Abstract. A new lithographic technique has been developed and applied to cell adhesion studies and electro-optical material development. Attachment of 6 nm Au particles, in periodic and non-periodic pattern, onto non-conductive substrates has been achieved. This was performed via a combination of diblock copolymer self-assembly and electron beam lithographic techniques. To optimize e-beam resolution on non-conductive materials, an additional carbon layer was thread-coated onto the substrates. This carbon coating and the diblock copolymer used in the self-assembly step were simultaneously removed by a final hydrogen plasma treatment to reveal Au nanodot patterns of unprecedented pattern quality. These optically transparent substrates (glass cover slips) were bio-functionalized via the Au-dot patterns to yield a platform for unique cell adhesion studies. The same Au-dot patterning technique was applied to sapphire substrates, which were subsequently employed to nucleate electro-optically active ZnO nanopost growth.
1. Introduction

A key issue in the fabrication of functional nanostructures is the ‘exact’ location of nanometre-sized objects in periodic or aperiodic arrangements on surfaces with different chemical, electrical and opto-electrical properties [1]–[4]. In the field of nano-bioscience, the positioning of chemical anchors on a substrate, to immobilize substances such as cells or proteins, with submicron resolution, is of significant interest [5]–[7]. Such a chemical platform, which may be regarded as a nanoscopic tool, offers the possibility to laterally separate single objects or to manipulate cell adhesion on a molecular level [8, 9]. This is a powerful approach since it offers the ability to test the effect of ligand presence on specific cellular responses. Biological experiments are usually performed in a liquid environment and the most common method of observation is using optical microscopy in combination with an optically transparent substrate, e.g. glass cover slips. In addition, the handling of such substrates and the localization of nanostructures must be inexpensive and not time-consuming. This limits the choice of substrates and applicable techniques for the generation of nanostructures in this particular field.

Photolithography and electron-beam (e-beam) lithography are the most frequently used techniques for structuring substrates. Different variations of these techniques, such as the use of short wavelength light sources, e.g. deep UV or X-ray, and chemical modification of polymer resistive materials allow researchers to reduce structure dimensions to a sub-micrometre scale [10, 11]. Structures as small as a few nanometres are produced nowadays by e-beam lithography and with photolithography down to 50 nm. Photo lithography is usually applied to insulating substrates such as glass. However, the cost of apparatus is considerably high if one aims for 50 nm resolution and this magnitude of structure is still too large to be used in combination with single proteins or molecules. E-beam lithography offers the magnitude of structure required, but is usually limited to small surface areas. Furthermore, it is a time-consuming technique and is limited to conductive substrates such as silicon wafers. There have been a few examples in which e-beam lithography has been applied to insulating substrates [12]–[15].

Pure self-assembly techniques provide the means of controlling feature size, even down to several nanometres, and are advantageous since the resulting structures are pre-programmed in their molecular and colloidal properties. In principle, this technique allows for immense flexibility in terms of control of architecture and resulting functionality on a molecular scale. The properties of the resulting materials depend on control of localization and pre-programmed interactions between the molecular building blocks. Self-assembly, however, does not cover
requirements regarding aperiodic structures or dimensions that are truly greater than 50 nm in terms of feature size and separation distance [3, 4, 16].

Amphiphilic diblock copolymers such as polystyrene(\(x\))-block-poly(2-vinylpyridine)(\(y\)) (PS(\(x\))-b-P2VP(\(y\))), aggregate into uniform micelles if in toluene, which preferentially solvates the polystyrene (PS). PS blocks form a shell around the less soluble poly(2-vinylpyridine) (P2VP) blocks to reduce energetically unfavourable interactions with the solvent [17]. The diameter of the micelles is controlled by the molecular weight of the block copolymers, the interactions between the polymer blocks and the blocks with the solvent. The micellar core-shell structure forms a nanoreactor, which enables selective dissolution of metal precursor salts into the P2VP core. Subsequently, mono-disperse metal particles are generated within each core following an additional chemical reduction step [18]. The particle size is predominantly controlled by the amount of metal precursor added to the micellar solution. The freedom in choosing the metal type for the formation of nanoclusters by diblock copolymer micelle confinement is rather large. This allowed the formation of, e.g., Au, Ag, Pt, Pd, Ni, Fe or TiO\(_x\).

Over the last few years, we have developed a more ‘hands-off’ method to generate small-scale aperiodic patterned structures, in which nanometre-sized objects are separated by microscopic length scales through the so-called guided self-assembly [18]–[20]. Here, self-assembled diblock copolymer micelles, containing a metal precursor core, are guided by topographically pre-structured surfaces or, alternatively, monolayers of diblock copolymer micelles are directly used as the resist [21]. Thus, the combination of macromolecular self-assembly with conventional lithographic techniques, such as photo or e-beam lithography, allows for symmetry breaking in surface structures and directed location of nanometre-sized features in specific areas. So far, this fabrication process has been limited to large-size features on glass, created using a combination of photo lithography and micellar self-assembly. Small-size features have been generated on conductive, but optically opaque substrates by employing e-beam lithography [22]. Here, we demonstrate how lithography can be extended to create nanometre-sized patterns, even on non-conductive interfaces. Furthermore, we demonstrate how these nanostructured interfaces can be utilized in cell adhesion studies on glass cover slips, and also in the growth of electro-optically active ZnO nanoposts on sapphire substrates.

2. Diblock copolymer micelle nanolithography on non-conductive substrates

The preparation of a diblock copolymer micelle solution in toluene is straightforward. The PS(\(x\))-b-P2VP(\(y\)) is dissolved to 5 mg ml\(^{-1}\) in toluene (p.a.) and subsequently stirred for 24 h. Then, in the case of Au-nanoparticles, HAuCl\(_4\) is added to the micellar solution and again stirred for 24–48 h. By then, each micellar core is filled with equal amount of Au. Here, the amount of HAuCl\(_4\) is chosen such that stochimetrically every second 2-VP unit forms a complex with HAuCl\(_4\), i.e. PS(\(x\))-b-P[2VP(HAuCl\(_4\))\(_{0.5}\)](\(y\)). A highly regular monomicellar film is prepared by immersing a suitable substrate into this solution and retraction of the substrate after a few seconds at a speed of 12 mm min\(^{-1}\). Air-evaporation of the toluene solution from the substrate completes the process [19].

As described previously, by using a conductive substrate, such as a Si-wafer, a monomicellar layer may be used as a negative electron beam resist [22]. The electrons locally modify the chemical properties of the micellar monolayer. This alters the polymer’s solubility and only non-modified polymer micelles can be washed away using a suitable solvent. Subsequent treatment with an isotropic hydrogen plasma results in the remaining Au nanoparticles to be confined to
the areas exposed to the electron beam. Thus, we have the ability to group a flexible number of Au nanoparticles into any desired pattern.

The structural dimensions of the patterns were varied between 100 nm × 100 nm (containing only two Au nanoclusters, each 5 nm in diameter) and 2 µm × 2 µm (containing ∼400 Au nanoclusters). This process was achieved using the combination of two techniques with different magnitudes of scale, i.e. self-assembly of block copolymers (<100 nm) and e-beam writing (>50 nm). It allows the patterning of nanometre-sized clusters of Au-dots, consisting of any given number, even in aperiodic patterns. These groups may be separated by distances not normally accessible by pure self-assembly.

Thus far, this technique has been limited to conductive substrates. This paper describes how the application of this lithographic technique can be applied to non-conductive, plasma-stable substrates, such as standard and cheap glass cover slips or sapphire substrates.

Initially, a glass cover slip was coated with a ∼5 nm thick layer of carbon by carbon-thread coating.1 This introduces the necessary electrical conductivity to the glass cover slip to prevent charging effects during writing of the surface pattern with the e-beam. In addition, carbon films offer low chemical reactivity, thermal and mechanical stability, low surface roughness and the possibility of removal by oxygen or hydrogen reactive-ion etching [23]. In the next step, e-beam lithography is applied to chemically modify a defined number of micelles in a selected area of the glass cover slip. Thus far, this has only been possible on conductive substrates such as Si-wafers [22]. The carbon layer, however, provides sufficient electrical conductivity to prevent substrate charging due to writing with the e-beam. This powerful approach allows precise location of a defined number of nanoparticles, or even a single nanoparticle, on a glass cover slip. Nanoparticle spacing depends on the molecular weight of diblock copolymer micelles, which can range between 30 and 150 nm, and hence the resolution of the e-beam, used to fix the micelles to the substrate, must only reflect these diameters. Exploitation of this fact enables utilization of a low e-beam resolution, to obtain the precise localization of particles, to as small as only 1 nm. This process is depicted in figure 1.

In the following patterning processes, micellar monolayers of PS(500)-b-P[2VP(HAuCl₄)₀.₅](270) or PS(990)-b-P[2VP(HAuCl₄)₀.₅](385) were deposited onto a carbon-coated glass cover slip and then were processed with an e-beam at 1 eV and exposed to an electron dose of 5000 µC cm⁻². The characteristics of the different diblock copolymers are listed in table 1.

In the structures presented, the irradiated areas of micellar monolayer take a square formation, with a width ranging between 100 and 500 nm. These monolayers were treated with a dimethylformamide (DMF) ultrasound bath for 5 min.2 The scanning electron micrographs in figures 2(a)–(d) and figures 3(a)–(d) show the respective diblock copolymer micelles on a carbon-coated glass cover slip after lift-off of non-irradiated micellar monolayers. Non-irradiated areas are completely free of the polymer, whereas the areas of micellar monolayers which were irradiated by e-beam retain the same micellar pattern prior to e-beam exposure and the lift-off step. To deposit Au nanoclusters from these films onto the glass cover slip, the substrate was exposed to a hydrogen plasma (150 W, 0.4 mbar, 30 min). This causes deposition of Au nanoparticles, and also results in a loss of the micelle polymer. This step also yields complete

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1 Carbon thread coating was carried out on a Bal Tec MED 020 coater at 5 × 10⁻⁵ mbar.
2 To remove non-irradiated areas from the solid interface DMF p.a. from Merck or toluene p.a. from Acros were used in combination with an ultrasound bath (Bandelin Sonorex Super RK102h).
Figure 1. Nanolithography process on insulating substrates. A substrate, e.g. a glass cover slip or a sapphire substrate, is coated with an ~5 nm thick carbon layer. Subsequently, the substrate is dip-coated with a monomicellar film. The monomicellar layer is applied as the negative resist, an e-beam then locally modifies chemical properties of the monolayer. In the pursuing step, the non-modified diblock copolymer micelles are lifted-off using an organic solvent. The subsequent gas plasma treatment removes the diblock copolymer and the carbon coating completely from the non-conductive substrate. Au nanoparticles are deposited in e-beam modified areas of the micellar monolayer.

Table 1. Molecular weights of diblock copolymers.

| Polymera | PS Mn(b) (g mol⁻¹) | PS Mw/Mnb | Block Mn(c) (g mol⁻¹) | Block Mw/Mnc |
|----------|--------------------|-----------|----------------------|-------------|
| PS(x)-b-P2VP(y) | 52 400 | 1.05 | 80 500 | 1.07 |
| 990-b-385 | 103 000 | 1.07 | 143 500 | 1.09 |

a $x$ and $y$ give the numbers of theoretical repeat units as calculated by the initial monomer/initiator feed ratio.
b Number average molecular weight and polydispersity of the PS block obtained from size exclusion chromatography (SEC) in tetrahydrofuran (THF) and calibrated to poly(styrene) standards.
c Number average molecular weight and polydispersity of the block copolymer obtained from SEC in dimethylacetamide (DMA) and also calibrated to PS standards.

carbon layer removal. The Au-dot pattern shown in figures 2(e)–(h) and figures 3(e)–(h) show Au-particles as white dots in scanning electron micrographs. Au-dots are found only in the areas in which the micellar monolayer has been modified by the e-beam. The small defects (white arrows in figure 2) typically observed on standard glass cover slips do not affect the quality of the nanopattern. Identical patterns may also be formed on sapphire $a$-plane substrates, they are also non-conductive, but differ in chemical composition (figure 4).
Figure 2. (a)–(d) SEM images of PS(990)-b-P[2VP(HAuCl₄)₀.₅](385) micellar monolayers after lift-off of non-irradiated areas of the monomicellar layer. White arrows indicate holes found typically on glass cover slips. (e)–(h) Micrographs of the corresponding pattern after hydrogen plasma treatment in which the diblock copolymer is removed from the Au nanoparticles and also the carbon coating from the glass. The particle–particle distance is \(~73\) nm in a single domain.

Figure 3. (a)–(d) SEM images of PS(500)-b-P[2VP(HAuCl₄)₀.₅](270) micellar monolayers after lift-off of non-irradiated areas of the monomicellar layer. (e)–(h) Micrographs of the corresponding pattern after hydrogen plasma treatment in which the diblock copolymer is removed from the Au nanoparticles and the carbon coating from the glass. The particle–particle distance is \(~57\) nm in a single domain.
Figure 4. (a)–(d) SEM images of PS(990)-b-P[2VP(HAuCl₄)₀.₅](385) micellar monolayers after lift-off of non-irradiated areas of the monomicellar layer from a sapphire a-plane substrate. (e)–(h) Micrographs of the corresponding pattern after hydrogen plasma treatment in which the diblock copolymer is removed from the Au nanoparticles and the carbon coating from the sapphire. The particle–particle distance is ∼73 nm in a single domain. There are no defects in the substrate in the case of sapphire.

Homogeneously Au-dot covered glass substrates were probed by scanning force microscope (SFM). The Au-dot height does not differ in comparison to e-beam generated structures and is determined as 5 nm for PS(500)-b-P[2VP(HAuCl₄)₀.₅](270) and 6 nm for PS(990)-b-P[2VP(HAuCl₄)₀.₅](385). Figure 5 depicts scanning force micrographs of the structures shown in figures 2 and 3. Figures 5(a) and (c) show micellar packing after lift-off of non-irradiated structures. The respective structures following plasma treatment are indicated in figures 5(b) and (d).

The nanopatterns reported here are formed by the assembly of Au-clusters on different substrates. Other cluster materials, such as Ag, AgOₓ, Pt, Pd, ZnOₓ, TiOₓ, Co, Ni or FeOₓ are possible as well. Especially in the case of Au, the question arises concerning the nanopattern’s mechanical stability. In contrast to evaporated or sputtered gold layers on glass or silicon substrates, which can be easily removed by touching the layer with a rather soft tissue or in an ultrasonic water bath, these nanopatterns demonstrate enormous mechanical stability. Substantial chemical treatments with ‘piranha’ acid, different bases, alcohol or water does not affect the pattern. This pattern’s mechanical stability have opened many different approaches which are currently under investigation. These are applications in biology as templates for immobilizing single proteins and probing their interactions with living cells or as coatings for lenses and filters [8]. Thermal treatment of a pattern up to 800 °C did not show any cluster mobility or coalescence. But it resulted in Au evaporation from clusters which did not affect the cluster’s position. We assume that this remarkable mechanical stability which is essential for future applications is
Figure 5. Three-dimensional and top-view images from SFM investigations of (a)–(b) PS(990)-b-P[2VP(HAuCl4)0.5](385) and (c)–(d) PS(500)-b-P[2VP(HAuCl4)0.5](270) after lift-off ((a) and (c)) as well as after plasma treatment ((b) and (d)).

Figure 6. (a) C 1s, (b) O 1s and (c) Au 4f XPS-signal: – – –, clean cover slip; – · –, micellar monolayer on carbon-coated glass cover slip; ——, micellar monolayer on carbon-coated glass cover slip and e-beam treatment (300 eV, 10 000 µC cm−2); ····, micellar monolayer on carbon-coated glass cover slip, e-beam and H2-plasma treated.

well-founded in the plasma processes described above as the polymer removal step. Also O2−, H2−, or Ar-plasma application did not affect the cluster supporting substrates substantially, and SFM displayed slight substrate roughening. This leads to the hypothesis that the edge formed by the substrate–cluster borderline is partly wetted by surface atoms of the substrate activated by the plasma process.

X-ray Photoelectron Spectroscopy (XPS) investigations were utilized to monitor changes in chemical composition of the system after each preparation step. In figure 6(a), the C 1s signal is broadened after electron irradiation (300 eV, 10 000 µC cm−2) of the micellar layer (on top of the carbon layer on glass). This is not the case with the non-irradiated layer. The signal areas are approximately the same in both cases, which indicates detection of a constant amount of carbon
Table 2. XPS studies of photo electron binding energies, signal intensity and signal area of C 1s and O 1s.a

|            | C 1s       | O 1s        |
|------------|------------|-------------|
|            | Energy     | Area        | Energy     | Area        |
| Reference  | 285.1 ± 0.2| 151         | 532.5 ± 0.2| 3985        |
| Monolayer + C | 284.6 ± 0.2| 1905        | 532.4 ± 0.2| 109         |
| Monolayer + C + e-beam | 284.4 ± 0.2| 1865        | 532.5 ± 0.2| 524         |
| Monolayer + C + plasma treatment | 284.7 ± 0.2| 158         | 532.4 ± 0.2| 3885        |

a Apparatus error: binding energy: ±0.2 eV; intensity: ±10%.

Table 3. XPS studies of photo-electron binding energies, signal intensity and signal area of Au 4f signal.a

|            | Au 4f7/2 | Au 4f5/2 |
|------------|----------|----------|
|            | Energy   | Area     | Energy   | Area     |
| Reference  | --       | --       | --       | --       |
| Monolayer + C | 85.3 ± 0.2| 30       | 89.0 ± 0.2| 22       |
| Monolayer + C + e-beam | 87.9 ± 0.2| 102      | 91.5 ± 0.2| 81       |
| Monolayer + C + plasma treatment | 84.3 ± 0.2| 121      | 87.9 ± 0.2| 106      |
| Monolayer + C + plasma treatment | 83.8 ± 0.2| 127      | 87.4 ± 0.2| 93       |

a Apparatus error: binding energy: ±0.2 eV; intensity: ±10%.

at the interface as is documented in table 2. Hence, the broadening of the peak must be due to the formation of different carbon derivatives. Hydrogen plasma-treated surfaces (30 min, 0.4 mbar, 150 W) are nearly free of carbon, which can be seen by comparison with the spectrum of a reference sample (figure 6(a)). The O 1s signal is dominant for both the reference and plasma-treated samples. This originates from the oxygen present in glass. No oxygen could be detected in substrates coated with both a carbon layer and a micellar monolayer, although this may be due to an attenuation factor. After electron irradiation of the micellar monolayer (on top of the carbon layer) and its subsequent exposure to air, a significant O 1s signal is generated. This indicates the formation of radicals, which bind oxygen upon exposure to air. We speculate that this results in the formation of carboxylic acids, ketones, aldehydes and ethers coordinated to the polymer as well as causing polymer cross-links [24]. Formation of these carbon species and cross-links causes the polymer to become insoluble in DMF and stabilized on the surface against its treatment. Since micelles are immobilized on chemically distinct substrates (glass and sapphire) we propose that chemical modification of the diblock copolymer is the only requirement for stabilization on a substrate against DMF treatment, this modification also prevents loss of the HAuCl4.

Formation of elemental Au after electron irradiation is shown in figure 6(c) where the Au 4f signal is monitored following each preparation step and values are documented in table 3. The XPS data show a shift of the Au 4f signal to lower energies if the monomicellar film has been exposed to electrons. This indicates that electron irradiation of the HAuCl4, incorporated in the monomicellar film, causes reduction of Au(III). However, these signals are shifted to higher energies than expected from bulk Au (Au 4f5/2 at 87.3 eV and Au 4f7/2 at 83.6 eV, indicated as
Figure 7. SEM images before ((a) and (c)) and after plasma treatment ((b) and (d)). Diblock copolymer micelles from PS(500)-b-P[2VP(HAuCl₄)₀.₅](270) and PS(990)-b-P[2VP(HAuCl₄)₀.₅](385) were deposited consecutively on the carbon-coated glass cover slip followed each time by e-beam writing and DFM lift-off processing. Finally, samples were exposed to a hydrogen gas plasma. This resulted in deposition of Au-particles (~5 and 6 nm diameter), in two differently spaced patterns (57 or 73 nm).

vertical lines in the graph). This is quite probably due to the formation of many ultra-small Au nanoparticles (<1 nm) contained within the polymeric shell after e-beam exposure. The electronic properties of such small Au particles are known to be affected by their size [25, 26]. In addition, charging of Au-particles is highly likely, since these particles are encapsulated by the isolating polymer. Hydrogen plasma treatment of the micellar monolayer gives rise to an identical spectrum to bulk Au, indicating complete loss of all polymers and complete reduction of Au.

The demonstrated technique of positioning single Au nanoparticles onto various surfaces allows the formation of more complex structures. Successive depositions of various micellar monolayers, exposures to e-beam writing and DMF treatments enable the immobilization of different Au-particle patterns on the same substrate. Initially, a micellar monolayer from PS(500)-b-P[2VP(HAuCl₄)₀.₅](270) was deposited onto a carbon coated glass cover slip followed by e-beam patterning of discs (figure 7(a)) and stripes (figure 7(c)). The non-irradiated areas were removed by DMF treatment. A second monomicellar layer from PS(990)-b-P[2VP(HAuCl₄)₀.₅](385) was then deposited onto the same glass cover slip followed by a second e-beam patterning of squares (figure 7(a)) and stripes (figure 7(c)) oriented perpendicularly to the initially deposited stripes. Lift-off of non-irradiated areas by DMF resulted in adjacent immobilization of different micelles. This becomes evident after an additional
3. Growth of luminescent ZnO nanoposts from Au nanoparticle patterned sapphire substrates

In the year 2000, Fons et al demonstrated the growth of ZnO epilayers on $a$-plane sapphire substrates [27]. This knowledge was exploited by Huang et al in 2001, using the vapour–liquid–solid (VLS) phase transport process to grow epitaxially ZnO nanowire arrays [28]. It requires a seed for nucleation of growth and the location of this seed on the surface dictates the position of the resulting ZnO nanopost. ZnO has had an explosion of interests during the past few years due to its intriguing optical properties, such as its characteristic direct and wide bandgap, with a large exciton binding energy of 60 meV [29]. In this study, Au nanodots were localized on to an $a$-plane sapphire surface using the method described in section 2. In a tube furnace, ZnO was generated from bulk Zn at 500–900°C. Argon flow was used to transport the vapour through the tube furnace to the $a$-plane sapphire substrate. The temperature of the substrate was 700–800°C. At this temperature, the Au clusters form an alloy with Zn [30]. Saturation of the Au clusters by Zn and the subsequent formation of Zn-suboxides [31] causes deposition of ZnO onto the sapphire substrate. ZnO posts are, subsequently, epitaxially grown in a perpendicular orientation to the sapphire substrate (due to its surface orientation). Figures 8(a) and (b) show

![Figure 8.](http://www.njp.org/)
scanning electron microscopy (SEM) images of \( a \)-plane sapphire substrates decorated with a homogeneous layer of ZnO nanoposts generated using a Au-dot covered sapphire substrate as described previously. The crystal lattice structure gives rise to the predominantly perpendicular orientation of the ZnO posts. This was investigated by small angle x-ray scattering as reported in [32]. Figure 8(c) shows a standard fluorescence optical microscopy image of such a surface, taken at room temperature. The excitation wavelength was \( \sim 350 \text{ nm} \) generated from a Hg lamp and the emission wavelength was \( \sim 405 \text{ nm} \). A clear borderline can be visualized between the Au-nanopatterned surface (bottom, shown in blue) and the non-patterned surface (top, shown in black). Even at room temperature the surfaces are highly luminescent.

The ability to control Au-pattern flexibility, enables one to position single ZnO nanoposts in a specific orientation with respect to one another. If this technique could be used to produce uniform layers of ZnO nanoposts at separation distances equal to half the wavelength of the emission light, they may have the possible application as sources of coherent light [33]. The optical properties may also be altered by controlling the diameter of nanoposts by the size of the Au nanoparticles [34]. Initial experiments on the control of Au-dot patterns show that this magnitude of precision may be possible (figure 9).

Figures 9(a)–(d) show SEM images of \( a \)-plane sapphire surfaces, which were decorated with Au-dots arranged into disc or stripe patterns. The discs were made up of \( \sim 100 \) Au nanoparticles, each with a diameter of 6 nm and a separation of \( \sim 73 \text{ nm} \). The stripes have a width which corresponds to \( \sim 5–15 \) Au nanoparticles of \( \sim 6 \text{ nm} \) diameter. However, uniform perpendicular growth of ZnO posts has not been obtained with the same quality as was demonstrated with the lateral spacing of Au-dots, shown in figure 8. Irrespective of the post orientation, fluorescent optical microscopy shows ZnO posts patterned into arrays of discs and stripes, demonstrating the high control of their lateral spacing (figure 9(e)).

4. Cell adhesion studies on bio-functionalized, nanopatterned glass

The application of nanopatterning on insulating, optically transparent substrates, such as glass cover slips, is very profitable in combination with inverted optical microscopy techniques, since these are unavailable with the use of non-transparent substrates. Upright microscopy techniques may be applied to monitor cell activity, but resolution is not of sufficient quality with use of an air objective in combination with a sealed cell environment due to restriction in the \( z \)-direction of the focal plane. Water immersion objectives overcome this problem, but are invasive and may contaminate or disturb the cell environment. Inverted microscopy for cell studies overcomes these two problems, but it requires a transparent substrate, which is also true for phase contrast and reflection interference contrast microscopy imaging (RICM) [35]. Use of non-transparent substrates, such as a Si-wafers, as platforms for the adhesion mediating nanodots therefore cannot be used in combination with inverted microscopy for cell imaging. The technique herein described overcomes problems of non-conductivity associated with glass cover slips, which provide the transparency required of a substrate for inverted microscopy. Advantages of employing these substrates in cell adhesion studies are now further described.

Figure 10 shows a phase contrast microscopy image of rat embryonic fibroblasts (REF52) adhering to an area marked by a white 250 \( \mu \text{m} \) \( \times \) 250 \( \mu \text{m} \) frame. The area within this frame is patterned with 500 nm \( \times \) 500 nm squares, squares separated by 1 \( \mu \text{m} \) on all sides. The squares are patterned with hexagonally organized Au nanoparticles \( \sim 5 \text{ nm} \) diameter, each separated
Figure 9. (a)–(d) SEMs of ZnO posts grown from Au nanodots arranged in disc and stripe patterns. (e) Fluorescent optical microscopy of surfaces shown in (a) and (b). by ~57 nm. Examples are shown in figure 3(e). Glass cover slips were bio-functionalized using the procedure described previously in [8]. Tri-ethoxy silane terminated polyethylene glycol (PEG) was immobilized to prevent non-specific protein adsorption onto the glass substrate. RGD-thiol peptides [36]–[38], the adhesion mediating motifs of fibronectin, were linked to the Au nanodots. This created Au nanodots with the required functionality to mediate cell adhesion. Cells were introduced to this nanostructured substrate and allowed to adhere.\textsuperscript{3} Cells localized

\textsuperscript{3} Cells plated at a density of 100 cells mm\textsuperscript{−2} in DMEM (Dulbecco’s modified Eagle’s medium) containing 1% fetal bovine serum for 22 h.
Figure 10. (a) Phase contrast microscopy image of REF52 cells adhering to an area marked by a white $250 \mu m \times 250 \mu m$ frame. This frame is patterned by $500 \text{nm} \times 500 \text{nm}$ squares each separated by $1 \mu m$. Each square contains hexagonally organized Au nanoparticles of $5 \text{nm}$ diameter, each separated by $\sim 57 \text{nm}$. This substrate was bio-functionalized as described in [8]. Cells outside the frame cannot spread on the interface in this area due to a lack of RGD-peptides (marked by white arrows). (b) An enlargement of the image of cells adhering to the substrate, from the marked square in image (a). The insets show fluorescent optical micrographs of two different cells. The actin filaments are shown in red and vinculin clusters of focal adhesions and RGD patterns are both shown in green, since they are stained with the same dye. Note that actin fibre organization linearly reflects the pattern of the squares (each separated by $1 \mu m$).
in areas of the substrate which are not RGD-functionalized are unable to spread. This behaviour can be seen to occur in the two cells in figure 10(a) in the upper right-hand side section of the micrograph (marked by white arrows). The protrusion of these two cells upwards, into the liquid, causes them to appear in images as bright spots, when compared with adhesive, well-spread cells.

The insets in figure 10(b) show fluorescent optical microscopy images in which visualization of actin filaments was enabled using red immunostaining. Vinculin, a protein associated with focal adhesion formation, was immunostained green. This process also caused partial staining of the RGD-peptides (green fluorescence), resulting in all squares of the bio-functionalized Au-dot pattern being visible in the fluorescent micrograph. Integrin clustering is expected to occur only on top of the RGD bio-functionalized Au-dots, as focal adhesions are formed. This was monitored via the presence of integrin-associated Vinculin molecules. Actin filaments form from the focal adhesion sites, extending towards the centre of the cell. In figure 10(b), red actin filaments can be seen extending from the focal adhesions. At one end of each of the actin filament bundles, where focal adhesions have formed, there is an increase in green fluorescence intensity, caused by the presence of Vinculin (examples marked by white arrows). This is only seen to occur in the sites where the bio-functionalized Au anchors have been positioned. Such nanoadhesive patterns offer the opportunity to tune separation between focal adhesions in multimolecular complexes with unprecedented resolution, even down to sizes of individual proteins. Control of the patterning of the bio-functionalized Au-dots can be applied in the form of small clusters. Variation in the number of Au-dots, and hence the number of RGD–Integrin bonds within the clusters, can be applied to discover the minimum number required to form a focal adhesion during cell attachment, spreading or migration. With this knowledge it would be possible to pattern specific features which would create the so-called ‘adhesion keys’. These keys could be used to mediate cell adhesion, to trigger certain events within the cell.

5. Conclusion

Precise Au-dot patterning of substrates has been realized using a combination of two different magnitude techniques, i.e. self-assembly of block copolymers (<100 nm) and e-beam writing (>50 nm). E-beam lithography was applied to homogeneous monomicellar films to pin micelles of selected patterns onto their substrates. This technique was extended to non-conductive substrates, such as glass cover slips or sapphire, by application of carbon-thread coating. This carbon coat is stable to multiple e-beam treatments, and may be removed when desired, allowing intricate patterns to be created, layer-by-layer. Removal of both the carbon coating and the micelle template may be achieved in one simple step: exposure to a hydrogen plasma.

This combination of techniques allows precise localization of clusters (of any desired number) of nanometre-sized Au-dots in periodic or aperiodic patterns, and these patterns may be separated by distances too great to be accessible by pure self-assembly. The size and geometry of clusters is much smaller than what could be obtained with purely lithographic techniques. Variation of particle diameter and investigation into alternative materials to Au are possibilities for future work.

This novel technique offers a powerful tool for cell adhesion studies, allowing control of location of cell focal adhesion sites, directly on a substrate, which may be used in combination with many inverted microscopy techniques. In future work, RICM and live-cell imaging will be carried out using these substrates. This patterning technique has also been applied to control the
growth of patterns of ZnO posts with the same exact location. This has an exciting application with respect to coherent light generation and production of arrays of posts with the required lateral positioning is currently underway.

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