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Mimicking Cellular Environments by Nanostructured Soft Interfaces

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ABSTRACT

We present herein an innovative technique for decorating soft polymer surfaces with metallic nanostructures fabricated by diblock copolymer micelle nanolithography. Thus far, such nanolithography has been limited to plasma-resistant inorganic substrates such as glass. Our new development is based on the transfer of nanopatterns from glass to soft substrates. Special emphasis is given to hydrogel surfaces with respect to their properties for tailoring cell adhesion. Besides planar surfaces, periodic gold nanopatterns on curved surfaces have been fabricated, as demonstrated on the interior surface of a tubelike hydrogel, which potentially mimic situations of vessels in vivo.

In many cases, high-precision nanopatterning of surfaces is restricted to inorganic substrates such as silicon, or gallium arsenide, wafers due to their electrical properties and resistance against cleaning processes.[1,2] As far as polymer-based substrates are concerned, necessary cleaning processes as those involving organic solvents or gas plasmas are, however, often impractical because the substrates are affected by these processes. This letter reports a transfer nanolithography technique to fabricate nanopatterns on various polymeric substrates. Polymer-based substrates are significantly advantageous over inorganic substrates in certain instances. Usually, these substrates are more flexible, offer a wider range of rigidity, can be stretched dynamically, and may adopt different shapes. Moreover, the properties of polymer materials may be tailored to specific purposes, either by chemically modifying the polymers or by varying the polymerization and/or crosslinking conditions.[3] Micro- and nanostructures on a polymeric support might be useful for a variety of applications such as electrically conductive circuits,[4] electrodes for neurons,[5,6] or as a substrate for the regulation of cell adhesion.[7,8] In particular, cell adhesion to synthetic interfaces has been found to be quite sensitive with respect to ligand chemistry, density, and pattern geometry[7−11] as well as the rigidity of the substrate.[12−14]

The smaller the pattern dimensions is required, the greater is the challenge to produce them and the more restricted is the choice of technique. A number of approaches for the fabrication of nanostructures have been successfully established on inorganic wafers such as those fashioned from silicon. The most common methods involve photo or electron beam (e-beam) lithography.[1] Less conventional lithographic techniques such as microcontact printing,[15] dip-pen lithography,[16] or imprinting technologies[17] have proven to be highly flexible with respect to substrate choice and patterning geometry. Polymer surfaces have been successfully patterned by these techniques.[15,18] Nevertheless, it is difficult to pattern macroscopic surface areas with dimensions smaller than 20 nm using those methods.

More recent developments in this field are based on pure molecular self-organization such as seen in block copolymer micelle nanolithography.[19,20] This patterning technique enables the extensive decoration of inorganic surfaces with nanoscopic lines and dots made of, e.g., gold, silver, platinum, palladium, or iron. Thus far, polymer supports were not utilized for block copolymer nanolithography because the gas plasma treatment, essential for the deposition of inorganic nanoparticles from their polymeric shell, also results in the corrosion of the underlying polymer support. As a result, the application of diblock copolymer micelle nanolithography has thus far been limited to inorganic supports.

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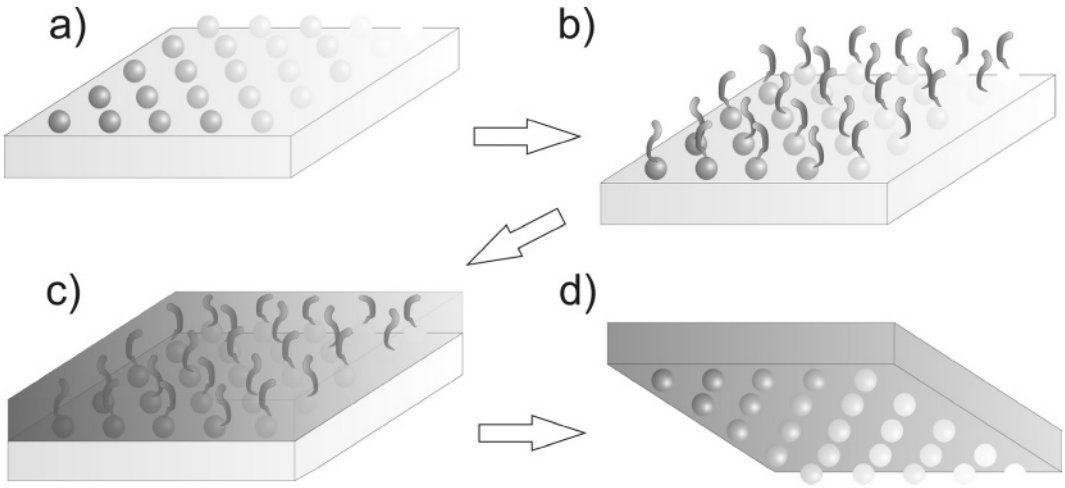

Figure 1. Schematic presentation of the transfer nanolithography. (a) Gold nanostructures are deposited on glass supports by means of diblock copolymer micelle nanolithography.[19] (b) The nanostructures are chemically functionalized by linker molecules. (c) A polymer covers the nanostructured glass support; linker molecules attach gold nanostructures with the polymer coat. (d) Mechanical separation of the inorganic support and the polymer layer, which transfers the inorganic structures from the glass to the polymer support.

Table 1. Polymer−Linker Combinations

polymer	PS	PDMS	PEGDA 700 g/mol	PEGDA 20 000 g/mol
linker	propene thiol	propene thiol	propene thiol	cysteamine + acryloylchloride

We describe herein the transfer of nanopatterns, fabricated by diblock copolymer micelle nanolithography on glass, to surfaces varying in rigidity from glassy polymers to soft and elastic hydrogels, with no constraints on either pattern precision or on the functionality of these supports for cell experiments. Our novel technique paves the way toward the flexible design of nanostructured material, including polymers as a materials class and hydrogels in particular as well as quasi-three-dimensional surfaces. This method turns out to be especially useful for cell adhesion applications because poly(ethyleneglycol) (PEG) hydrogels provide a cellular inert and repellent surface. This approach is quite powerful because it enables testing the effect of ligand presentation on specific cellular responses entirely to the interaction with specific ligands and how lateral positioning of single receptors effects cell functions.

The technique of transfer lithography is schematically depicted in Figure 1. Inorganic nanostructures such as those made from gold are first arranged on an inorganic support, e.g., glass slides or silicon wafers, by means of diblock copolymer micelle nanolithography (Figure 1a). Afterwards the gold nanostructures are chemically functionalized by linker molecules (Figure 1b). Subsequently, the structured surface is coated with a solution or melt of a polymer, which in turn is solidified by either evaporation of the solvent, polymer cooling, or crosslinking (Figure 1c). The linker molecules, which are covalently attached to the gold nanostructures, couple the nanostructures to the solidified polymer. Separation of the polymer from the original support (Figure 1d) results in a polymer surface decorated with the structures originating from the diblock copolymer nanolithography, in essence transferring the inorganic structures from the glass to the polymer support.

Block copolymer micelle nanolithography has been previously described in detail (see ref 19). In brief, polystyrene−poly(2-vinylpyridine) diblock copolymers self-assemble in nonpolar solvents to inverse micelles. The cores of the micelles are loaded by tetrachloroaurate ($HAuCl_4$) by adding adequate amounts of $HAuCl_4$ to the micellar solution. All gold is uniformly distributed to the micellar cores after stirring the suspension for 24 h. A monolayer of micelles is then deposited on a glass surface by dip coating. Reduction of the gold and removal of the polymer is carried out by means of isotropic hydrogen or oxygen plasma etching. Finally, the glass substrate is hexagonally patterned with gold particles 2−15 nm in diameter, with lateral separation between 15 and 200 nm. In principle, the lateral particle separation is governed by the molecular weight of the diblock copolymer.

In our experiments, we transferred the gold nanostructures from glass onto various polymeric matrices, namely polystyrene (PS), poly(dimethylsiloxane) (PDMS), or the macromolecular network resulting from crosslinking macromers of polyethylene glycol diacrylate (PEGDA). The respective linkers (see Table 1) were chosen such that they are most compatible with the polymer used for coating the functionalized nanostructures. Miscibility of the linker with the polymer is affected by the polymer's molecular weight. The linkers were covalently bound to the gold particles via a terminal thiol group. The samples carrying the gold particles were then kept for 1 h in a linker-contaminated atmosphere at 5×10^{-2} mbar. In the case of PEG gel with a macromolecular mass of 20 000 g/mol (PEGDA 20 000), a linker system composed of two molecular parts was applied. Cysteamine molecules immobilized on the gold particles were converted to methacrylates at their terminal amino

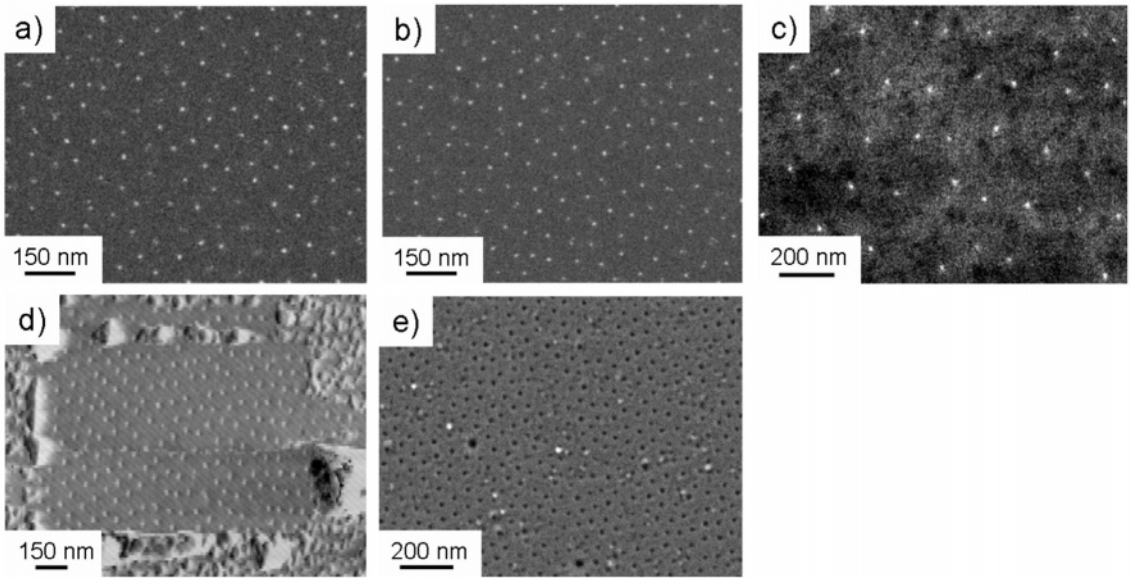

Figure 2. Gold nanostructures on various polymer substrates as patterned by transfer lithography. (a, b) Scanning electron microscopy (SEM) images of gold nanoparticles transferred from glass to PS (a) and PDMS (b). (c) A cryoscanned electron micrograph of a gold nanopattern on a PEGDA 20 000 hydrogel. (d) Atomic force microscopy (AFM) micrograph of gold nanoparticles on a PEGDA 700 hydrogel immersed in water. (e) Imprint from nonchemically functionalized gold nanoparticles on a PS substrate, which indicates that, the particles without linker were not transferred to the substrate.

groups, using a solution of 30 mL of dichloroethane containing 1 mmol *N*,*N*-diisopropylethylamine and 81 μL of acryloylchloride. The samples were added to the mixture and stored at 0 °C for 2 h in a refrigerator.

In the case of polymers which are solid at room temperature, the polymers are dissolved in a good solvent for coating the nanoparticle carrying glass support; i.e., water for PEGDA 20 000, or isopropanol for PS. The linker-functionalized gold nanostructures are then covered with the respective polymer solution. In the case of PS, evaporation of the solvent causes the polymer to solidify and physically attach to the linker. In the case of PEG gel, the macromolecules are crosslinked by the linker molecule in the presence of the photoinitiator 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone; UV irradiation (at $\lambda = 365$ nm) initiates the photo crosslinking.[21] This process results in a water-containing hydrogel covalently bound to the gold nanostructures.

Then, the gold nanostructures are separated from the glass substrate by different procedures: etching the glass in HF, mechanically peeling the polymer layer from the glass substrate, or in case of hydrogels as PEG, swelling in water. Swelling in water turned out to be the most appropriate method for hydrogels while mechanical peeling was simplest in the case of polymers such as polystyrene or epoxy. Figure 2a−c show scanning electron micrographs of gold nanostructures 6 nm in size, represented as white spots on substrates made of either PS (a), PDMS (b), or PEGDA 20 000 hydrogels. The micrographs were produced by an Ultra 55 field emission electron microscope with a Gemini column (Carl Zeiss); Figure 2c represents a cryoscanning electron micrograph of a PEG hydrogel frozen in the water-swollen state at approximately −120 °C. Independently of the polymer, the nanostructures were successfully transferred one-to-one, with nanoscale precision, from the glass to the respective polymer substrate. These results demonstrate that transfer lithography is applicable to various polymers from rigid hydrophobic polystyrene, elastic silicone to soft hydrogels.

The nanostructured hydrogel surface was further characterized by scanning force microscopy (SFM, MFP-3D-Bio, Asylum Research). Figure 2d illustrates the hydrogel surface itself, made of PEGDA with a molecular weight of 700 g/mol (PEGDA 700), which was captured in an aqueous environment. The AFM cantilever applied varying forces to the surfaces. The square in the center of the image represents the area where a higher force was applied. This amplified force resulted in the uncovering of nanostructures immobilized to the hydrogel interface. Nanostructures were seen as bright dots. Scanning with minimal force revealed that the nanostructures are covered by material. This finding held true only for the PEG hydrogels, indicating that PEG chains, extending from the surface and loosely covering the gold nanostructures, are removed by the scanning cantilever at higher forces. In contrast to the one-to-one transfer of nanostructures to the PS substrate, in the presence of propenethiol (Figure 2a), the gold particles were not transferred to the PS layer if they were not chemically functionalized with the linker. As shown in Figure 2e, imprints of the nonchemically functionalized gold nanoparticles on the PS surface were observed as dark spots in the scanning electron micrographs.

The gold nanostructured PEG hydrogels make it possible to position individual signaling molecules such as cyclic RGD peptides on a biologically inert background. This approach is very powerful because it enables the testing of cellular responses entirely due to individual, specific signaling molecules, their spatial ordering as well as material

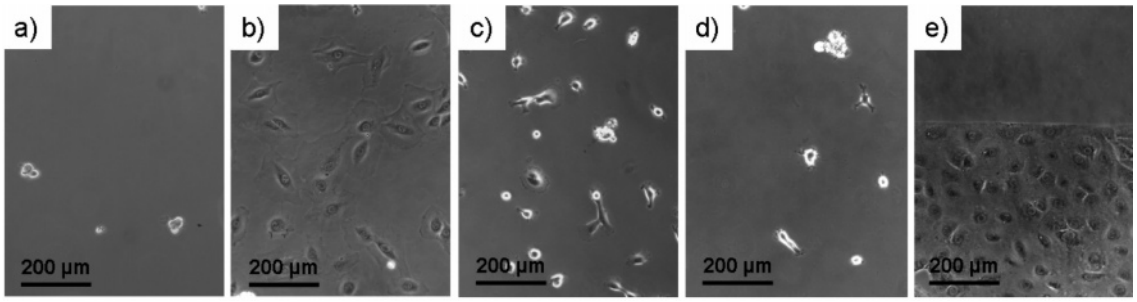

Figure 3. Phase contrast optical micrographs of 3T3 fibroblasts on PEGDA 700 hydrogels. (a) Cells on a non-RGD-functionalized gold nanoparticle pattern. (b−d) Cells on cyclo(-RGDfK-)-functionalized gold particles; cyclo(-RGDfK-) patches are separated by varying distances (b) 40 nm, (c) 80 nm, and (d) 100 nm, after 24 h in culture. (e) Dense cell layer on a PEG support after 14 days in culture. The bottom part of the sample was patterned with cyclo(-RGDfK-) peptide-functionalized gold nanoparticles spaced 40 nm apart.

stiffness. In other biological applications such as drug eluting stents or biosensors, PEG hydrogels provide a most useful biologically inert background at interfaces, which successfully limits adsorption of biomolecules.[7,22−24]

For our cell adhesion studies, gold nanoparticles were transferred to PEG hydrogels and functionalized by cyclo(-RGDfK-) peptides.[25−27] The gold nanostructured hydrogels were incubated in a 0.2 mmol solution of the cyclo(-RGDfK-)−thiol (the cyclic pentapeptide was linked via an aminohexanoic acid spacer to mercaptopropionic acid) in water for 1 h. The hydrogels were then intensively washed with water to remove peptides, which might have penetrated the gel. Subsequently, 3T3 fibroblasts were cultured for 24 h under standard cell culture conditions (in DMEM with 1% FBS) on PEGDA 700 hydrogels equipped with gold nanoparticles separated by distances of 40 nm.

Figure 3a shows gold nanostructures that were not functionalized by cyclo(-RGDfK-). Only a few cell aggregates are visible in Figure 3a, which indicates that cells neither spread nor survive. After functionalization of the gold nanostructures with cyclo(-RGDfK-) cell attachment and spreading are clearly visible in Figure 3b. Cells did not spread on the undecorated hydrogel; rather, only on areas with gold particles, as illustrated by the partially gold-decorated substrate in Figure 3e. With this substrate, the long-term biological activity of the gold-decorated hydrogel was investigated by culturing fibroblasts for more than 14 days on a PEGDA 700 hydrogel partially decorated with cyclo(-RGDfK-)-functionalized gold particles. A dense layer of cells was observed only in the region with a cyclo(-RGDfK-)-functionalized nanopattern. These observations are in agreement with former work where cyclo(-RGDfK-) was covalently attached to nonpatterned polymethylmethacrylate (PMMA) surfaces.[26] In ref 26, adhesion and proliferation of Osteoblasts was reported. The observations on cell adhesion are also in agreement with previous studies of nanopatterned glass surfaces,[7] where cell adhesion and spreading failed if cyclo(-RGDfK-)-functionalized gold nanoparticles were separated by a critical distance near 80 nm (Figure 3c). In the case of larger distances (Figure 3d), cells could attach to the functionalized nanodots, but an efficient cell adhesion and spreading[28] did not happen. Our preliminary cell experiments illustrate that our nanolithography technique is biocompatible, and the results meanwhile imply effects of cell adhesion on the nanopatterned polymer surfaces.

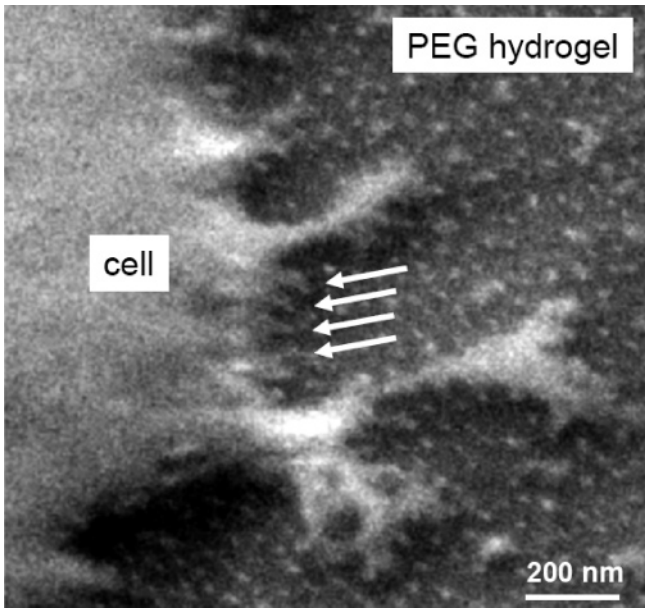

Figure 4. A cryo-SEM image of 3T3-fibroblasts on PEGDA 700 hydrogels decorated with RGDfK-functionalized gold nanoparticles.

The adhesion of cells to the nanopatterned hydrogels was further observed in a cryoscanning electron microscope. In this case, 3T3 fibroblasts were plated for 24 h on a cyclo(-RGDfK-)-functionalized, nanopatterned PEGDA 700 hydrogel. The samples were carefully rinsed with phosphate buffer saline (PBS) solution and immediately shock frozen by immersing them in liquid nitrogen. The cryo-SEM micrograph presented in Figure 4 depicts the adhesive contacts between the cell membrane and single cyclo(-RGDfK-)-functionalized gold nanoparticles (Figure 4). It is noteworthy that this is not a trivial observation because the substrate is a polymeric hydrogel instead of an inorganic glass slide.

Another challenge regarding nanofabrication techniques is to prepare nanopatterns beyond planar surfaces. Curved surfaces are obviously meaningful for studies of cell behavior because most cells are three-dimensionally embedded in the extracellular matrix. This study confirms the feasibility of

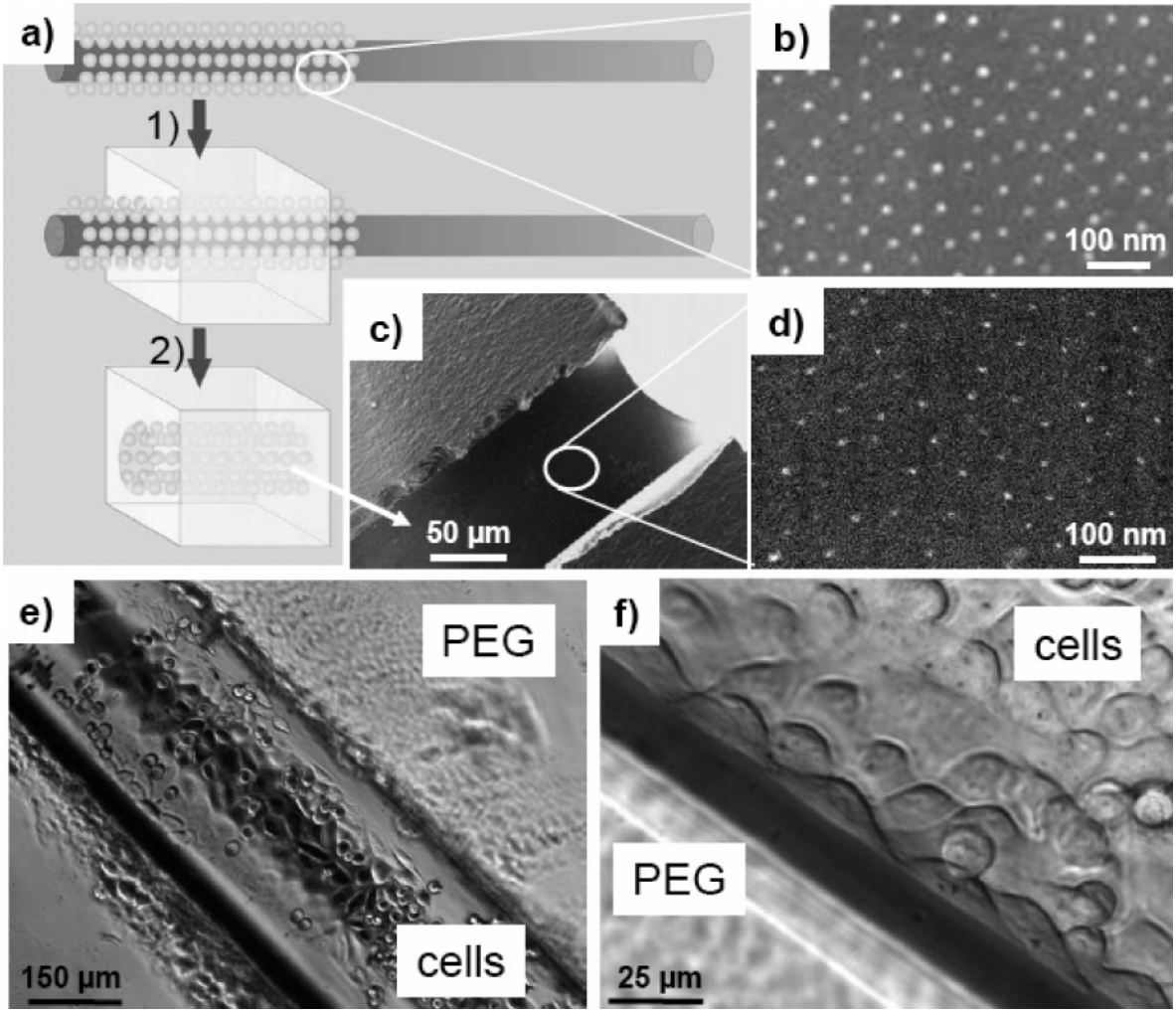

Figure 5. Formation of nanostructured hydrogel microtubes. (a) Schematic drawing of the transfer lithography technique applied to curved surfaces, e.g., optical fibers. (b) SE micrograph of a glass fiber decorated with gold nanoparticles by means of block copolymer micelle nanolithography. (c, d) Cryo SEM images of a PEGDA 700 hydrogel channel decorated with gold nanoparticles. (e, f) HeLa cells cultured in PEGDA 700 hydrogel. The inside of the tube is decorated with cyclo(-RGDfK-) functionalized gold nanoparticles.

the block copolymer micelle nanolithography technique for nonplanar surfaces. As a demonstration, a tubelike hydrogel with a nanopatterned internal surface was prepared.

The diblock copolymer micelle nanolithographic technique was extended so as to decorate glass fibers with gold nanoparticles. Accordingly, glass fibers with diameters ranging from 80 to 300 μm were coated with nanostructures by dip-coating. The fibers were then treated with a hydrogen plasma to remove the micellar diblock copolymer from the gold particles as well as the glass fiber and to deposit the gold nanoparticles along the fiber. The remaining metal particles showed a typical hexagonal arrangement like that seen on the planar substrates (Figure 5a). As with the transfer process of particles to a planar substrate, the gold particles were functionalized with a linker molecule. After embedding the fibers in PEGDA 700 and crosslinking PEGDA (Figure 5b, step 1), the glass fiber was dissolved with hydrofluoric acid (Figure 5b, step 2). This yielded channel structures internally decorated with gold nanoparticles (Figure 5d), as revealed by cryo-SEM of frozen and halved channels (Figure 5c,d).

To make the inside of the PEG hydrogel microchannels adhesive for cells, the tube was further biofunctionalized by a 0.2 mM water solution of cyclo(-RGDfK-) which was injected with a syringe into the channel; the microchannels were then incubated for 1 h. After the microchannels were intensively rinsed with water, HeLa cells were seeded into the channel and cultured under standard conditions for 24 h. Parts e and f of Figure 5 depict cell spreading along the nanopatterned interior wall of the hydrogel tube.

In conclusion, the transfer nanolithography method has been successfully employed to decorate polymeric substrates from rigid hydrophobic polymers to soft hydrogels with gold nanostructures prepared by diblock copolymer micelle nanolithography. This method is utilized in cell adhesion studies for the design of two- or three-dimensional gels with tunable chemistry and elasticity. Because of the high spatial resolution of diblock copolymer nanolithography, these materials enable the positioning of large proteins on an inert matrix with a precision that is comparable to the size of the protein itself. Thus, the transfer technique described herein is a powerful tool for tailoring artificial matrices with highly

defined properties, to applications in cell biology. In the future, we plan to examine cellular behavior as a function of peptide spacing, substrate rigidity, and shape of the substrate.

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