Two Dimensional Patterning of Fluorescent Proteins in Hydrogels

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This work describes the successful micropatterning of hybrid systems consisting of hydrogel-dispersed optically active and controllable proteins on solid surfaces without degradation of the photophysical properties of the light-emitting biomolecules. It demonstrates the preservation of the luminescence properties of proteins entrapped into isolated microstructures of poly(acrylamide) gel. This way we can exploit both the structural and function-preserving properties of the hydrogels and the functionality of light-emitting proteins. We believe that this approach can open the way to the realization of nanopatterned optical memories based on photochromic biomolecules.

Among photoactive proteins, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is the most widely used in cell biology, biochemistry, and biotechnology.[1−3] In this class of proteins, the chromophore is formed by an autocatalytic cyclization and subsequent oxidation of three adjacent residues (Ser65, Tyr66, and Gly67) leading to the optical activity without the need of any external cofactor. Crystallographic studies revealed a β-barrel structure with the chromophore in the center (Figure 1a).[4,5] This structure is responsible for (i) the protein stability, (ii) resistance to heat, denaturants, and quenchers such as oxygen,[4] and (iii) high fluorescence efficiency because of the chromophore mobility restriction. Generally, GFP is used as a noninvasive fluorescent marker for gene expression,[6] protein localization,[7] and intracellular protein interaction; as a pH indicator;[8] or to study different biological process (such as enzymatic kinetics[9] and protein structural dynamics)[10] by single-molecule spectroscopy. In addition, GFP can be tailored by genetic engineering to obtain optimized mutants in terms of brightness, heat resistance, photostability, etc.[5]

Despite its unique properties, the applicability of this fluorescent protein for bioelectronics is widely unexplored. Remarkably, the fluorescence dynamics of some GFP mutants is characterized by the light-induced transition between distinct ground states (nonfluorescent and fluorescent, Figure 1b). Indeed, repeated photochromicity cycles were observed in GFP mutants in bulk studies as well as on single molecules.[3,11−13] The use of GFP photochromic mutants appears to be an effective way to store and manipulate data down to the single molecule level, thus allowing the development of high-density optical memories and photonic switches.[2,3] For this reason, the GFP is an excellent prototype molecule for nanotechnology processes that can lead to the realization of optoelectronic devices. Another important requirement to this application is achieving high spatial resolution

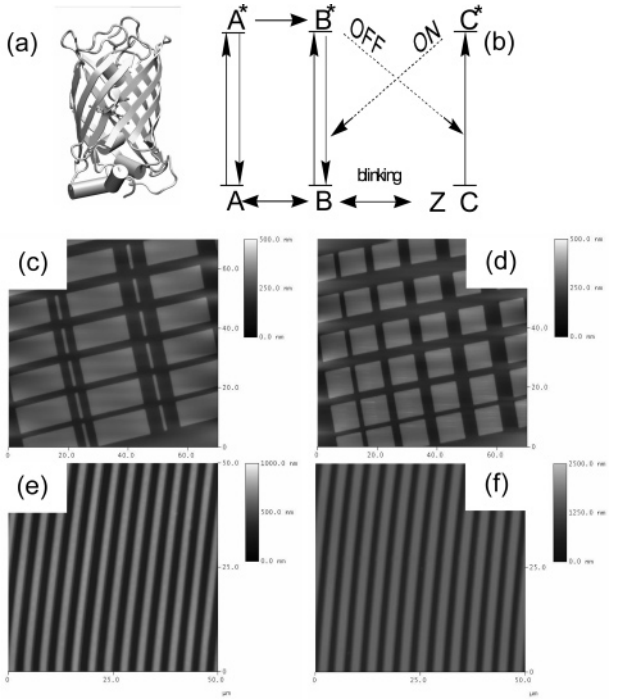

Figure 1. (a) Structure and (b) photophysical model of the E-GFP. A and B indicate the E-GFP bright states, whereas Z and C indicate the dark ones. The stars stand for the excited states. AFM images of PAAG pattern at different geometries (c, d) and comparison between the gel structure (e) and the master (f).

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by patterning. Consequently, the development of new lithography strategies for the immobilization of GFP molecules appears to be a necessary step toward the realization of optical devices based on the protein photochromicity.

In this work, we report for the first time on a soft-molding technique (SM) to pattern wild-type GFP (wt-GFP) and its enhanced mutant EGFP (S65T GFP), entrapped in isolated microstructures of poly(acrylamide) gel (PAAG). By this technique, we successfully preserved the optical functionality of immobilized biomolecules, as displayed by the unaltered fluorescent response detected by confocal microscopy before and after the patterning procedure.

The PAAG is a polymeric hydrogel widely used in biomedical research and in various scientific applications (electrophoretic separation and detection of molecules). It offers a number of interesting properties by virtue of its high degree of hydration,[14] permeability (favoring mass transport),[15] and biocompatibility, that well mimic a physiological environment.[16] These properties prevent structural deformation of embedded proteins (often driven by the interaction with a hydrophobic solid surface or by the drying process required by lithographic processes)[17] and their subsequent loss of functionality. We used here gels with an acrylamide concentration of 15% (w/v) and *N*,*N*-methylene-bisacryl-amide concentration of 2% (w/v) (3% cross-linking ratio) in aqueous solution. The resulting pore size was shown to immobilize the proteins while preserving their native conformation,[18] without quenching fluorescence emission in a pH range 5−7.[8] However, we noticed that adding the protein solution to the aqueous polymer gel could compromise the mechanical stability of the resulting matrix. To overcome this problem, we preserved the water content of the poly(acrylamide), by substituting the water component employed for the gel preparation with the protein solution (0.7−4 μg/mL).

Indeed, due to the fast evaporation of the low molecular-weight monomers, the use of conventional photolithographic techniques would require particular sandwich arrangements.[19] Differently, our SM technique is based on a combination of spontaneous upward capillarity and long-range horizontal polymer transport induced by the pressure manually applied to the fluid gel. This favors the polymer flow into the recessed features of the poly(dimethylsiloxane) (PDMS) copy of the master.[20]

Following polymerization in N_2 atmosphere to avoid the strong inhibitory action of oxygen on PAAG cross-linking,[19] we obtained protein-embedding patterns on Si or glass, stable for several days at $T < 0$ °C. Differently from collagen and sugar-based gels,[21] the weak interaction between PAAG and PDMS permits an easy peeling off of the stamp without any chemical surface treatment, which could irreversibly interact with the immobilized biomolecules. In a single step SM allowed to realize patterns over large areas (cm^2) reproducing microstructures down to 2 μm (Figure 1c−f) in about 30 min. The high quality of the pattern suggests that the resolution can be further increased, down to sub-μm scale, depending on the starting master.

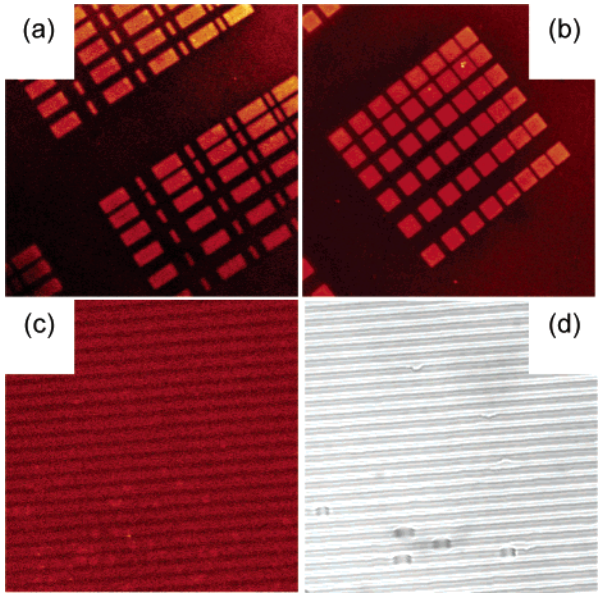

Figure 2. Confocal images of the GFP entrapped in microstructures PAAG with different geometries (a, b) and with periodicity of 4 μm (c). (d) Optical micrograph of the 4 μm period molded PAAG.

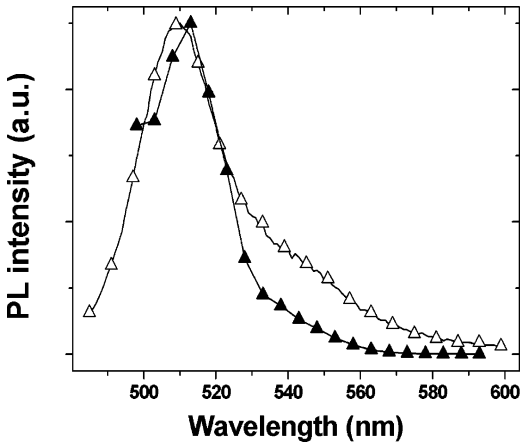

Figure 3. Emission spectra of GFP in a buffer solution (empty triangles) and entrapped into PAAG microstructures (full triangles). No significant differences are observed after the patterning process.

The initial pressure onto the stamp ensures a good conformal contact and allows the extension of the application of SM to hydrophilic solutions. In fact the resulting polymer transport does not directly depend on the mutual interfacial free energies of the target polymers and the mold. Such long-range transport can also determine the complete exposure of the substrate surface between adjacent lines, affecting the volume variation in the different areas of the mold A (recessed) and B (protruding) upon patterning. This variation is given by $\Delta V = wl\Delta z$, where w and l are the width and the length of the features, respectively, and Δz is the height variation due to the capillary transport ($\Delta z_A > 0$ and $\Delta z_B < 0$). We measured for our 50% duty-cycle patterns values, Δz_A significantly larger than $|\Delta z_B|$. This confirms that the nearby polymeric material is not enough to fill the pattern, hence being completely moved from the capillary flow, till the exposure of the substrate surface.[22] The absence of a bottom layer is also confirmed by the root-mean-square roughness of the regions between the PAAG features (≅6.4 nm), that is very similar to that measured on bare substrates (≅6.3 nm), and remarkably larger than that of un-textured PAAG regions (≅2.5

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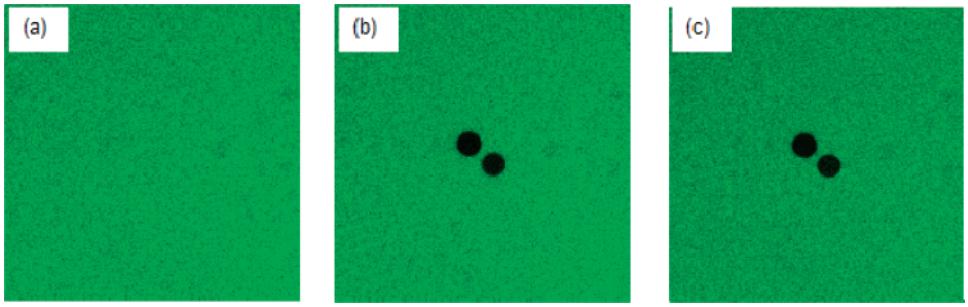

Figure 4. (a) EGFP prebleach: uniform distribution of EGFP in polyacrylamide gels ($C = 1\ \mu$M). Excitation at 488 nm, emission at 500−600 nm. (b) EGFP postbleach at $t = 0$: two circular areas in the gel were photobleached by 514 light (top left) and 488 nm (bottom right). Laser power for imaging 15.5 μW (488 nm), laser power for bleaching = 350 μW (514 nm) and 345 μW (488 nm), scanning speed 50 Hz, image 512 × 512 pixels, objective 40×, 1.25 N. A. oil, digital zoom 2.5×. (c) EGFP postbleach at $t = 30'$. The same bleaching areas of EGFP postbleach were imaged after 30′. No significant variation of fluorescence in the bleached areas was detected.

nm). The absence of any detectable residual polymer layer at the bottom of the imprinted pattern yields a very high optical contrast (Figure 2).

A few soft lithographic methods have been to date proposed for micropatterning hydrogels, such as reactive injection molding,[23] including the possible encapsulation of living cells within peptide-modified poly(ethylene glycol),[24] casting, and molding, for the fabrication of agarose stamps for microcontact printing,[25] and microfluidic networks, for the selective binding of histidine-tagged proteins to nickel-nitriloacetic acid modified 2-hydroxyethyl methacrylate surfaces.[19,26] Our process exhibit some peculiar advantages in terms of operational simplicity, flexibility, and achievable results, since (i) directly processing the protein/hydrogel blends, instead of needing the binding or the inking with the biomolecules after patterning, (ii) not demanding any specific binding chemistry or treatments of the elastomeric elements, (iii) not being limited to hydraulically connected patterns, such as injection or microfluidic approaches, and (iv) retaining a very high resolution, up to the scale of 1 μm.

To assess in depth the possible influence of the patterning process on the optical properties of the embedded proteins, we collected the PL spectra before and after the lithography process (Figure 3). Indeed, it is well established that the fluorescence properties of the GFP are directly affected by the protein structure and that the denatured biomolecules are nonemissive.[27,28] In this frame, checking the fluorescence of intrinsically fluorescent proteins after the patterning process is a much more effective test than measuring the PL of dye-marked biomolecules, whose fluorescence may still be active after a structural unfolding of the proteins.

Remarkably, we found the emission of wtGFPs and EGFPs (S65T GFP) in the pattern is similar to that of the same proteins in solution (Figure 3); the detected minor differences in the emission spectra might be attributable to the local heating of the scanning laser of the microscope. Similar results were found on other fluorescent proteins, such as the F64L/S65T/T203Y (E^2GFP) and the F64L GFP, thus indicating the general validity of the suggested patterning approach and their wide compatibility with the functional protein folding. In addition, the photobleaching behavior and the solvent stability of the patterned protein resulted similar to those observed for the same protein entrapped in nonpatterned gels.

Further experiments showed that immobilized EGFP can be effectively bleached at moderate laser power (around 1 W/cm^2). Furthermore, the photobleaching experiment clearly showed that no spontaneous recovery of the fluorescence occurs after bleaching, thus indicating that the protein diffusion in the gel network is abolished (Figure 4). These results indicate that the proteins are fully functional upon the lithographic process, and they can be employed in micropatterned optical biodevices. In particular, the possibility to bleach easily the protein entrapped in selected areas of the gel, without subsequent spontaneous recovery of fluorescence constitutes a fundamental requirements for the realization of biomolecular memories based on photochromic GFP mutants. Remarkably, by single molecule experiments on the photochromic mutant E^2GFP,[3,12] we have the evidence that the protein in the gel maintains its folded structure also in the bleached state, as it recovers its typical optical emission upon illumination at 360 nm.

In conclusion, we demonstrated that room-temperature SM is a promising approach to realize patterns of functional biomacromolecules embedded in hydrogels, without degradation of the optical properties of the fluorescent proteins. Patterning the hybrid system made by a fluorescent protein and a supporting hydrogel will be useful for a wide class of nano- and biotechnology applications. Our next step will be the conjugation of this soft lithography with the photochromic characteristics of GFP mutants, to realize a novel class of bioelectronic devices, and particularly micropatterned optical memories.

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