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Functionalization of polymerized 3D microstructures for biological applications

Ph.D. Thesis

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Introduction

There is an increasing interest in functionalized complex 3D microstructures with submicrometer features for micro- and nanotechnology applications in biology. Depending primarily on the material of the structures various methods exist to create functional layers of simple chemical groups, biological macromolecules or metal nanoparticles. Using the state of the art microfabrication technology physical objects with dimensions in the micrometer range can be easily made. Microfabrication via lithography of photosensitive materials using spatially selective light exposure is an active research field that utilizes the relatively easily achievable electromagnetic radiation as a source. Two-Photon Polymerization (TPP) is one of the suitable methods for micro-structural patterning that employs non-linear interaction that can help improve pattern resolution into sub-micrometer scale. In this thesis I explore the functionalization of 3D microstructures made my two photon polymerization and their biological application.

3D microstructures produced by TPP have the potential of using them in conjunction with optical tweezers in biological experiments for precise, localized manipulation or probing of various target objects (proteins, DNA, single cells). A key element in the effective application of these microstructures is the functionalization of their surfaces. In my thesis I introduce protein as well as metal nanoparticle (NP) functionalization of TPP microstructures based on amine-terminated linker molecules. The primary goal of the protein coating on the structures is their further use in indirect manipulation of live cells, while the NP coating was used to achieve metal-enhanced fluorescence (MEF) observation. In the thesis I present examples for both applications.

Aims of the thesis

The objective of my work is to enable the use of two-photon polymerized 3D microstructures made of the photoresist SU-8 in biological applications such as fluorescence enhancement or indirect cell manipulation by functionalizing their surface. Therefore we proposed the following aims:

a) Effective silane-mediated functionalization of TPP 3D microstructures with streptavidin and gold nanoparticles.

- Characterization the effects of the different process parameters on the quality, integrity and surface morphology of the SU-8 structures.
- Implementation of the efficient biotin-streptavidin linkage on the microtools by covalently binding biotin to the SU-8 surface.
- Coating the surface of the polymer microtools with gold nanoparticles with controlled NP density.

b) Fluorescence enhancement by gold nanoparticle-functionalized TPP 3D microstructures.

- Determination of the degree of localization of the fluorescence enhancement that can be achieved by the coated TPP structures.
- Resolving the effect of NP densities on the enhancement factor.
- Demonstration of the enhancement in a general geometrical arrangement.
- Evaluation of the enhancement at different excitation wavelengths.
- Exploring the origin of the enhancement.

c) Indirect optical manipulation of single live cells by protein-coated TPP 3D microstructures.

- Binding live cells effectively to complex 3D microtools taking advantage of the high-affinity biotin-streptavidin binding.
- Demonstrating simple manipulative tasks with the coated microtools, such as translation of the cell within the sample volume.

Material and Methods

1. Fabrication of SU-8 layers by UV lithography and microstructures by TPP

Large-area SU-8 layers were prepared from 2–3 µm thick layers by illuminating them entirely or via circular masks of 1 or 4 mm diameter with a UV photolithography flood exposure source (365 nm line). The two-photon polymerization of the microstructures was carried out by a system built around a Zeiss Axiovert 40 CFL microscope using a 100 fs

pulse length laser (Menlo C-Fiber A) at 785 nm as light source. For all experiments we prepared TPP microstructures with sizes less than 20 μm in any dimensions. For the protein functionalization experiments, we designed TPP structures in the forms of block, microspiral and 3D optical tweezer microtool. For the metal-enhanced fluorescence studies enhancer microstructures were designed: flat enhancers with parallel or with rounded sides and enhancers with two or four tips. For the optical trap-assisted cell binding experiments we polymerized ellipsoids and crosses as well as complex 3D optical tweezer microtools.

2. Functionalization of SU-8 with Proteins

The goal was to bind proteins specifically (using biotin) and nonspecifically (with gluteraldehyde) to the SU-8 structures. The process starts with creating –OH groups on the surface by epoxy ring opening, followed by introducing primary amines (–NH_2 groups) and then by incubating it in a biotinylation agent or in glutaraldehyde. The affinity of the surface toward protein was checked after each of these steps.

Coating with Streptavidin (STA) via silanization

The polymerized SU-8 structures and microstructures were first treated with the mixture of 1 M nitric acid and 0.1 M CAN. Next the samples were incubated in 2% APTES (3-Aminopropyl triethoxysilane) to create surface amine groups which were then used to bind biotin (1 mg/mL sulfo-NHS Biotin in PBS) and finally STA (10 or 100 nM in PBS).

Coating via PEGylation

The process of PEGylation starts with an acid treatment similarly as silanization. Then 10 μL of 15 mM PEG-diamine solution in methanol was applied on the samples and the solvent was allowed to evaporate slowly. Then the slides were incubated at RT for 20 mins, rinsed in milli Q water and dried. For specific protein coating freshly dissolved sulfo-NHS biotin (1 mg/mL in PBS) was reacted with the PEGylated SU-8. After washing the slides were incubated with 10 nM or 100 nM streptavidin in PBS, washed and kept in PBS. For non-specific functionalization, the PEGylated sample was incubated in glutaraldehyde (2.5% in PBS). The surfaces were finally incubated in one of the two protein solutions: streptavidin (10 nM) or IgG antibody (7 nM).

Coating with gold nanoparticles via silanization and PEGylation

Gold nanoparticles were synthesized with citrate reduction. The diameter of the obtained NP was 80 nm, 41 nm, 24 nm and 16 nm, as confirmed by transmission electron microscopy; the VIS absorption maxima are at 545 nm, 530 nm, 525 nm and 519 nm, respectively.

When silanization was used for gold NP coating, the APTES coating was performed as before only the silanized layer was cured at 120^{0}C for 10 min. Then it was incubated with as prepared gold suspensions at RT for 5, 20 or 60 min and finally rinsed with milliQ water. The presence and status of the NP coating was verified by scanning electron microscopy and by VIS absorption spectroscopy.

The enhancer TPP microstructures were coated with gold NPs using APTES and PEG-diamine linker as described above. The tipped enhancers were always coated with PEG-diamine linker and incubated with the 80 nm gold NPs for 6 h. The flat structures were prepared with APTES linker and incubated with the 80 nm gold for 10 min and 30 min as well as with PEG-diamine linker and incubated with NPs for 5 min, 10 min, 20 min, 30 min and 6 hr; some flat enhancers were coated with 41 nm and 16 nm diameter gold NPs. The such-coated enhancers were placed into a home build microfluidic chamber, whose bottom surface was coated by a fluorescent protein layer.

3. Characterization of SU-8 surfaces

Characterization was done to check the quality, integrity and surface morphology of the SU-8 structures through contact angle measurements, FTIR, AFM, SEM and optical microscopy. The relative amount of the residual epoxide was checked by measuring the IR spectra of UV-polymerized SU-8 layers and that of adjacently positioned TPP microblocks; the spectra were compared by the SU-8 band at 914 cm^{-1}, after normalization by the 1608 cm^{-1} band.

The effect of acid treatment of the SU-8 surfaces was evaluated in two aspects: first, the presence of the surface –OH groups at different acid incubation time was confirmed with water contact angle measurements and the hydroxyl group density was quantified with the toluidine blue method. Second, the eroding effect of the acid treatment on the sub micrometer features of TPP-microstructures was studied using a series of bars bearing sub-

micrometer features. The bars of the very same structure were imaged before and after 30 min, 2 hr and 4 hr acid treatments by optical microscopy and checked for damage.

The surface morphology of untreated, acid-treated and streptavidin-coated SU-8 were characterized using atomic force microscopy (AFM, Asylum MFP-3D); the samples were compared through their roughness values.

The determination of protein density on the SU-8 surface was carried out in a custom built single molecule scanner based on a conventional inverted epi-fluorescence microscope. The DyLight650 and Alexa 568 conjugated, surface-bound streptavidin was illuminated at 647 nm by a Kr+ laser and at 514 nm by an Ar+-laser respectively; the emitted fluorescence was collected onto a back-illuminated CCD camera (NTE/CCD-1340/100-EMB, Roper Scientific) by a 100x oil immersion objective.

4. Fluorescence enhancement measurements

The extinction of the flat type, NP-coated enhancer was measured on a home-made microspectroscopic unit built on an Olympus BX43 microscope equipped with a QE6500 (Ocean Optics) detector.

The fluorescence enhancement and reflectance measurements were carried out on an inverted laser scanning confocal microscope (LSCM, Olympus Fluoview FV 1000) with an Olympus LUMPLFL 60X W objective, NA=0.9 and a 10x objective, NA=0.4.

5. Indirect optical trapping of cells

In these experiments the TPP microstructures were coated with STA using PEG-diamine linkers and sulfo-NHS biotin as before. K562 cell line was used and coated with biotin by incubating them in 1 mg/mL solution in HEPES for 20 min. The optical tweezers system was a holographic optical trap (HOT) system built on a Zeiss Axio Observer A1 inverted microscope with a continuous wave fiber laser as a light source (IPG-YLM-10, IPG Photonics) and an SLM as a beam splitter (PLUTO NIR, Holoeye). In order to determine the binding efficiency of the TPP structures to the cells attachment tests were performed: the cells approached the trapped crosses or ellipsoids and were pushed against them for up to 2 s and checked for successful binding. Eventually, we also used a complex OT-tools with four

spheroids to indirectly manipulate cells within the sample chamber. The flat probe part of such a trapped structure was approached by a cell and attached to it. Next, the sample chamber was translated along all three directions at constant speed to explore its actuation. According to our knowledge this is the first demonstration of a two-photon polymerized, optically trapped, complex (non-spherical) structure chemically modified in a specific way to manipulate a living cell.

Results and discussions

1. Characterization of SU-8 surface functionalization

Evaluation of the residual epoxy groups in SU-8 layers

The relative amount of the remaining epoxide groups after SU-8 crosslinking in the UV-, and TPP-polymerized structures was compared by FTIR spectroscopy; for the meaningful use of UV-polymerized layers as model system, this had to be as similar as possible. For the UV-polymerized layers the UV light dose was varied between 170 and 16320 mJ/cm^2. The area of the 914 cm^{-1} band in the IR spectra of the polymerized SU-8 layers was compared to that of the unexposed SU-8 layer. The band area in the TPP spectrum was 0.446, in the spectrum of 170 mJ/cm^2 dose it was 0.464, for 510 mJ/cm^2 it was 0.318. The layers exposed with 170 mJ/cm^2 dose were unstable therefore we used 340 mJ/cm^2 dose to model the functionalization of TPP surfaces.

2. Effect of acid treatment

The appearance of –OH groups on the SU-8 surface due to acid treatment renders it hydrophilic as followed by contact angle measurement: the angle decreased from the original 82^0 to 42^0 after 2 hr of acid incubation. The surface density of hydroxyl groups, quantified by the toluidine blue method, increased almost tenfold with the incubation time from 0.39×10^{-9} to 3.89×10^{-9} $molecule/cm^2$.

The possible damaging effects of the acid treatment on SU-8 surface morphology and on two-photon polymerized microstructures were explored with AFM measurements on flat SU-8 surfaces and with optical microscopy on test bars of submicrometer thickness. The AFM

measurements discovered practically unchanged surface morphology: the roughness of the untreated SU-8 is 2.6 nm, that of the 30 min acid treated is 1.8 nm and that of the final, streptavidin treated surface is 2.5 nm. It means that further protein or gold NP incubation steps will find the same smooth SU-8 surface after acid treatment as it was before. According to optical microscopy, 30 min acid treatment left the sub-micrometer features intact, however 2 hr treatment damaged them and 4 hr completely removed from the substrate.

3. Quantification of protein density on SU-8 surface

Streptavidin coating with silanization

The comparison of the treatment methods after each functionalization step showed that the method involving all the treatment yielded the highest protein density: of about 4544 ± 520 protein/μm^2 on UV-polymerized layers and 8156 ± 1496 protein/μm^2 on TPP structures; it is 1–2.5 orders of magnitude higher than the other methods can provide. These values mean an about 20% surface coverage for the UV polymerized layer and 35% for TPP structures. The small difference between the protein surface densities on UV- and TPP surfaces justifies our choice of the model system for the TPP microstructures. The treatment was successfully demonstrated with the coating of micrometer-sized spirals and optical trapping microtools.

Streptavidin and antibody coating with PEGylation

The comparison of the treatment methods using PEG linker revealed that the highest protein density is about 1.6×10^5 protein/μm^2, with regard to the biotin-assisted specific binding of streptavidin. This is orders of magnitude higher than that of the other biotin-based methods and about 20 times higher than that of the glutaraldehyde-based ones. In case of the biotin linker, there was no significant difference between the UV- and the TPP polymerized SU-8 surface, while for glutaraldehyde linker, the TPP structures were covered with about 5 times more streptavidin.

In case of the unspecific gluteraldehyde based functionalization, Alexa 568 conjugated IgG antibody was also used besides streptavidin. It was observed that the treatment involving all the steps gives more than 20 times higher surface protein density than the untreated SU-8 itself regardless the type of protein.

4. Characterization of gold NPs on SU-8 layers and microstructures

Gold particles of 24 nm average diameter were immobilized on SU-8 surfaces with APTES linker. The SEM images revealed that without APTES treatment only 4 NPs on average bind onto 1 μm^2 area, although with it 446±25 NP/μm^2 surface density was reached. The surface density was seen to increase with the incubation time, which manifested in the increase of the amplitude of the 530 nm band in the VIS absorption spectra of dry, NP-coated SU-8 layer . The spectra lack any further band above 600 nm, indicating the absence of aggregates. In order to achieve higher NP surface densities, PEG-diamine linker was used. Variously coated TPP microstructures (enhancers) were prepared and used to measure the VIS extinction spectra, the NP surface densities and reflectivity. The measured extinction spectra of the 80 nm diameter NP layers show a dominant band around 550 nm, the plasmonic frequency of the gold NPs and a broader band above 650 nm. The NP density increased from about 47 NP/μm^2 to about 120 NP/μm^2. The tip region of the relevant enhancers is also coated with high density and the resulted envelope of the tip has a radius of curvature of about 350 nm. The reflectance of the structures was measured to increase from 6.6% to 26.7% with the increasing NP density.

5. Application of functionalized TPP structures

Fluorescence enhancement

Fluorescence enhancement achieved with gold NP-coated tips over a layer of fluorescent strepatavidin was clearly localized to regions where the tips of the microstructure are in contact with the surface. The enhancement factor was between 2.5 and 4 with the average of 3.16 ± 0.46 and was restricted to an area less than 1 μm^2.

Fluorescence enhancement was also observed over larger area using flat enhancers. The enhancement factor was 5.05±0.58 as observed for the highest NP-density and it was 2.3±0.2 in case of the lowest. Regarding the whole range of NP densities, the enhancement factor depends on the extinction of the NP layer at the excitation wavelength, 633 nm, in a saturating manner. The enhanced fluorescence always showed strong patterns in the lateral directions. This dependence was related to the varying distance between the fluorescent layer and the structure's coated surface.

We further studied the fluorescent enhancement in two kinds of tilted arrangements with high gold NP density samples. First, the fluorescent layer was perpendicular to the optical axis and a flat enhancer made a 15° angle tilt with it. In this case each structure displays a series of intense fluorescent lines with an average distance of maxima of 990±67 nm. In the second scenario the fluorescent surface and the enhancer surface were in contact and parallel, but both were tilted relative to the observation by 30°. In this case no lines were observed and the enhancement was just slightly smaller than in the original arrangement. The fluorescence enhancement was also demonstrated with smaller (16 nm and 41 nm) diameter gold NPs. The enhanced fluorescence of smaller sized NP coatings shows the same distance-dependent periodic intensity pattern and the measured enhancement factors are 3.23±0.38 for the 16 nm and 4±0.46 for the 41 nm NP layer.

On the origin of the fluorescence enhancement

The fluorescence enhancement observed here takes place in a spatially structured manner. For the round-sided type structures the maximum observed intensity was underneath the center and the second maximum was at around 5 μm from it laterally. The NP layer to fluorophore distance at this point is about 250 nm and the enhancement is between 3 or 4. For the tilted structures, the periodic lines of enhanced intensity are present for fluorophore-NP layer distance of several micrometers. In addition, in the darker regions between the enhanced ones the fluorescence is often weaker than the background. Therefore we assume that the origin of the enhancement is primarily the formation of standing waves.

In our arrangement standing waves are generated by the reflection from the lower surface of the enhancers. We measured reflectance values at 633 nm (between 6.6% and 26.7%) resulting in 1.58–2.3 times higher intensities in the interference maxima. The observed florescence enhancement factor of 2.3–5 cannot be explained by only the intensity increase of the excitation beam. It is plausible to assume, which is consistent with the literature, that the emitted light is also reflected by the enhancers, which means that the observed fluorescence increase is due to the reflection and interference of the incoming excitation wave as well as that of the light emitted by the chromophore. We note that we cannot rule out the role of plasmonic enhancement at parts of the enhancers where the NPs are sufficiently close (10–30 nm) to the fluorophore layer.

6. Indirect cell manipulation

Binding efficiency of cells to TPP structures

For efficient indirect trapping of live cells with TPP structures it is crucial to achieve strong linkage in a reproducible manner that also forms fast between the two components. For this we performed attachment tests on biotinylated K562 cells with structures coated with streptavidin. The target cells were either freely floating or substrate-attached. The success rate for the various functionalization combinations was recorded. When the streptavidin-coated structures touched the biotinylated cells, the success rate was 88.4% for crosses and was 97.5% for ellipsoids; when the cells were not biotinylated, this number fell to 30%, showing some intrinsic binding ability of the streptavidin to the cell membrane. When the TPP structures were not coated with the protein, the success rate was always below 10%.

Indirect manipulation of cells

To further demonstrate the applicability of two-photon polymerized structures for indirect cell manipulation we constructed a complex structure to be used in a multitrap system. The main advantages of using such complex structure are the increased distance of the cell and the very intense trapping beam and the possibility of 6 degrees of freedom manipulation. We carried out successful translation of such a structure with a cell attached to it in all three spatial directions with a scan speed in the range of 10-20 μm/s without losing the cell or the structure. According to our knowledge this is the first demonstration of a two-photon polymerized, optically trapped, complex (non-spherical) structure chemically modified in a specific way to manipulate a living cell.

Summary

This study presents insights into different surface functionalization methods of SU-8 photoresist based 3D microstructures and their possible biological applications.

First, a functionalization with the protein streptavidin and gold NPs is shown that is based on CAN-catalyzed acid and subsequent aminosilane treatments. The straightforward method can be safely applied on any arbitrarily shaped microstructure because the careful choice of the duration of the acid incubation preserves their integrity including sub-micrometer sized free-

standing features and surface morphology. The protein surface density results show that a protein layer with about 20–35% surface coverage is formed on the surface of the TPP structures, rendering them applicable in optical tweezers experiments where biotin–streptavidin interaction is to be utilized. Evenly distributed gold NPs with controlled density were formed on the structural elements of the microstructures by adjusting a simple parameter, the incubation time. The NP density is a key condition in the further applications such as optical trapping and surface enhanced Raman enhancement.

Secondly it was successfully demonstrated that TPP can indeed be effectively used to make novel microcarriers with shape variability for metal NP layers to be used for fluorescence enhancement. It was shown that such NP-coated TPP microstructures can efficiently enhance fluorescence detection. Fluorescence enhancement was localized to less than 1 μm^2 area with a factor of more than 3. The microstructures could also enhance fluorescence even by a factor of 6 when larger areas were considered. The enhancement factor was found higher when NPs were applied at higher surface density. It is believed that the origin of the enhancement is mainly the formation of standing waves due to the reflection of the excitation field as well as the emitted light by the NP layer; plasmonic effect in the close contact areas may also play a role in the enhancement. The application of polymer microstructures as enhancers offers the possibility to combine fluorescence enhancement with optical tweezers in the future.

Lastly indirect optical manipulation of live cells was presented. In order to enable the manipulation with motions more complex than what can be achieved by a microbead, special-shaped intermediate objects are needed. Furthermore, the structures are needed to be functionalized efficiently to realize quick and stable cell-microstrcture binding. We showed that using a PEG-diamine based coating method the protein layer on the 3D microtools has ten times higher surface density than that achieved with the APTES-based method. Binding efficiency of about 90% was achieved with such-coated microstructures and a basic but successful indirect manipulation of live cells with optically trapped, protein-functionalized microtools was demonstrated.

List of Publications

Articles related to the subject of the thesis

I. **Aekbote B L**, Jacak J, Schütz G J, Csányi E, Szegletes Z, Ormos P, Kelemen L. Aminosilane-based functionalization of two-photon polymerized 3D SU-8 microstructures. *European Polymer J* 48 (**2012**) 1745–1754. IF:2.562

II. **Aekbote B L**, Ormos P, Kelemen L. Gold nanoparticle-mediated fluorescence enhancement by two-photon polymerized 3D microstructures. *Optical Materials* 38 (**2014**) 301–309. IF:2.075

Conference proceeding related to the thesis

I. **Aekbote B L**, Kelemen L, Buzas A, Ormos P. Optical Tools For Localized Fluorescence Enhancement and Single Cell Studies. *European Biophysics Journal with Biophysics Letters* 42, (**2013**) S139-S139. IF: 2.47

Conference proceedings and manuscript not related to the thesis

I. Vizsnyiczai G, Kelemen L, **Aekbote B L**, Buzas A, Ormos P. Indirect optical manipulation of live cells with functionalized polymer microtools. *European Biophysics Journal with Biophysics Letters* 42, **(2013)**, S114-S114. IF: 2.47

II. Kelemen L, **Aekbote B L**, Buzas A, Ormos P .Microtools made by two-photon polymerization for optical tweezers systems used in biological manipulation experiments. *European Biophysics Journal with Biophysics Letters* 40, **(2011)**, 228. IF: 2.1

III. Vizsnyiczai G, Lestyán T, Joniova J, **Aekbote B L**, Ormos P, Miskovsky P, Kelemen L, Bánó G. Optically trapped surface-enhanced Raman probes prepared by silver photo-reduction to 3D microstructures *(under Review)*.

Conferences (Oral and Poster presentations)

- **Aekbote B L**, Chorvat D, Ormos P and Kelemen L 'Optical tools for localized fluorescence enhancement and cell studies' at PolyNano Summer School in collaboration with COST Action MP1205,Denmark, 2014
- **Aekbote B L**, Kelemen L, Buzás A and Ormos P, 'Optical tools for localized fluorescence enhancement and single cell studies' at 9th European Biophysics Congress, EBSA,Lisbon,2013
- **Aekbote B.L**, Jacak J, Schütz G J, Szegletes Z, Kelemen L and. Ormos P, 'Two-photon polymerized and functionalized 3D microstructures for biological applications' at the 11th Greta Pifat-Mrzljak International School of Biophysics, Primosten, Croatia,2012
- **Aekbote B L**, Vizsnyiczai G, Kelemen L, Buzas A, Ormos P, 'Indirect optical manipulation of live cells with functionalized polymer microtools' at the COST action TD0906 school on Nanomechanics of biomolecular adhesion,Venice, Italy,2014
- **Aekbote B L**, Jacak J, Schütz G J, Szegletes Z, Kelemen L and. Ormos P, 'Two-photon polymerized and functionalized 3D microstructures for biological applications, at the 11th Greta Pifat-Mrzljak International School of Biophysics, Primosten, Croatia,2012
- **Aekbote B L**, Jacak J, Schütz G J, Szegletes Z, Kelemen L and Ormos P 'Functionalization of 3D photopolymerized microtools for biological applications'at the ' Joint ICTP-KFAS conference on Nanotechnology for biological and biomedical applications' at ICTP (The Abdus Salam International Centre for Theoretical physics),Trieste, Italy,2011