

Three-dimensional biomolecule patterning

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Abstract

A new method is demonstrated, where three-dimensional protein structures are made by employing multi-photon polymerization and photobiotin photolysis. The technique enables the construction of arbitrary two- and three-dimensional shapes with submicron resolution. The integrity of the immobilized biotin is confirmed by derivatization with fluorescently labeled streptavidin. Fluorescence microscopy is used in order to visualize the distribution of fluorescent streptavidin on the 3D structure.

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1. Introduction

The analysis of small and complex biological samples with biosensors has been the focus of much analytical research in recent years [1]. Micro-patterned surfaces have become increasingly important to biosensor systems, where patterned arrays of antibodies, DNA and enzymes have been proposed to monitor the functions of biomolecules *in situ* [2]. The construction of arrays with micrometer dimension allows the measurement of many different biological analytes using samples of very small volume. To this end, different approaches have been examined and a number of novel techniques have been applied to fabricate patterns of biological molecules.

Photochemical activation of light sensitive molecules is a very versatile method and several materials have been used to create patterned surfaces [3,4]. The use of photoactive biotin derivatives to create patterned surfaces is a very attractive technique for the following reasons; firstly, it offers the exploitation of a strong non-covalent biological interaction between biotin and the glycoprotein streptavidin [5]; secondly,

because the tetrameric nature of streptavidin allows for further binding of biotinylated ligands and therefore the creation of higher assemblies of molecules upon the sample surface [6,7].

We propose a novel rapid prototyping method of three-dimensional biotin–streptavidin patterning which enables the construction of arbitrary two- and three-dimensional shapes, not restricted to arrays. By going from 2D to 3D one can increase greatly the active surface area and thus the sensitivity of the system without sacrificing the size of the sensor. The method allows not only prototyping but direct device construction.

We constructed three-dimensional structures employing three-photon polymerization using the organic–inorganic hybrid glass ORMOCER and an Yb laser emitting pulses of 200 fs duration at 1.028 μm [8]. Biotin was subsequently immobilized on the surface of this structure by excimer laser photolysis of photobiotin and further derivatized by fluorescently labeled streptavidin. Fluorescence microscopy was used to visualize the distribution of fluorescent streptavidin.

2. Materials

The material used for the fabrication of the 3D nanostructures is the organic–inorganic hybrid ORMOCER obtained

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from MicroResist Technology (Fig. 1a), a biocompatible material that shows high transparency in the visible and near infrared ranges. ORMOCER contains a highly cross-linkable organic network as well as inorganic components leading to high optical and mechanical quality as well as thermal stability. The polymerization process is initiated by the reaction of the radical photoinitiator IrgacureTM 369 (Ciba) (Fig. 1b).

Photobiotin (Fig. 1c) and streptavidin labeled with Atto 565 (Fig. 1d) were both obtained from Sigma–Aldrich.

3. Construction of 3D structures by three-photon stereolithography

Non-linear optical stereolithography based on femtosecond laser polymerization of polymeric mixtures allows the fabrication of three-dimensional structures with submicron resolution [9]. When the beam of an ultra-fast infrared laser is tightly focused into the volume of a photosensitive material, the polymerization process can be initiated by non-linear absorption within the focal volume. By moving the laser focus three-dimensionally through the resin, 3D structures with submicron resolution can be fabricated. The technique has been used with a variety of acrylate and epoxy materials [9,10], for the fabrication of and several components and devices such as photonic crystal templates [11,12] and micromechanical devices [13,14].

The experimental set-up and procedure for building 3D structures using three-photon polymerization has been described in detail elsewhere [8]. The laser used was an Amplitude Systems *t*-pulse femtosecond oscillator operating at 1028 nm. A femtosecond laser is necessary in order to achieve the high intensity required for three-photon polymerization, which in the case of ORMOCER is 5×10^{11} J/cm² s. The photo-polymerized pathway was generated using an *x*-*y*-galvanometric mirror

scanner (Scanlabs Hurriscan II), controlled by SAMLight (SCAPS) software. The scanner was adapted to accommodate a high numerical aperture focusing lens (Edmund Scientific 100× N.A. 1.25). Immersion oil was used for index matching ($n_{oil} = 1.515$). The movement on the *z*-axis was achieved with a single-axis piezoelectric stage (PI). The beam was controlled using a mechanical shutter (Uniblitz). The piezoelectric stage and the shutter were computer-controlled via a National Instruments LabVIEW program. The laser power was controlled using neutral density filters. A CCD camera was mounted behind a dichroic mirror for online monitoring of the three-photon polymerization process.

After the completion of the process, the sample was developed for 3 min in a 50:50 solution isopropanol:4-methyl-2-pentanone and rinsed in isopropanol.

4. Biomolecule patterning

Once the 3D components were built, they were covered with a photobiotin solution consisting of 1 mg of photobiotin in 1 ml distilled water and 1 ml ethanol. To avoid thermal degradation, the solution was removed by drying the samples under vacuum at room temperature in the dark for 1 h. To this end, the samples were exposed to 20 pulses, 1 Hz repetition rate UV radiation from an excimer KrF laser (TUI Braggstar, 248 nm wavelength, 7 ns pulse duration). After the samples were rinsed with de-ionized (DI) water, they were covered in an Atto 565-Streptavidin solution (0.1 mg/ml) and left to incubate for at least 1 h. They were then washed in DI water by placing them in an ultrasonic bath for 10 min.

Subsequently, the 3D structures were imaged using a fluorescent microscope. The microscope used in these experiments is the Nikon Eclipse E800 equipped with a Nikon digital camera. The excitation of samples through the fluorescence

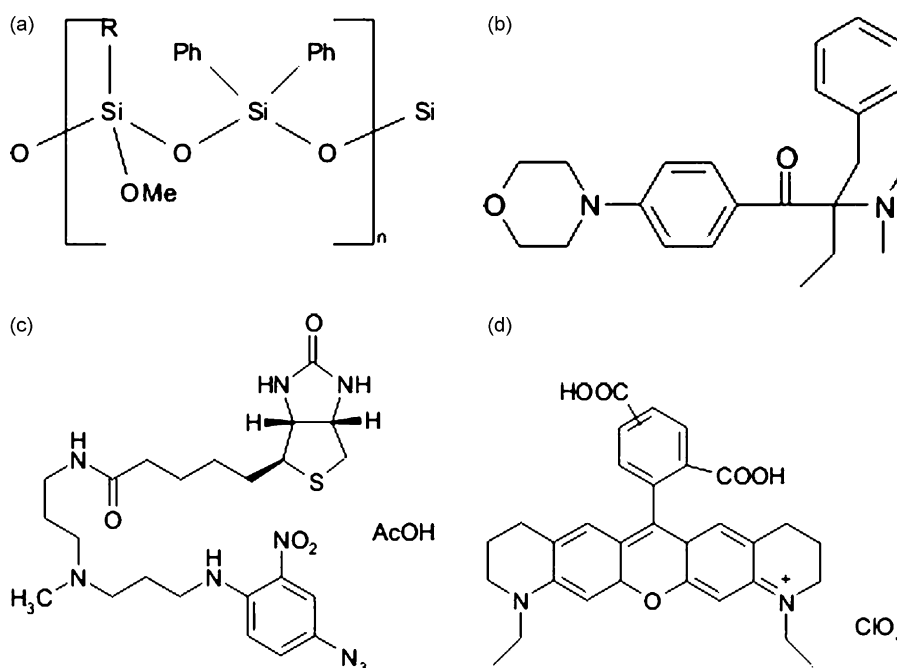


Fig. 1. (a) Ormocer; (b) Irgacure 369; (c) photobiotin; (d) Atto 565 fluorescent label.

microscope was done using the appropriate green filter (G-2A, 510–560 nm), according to the wavelength of the fluorescence substance's excitement.

5. Results and discussion

Fig. 2a shows the SEM image of a structure built employing three-photon polymerization. The structure consisted of five step-in squares, which serve the purpose of building a robust support structure and four vertical and horizontal lines, which serve as dividers. Fig. 2b depicts the image of a similar component that has been treated with photobiotin/streptavidin. It can be seen that the 3D component fluoresces very strongly when excited with green radiation. This fluorescence originates solely from the component and not from the glass substrate. As ORMOCER and photobiotin do not fluoresce in the green, presumably it can be concluded that streptavidin is responsible for the observed fluorescence.

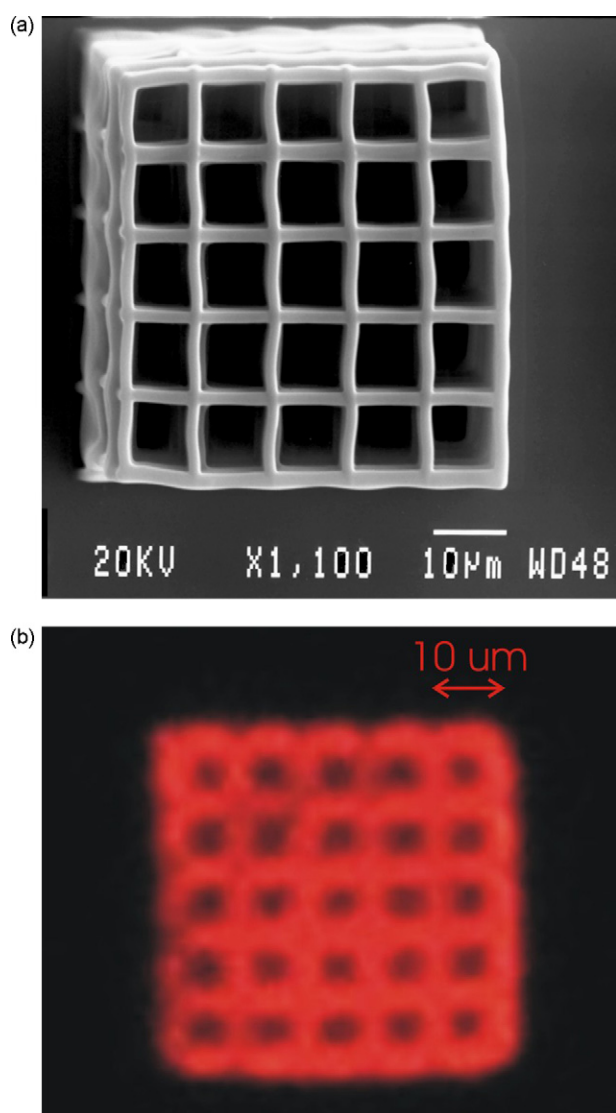


Fig. 2. (a) A 3D structure made employing three-photon polymerization. (b) Fluorescence microscopy image of a 3D structure with attached biotin/streptavidin.

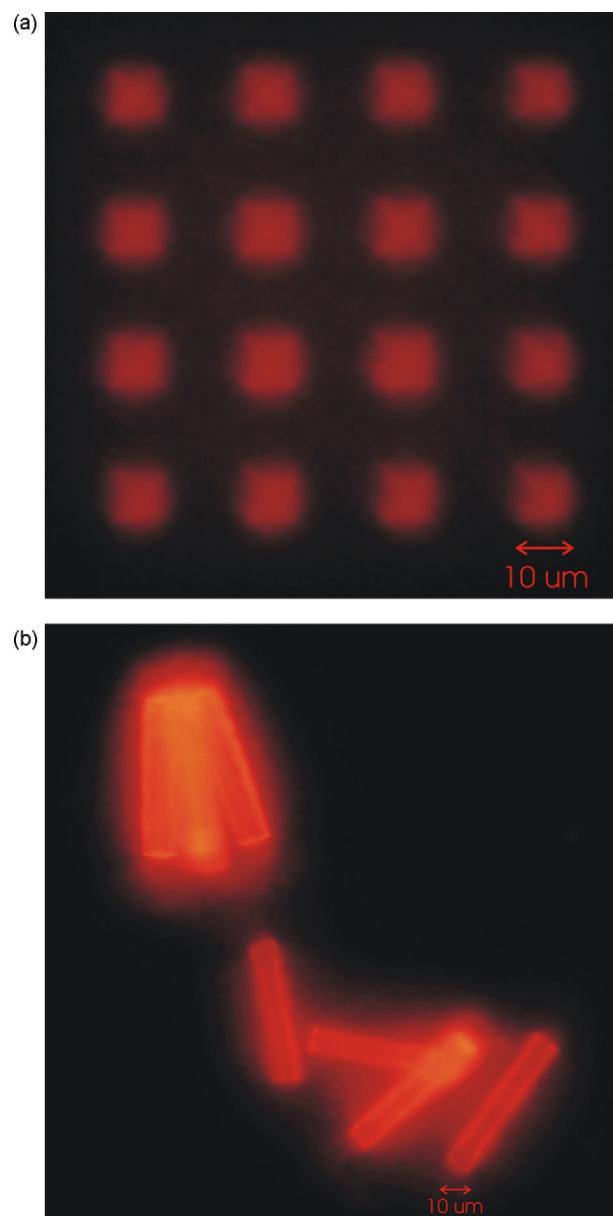


Fig. 3. (a) 3D protein array-top view and (b) 3D protein array-side view.

Fig. 3a shows the fluorescence microscope pictures of a 4×4 column protein array. The three-dimensional nature of the protein attachment is demonstrated in Fig. 3b, where a side view of similar columns is shown.

In control experiments, where samples were not pre-treated with photobiotin and are only treated with streptavidin, there was no fluorescence. Therefore, the incorporation of streptavidin is due to the biotin bound to the structure.

6. Conclusions

We have demonstrated here a method for fabricating a wide range of biopolymer structures by exploiting recent advances in materials processing by femtosecond laser technology. The method is straightforward and can provide the basis for developing a wide variety of biopolymer structures by

exploiting the binding capabilities of the biotin–streptavidin system.

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