40%, 60%, 80% and 100%) was prepared in order to create areas with different content of carboxylic groups. The array was then incubated with the same concentration of protein.

The presence of the proteins attached on the surface of the samples was then assessed through the characterization of the fluorescence with a spinning disk microscope (Figure 4). As showed in Figure 4a, a large image scan was performed (72 scans with a 20x objective) to provide a wider field-of-view. The wide field image clearly states the different intensities of the fluorescence emission signal, due to the increasing laser UV irradiation dose for the various squares (in triplicate). The red fluorescence signal, related to the presence of Alexa-546 conjugated protein, was really intense for the higher dose (top of the image) and was barely visible for the lower ones (bottom of the image). This behavior verifies the presence of -COOH groups at the surface, after the exposure to the UV laser beam, and their availability for protein grafting. Indeed, the acquired signal would not have been different if the protein would have only adsorbed on the surface. Instead, this test showed that the amount of protein increased at increasing UV doses, then with the increasing number of functional COOH groups, by forming stable amide bonds. To better highlight these results, the images were further analyzed by using ImageJ 1.50d.

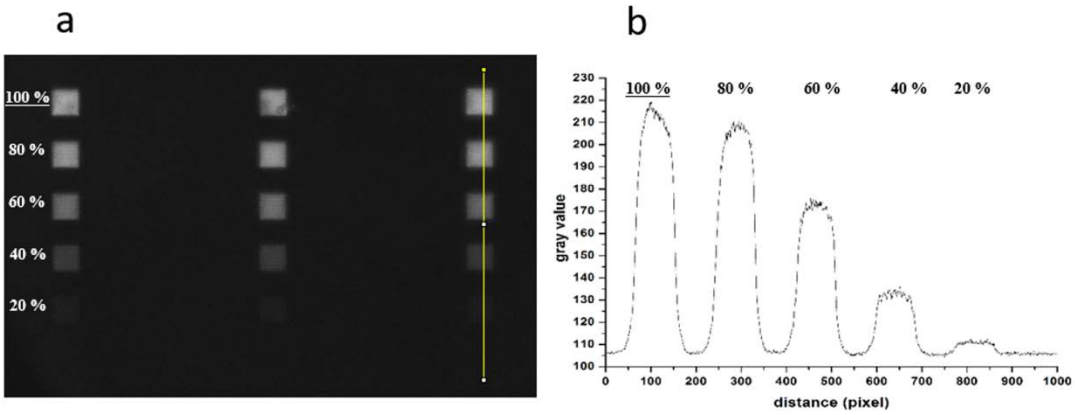


Figure 4: (a) Spinning Disk confocal Microscope of the TMPMP/vinyl-NBE sample (each square is 50 x 50 microns) patterned at different laser UV irradiation dose, functionalized with Alexa-546 conjugated Protein A. (b) Plot profile of the average signal (average of 10 profiles) along the yellow line in image (a) for the three replicates of each laser UV irradiation dose.

Figure 4b reports the plot profile obtained by taking an average of the signal along the y-axis (yellow line shown in Figure 4a). In particular, the image was converted to gray scale and then a line was drawn across all the different squares with different UV laser doses. To be sure that the final plot can be representative of the signal in the image, the profile along this line was acquired ten times in different positions, moving the line on x-direction. This procedure was repeated for all the replicates obtaining an average plot. The grey values in the plot are directly proportional to the amount of signal, enabling a quantification of grafted Alexa-546 conjugated protein A. Therefore, the pattern in Figure 4a clearly shows the possibility to selectively attach a conjugated protein on the laser irradiated areas, with a sharp surface definition. The localized presence of various concentrations of carboxylic acid is also underlined by the linear trend in the plot: the higher the laser dose, the larger the number of available –COOH groups and the higher the amount

of immobilized proteins. A similar value was obtained at 80 and 100 % of laser energy dose, confirming the saturation regime reached by this approach, as already reported by the XPS analysis. These results show even a better correlation between the laser energy dose and the presence of carboxylic acid moieties than the data obtained by XPS measurements, especially at low laser energy dose. This apparent mismatch could be explained considering that the typical XPS penetration is 10 nm:[31] it is then reasonable that on this thin surface layer the o-NBE reaction occurs already at low irradiation dose. In contrast to XPS spectra, the amount of immobilized protein correlates with the presence of functional and available carboxylic groups, even below the ~10 nm scanned by XPS. Another possible explanation could be hydrophilic recovery of the surfaces when immersed in water for functionalization.[32] In any case, a higher degree of protein immobilization is related to a higher amount of carboxylic groups generated at higher energy dose. From these results it can be concluded that this functional test witnesses a more linear correlation with the laser irradiation, demonstrating the potency of the proposed approach for producing 2.5D patterned functional surfaces.

3. Conclusions

In this work we successfully employed a laser beam source in order to control the patterning and surface properties of a thiol-ene network containing o-NBE groups. A formulation containing a trifunctional thiol and a bifunctional vinyl monomer bearing o-NBE groups was cured using a visible light source. Subsequently, a laser induced degradation of o-NBE moieties with UV light was exploited to create a 2.5D positive pattern with controllable generation of carboxylic acids as photoreaction products. Regarding the patterning, we observed the formation of sharp and defined

structures without the necessity of a developing step, enabling a dry-development step. Moreover, SEM analysis on the sample surface showed that it was possible to inscribe a defined pattern with narrow profile, modulating the z-depth by tuning laser energy dose. Regarding the photogeneration of carboxylic groups, we demonstrated by XPS that it was possible to tune the amount of free carboxylic groups as a function of the laser dose. These moieties were exploited for a functional immobilization test, by using Alexa-546 conjugated protein A, showing high linearity between laser energy dose and grafting of proteins. This last result proof the possibility to use ortho-nitrobenzyl ester chemistry combined with laser technology for developing 2.5D patterned functional structures, which could be fundamental building blocks for a wide range of bio-applications, such as active sensitive layers of biosensors, controlled surfaces for cell growth or Lab-on-Chips.

4. Experimental

Materials: Trimethylolpropane tris(3mercaptopropionate) (TMPMP) as multi-functional thiol and phenylbis (2,4,6-trimethylbenzoyl)phosphine oxide (BAPO) as photoinitiator were provided from Sigma-Aldrich (Milan, Italy) and were used without further purification. (2-nitro-1,4 phenylene) bis(methylene) acetate (vinyl-NBE) was synthesized as reported in previous work.[21]

For the post functionalization step, 2-(N-morpholino)ethanesulfonic acid (MES, 99.5%), sodium chloride (99.5%), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 99%), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS, 98%), Dulbecco's phosphate-buffered saline (PBS) and poly(oxyethylene) glycol sorbitan monolaurate (Tween™ 20) were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification. Protein A, Alexa Fluor™ 546

conjugated was purchased from Thermo Fischer Scientific (Monza, Italy) and used according to the manufacturer's instructions.

Sample preparation and laser writing: The photo-curable resin formulation was prepared by mixing vinyl-NBE and TMPMP in stoichiometric amounts (related to monomers' functionality) with 4 wt% of BAPO. The resin mixture was sonicated for 45 min at 50 °C to dissolve the photo-initiator. Once the mixture was homogeneous, it was coated by spin-casting 125 µL of the resin on a glass substrate with a SUSS microtec lithography GmbH Delta 6RC BM (500 rpm, 20 s), and irradiated by means of a Hamamatsu LC8 lamp equipped with visible bulb (cut-off filter below 400 nm), with an intensity of 4 mW/cm^2 for 20 minutes in order to produce a photocured film. Selected areas of the cured sample were then irradiated with a laser beam using different energy doses (20, 40, 60, 70, 80, 90 and 100 %). The laser source was a 375 nm CW Laser mounted on a µPG101, from Heidelberg. The writing parameters were set as follows: laser spot diameter: 0.6 µm; laser power: 70 mW; energy mode: 4x4 (i.e. 4 passes with a pixel exposure time that is 4 times the standard exposure time). The dose was adjusted by varying the dwell time, given in percentage of the pixel exposure time. CAD patterns were designed with Draftsight™ software and then transformed in STL files for laser processing. These samples were characterized by XPS and laser profilometry and used for subsequent grafting processes.

Methods: The surface topography has been characterized by an optical profilometer (S Neox 3D Optical Profilometer from Sensofar ™) in Active Illumination Focus Variation Mode with a NA 0.42 x20 objective. SEM images were taken in an Inspect F Scanning Electron Microscope from FEI operating at 30 kV. XPS characterization was performed by means of a PHI Versaprobe 5000 instrument, with a mono-chromated X-ray source (Al K-alpha line at 1486.6 eV). A pass energy of 187.85 eV was chosen for survey spectra scans (not reported), while 23.50 eV was used for high

resolution (HR) spectra. Data analysis was performed by using a dedicated CasaXPS Version 2.3.13 software. C1s peak maximum at 284.5 eV was chosen as Binding Energy (BE) axis reference. HR spectra background signals were removed by Tougaard functions. A 100 µm spot was used in order to collect the signal only from the inner part of each irradiated rectangle. C1s HR core lines were acquired with at least 50 cycles repeats each, with an energy step of 0.1 eV and an integration time step of 80 ms. Each peak was deconvoluted with Gaussian-Lorentian functions.

Fluorescent images were collected using a microscope (Eclipse Ti2 Nikon) coupled with a Crest X-Light spinning disk confocal and a Lumencor SPECTRA X light engine. All images displayed the same scaling and were collected using a Plan Apo 20x 0.75 NA (Nikon).

For the functionalization step on the array of three aligned squares, the laser-patterned thiol-ene photopolymer was incubated in a mixture of EDC/sulfo-NHS (4/10 mM) in MES buffer (0.1 M, pH 4.7) for 15 minutes.[28] After that, the sample was rinsed thrice in PBS for 5 minutes. In this way, the carboxyl groups become more reactive toward primary amines, which are always present in the structure of proteins, with the resulting formation of an amide bond. The Alexa-546 conjugated Protein A (50 µg/mL in PBS) was incubated overnight (O/N) by drop casting using a total volume of 50 µL. The activated carboxyl groups were exploited as anchor points for the immobilization of the incubated proteins. Finally, the sample was washed three times in PBS with Tween™ 20 at 0.05% wt.

Supporting Information. A characterization of the material including FT-IR analysis, Dynamic Mechanical Analysis, Differential Scanning Calorimetry of the blank sample, the detailed

mechanism of photocleavage of o-NBE as well as C1s XPS deconvolutions are reported in Supporting Information File

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