Title: ENZYME-ASSISTED NANOLITHOGRAPHY

Abstract: A method for enzyme-assisted nanolithography and devices for performing such methods are provided. Furthermore, supports with surfaces obtainable by the method according to the present invention are described as well as devices containing such supports, e.g. chips, microarrays and biosensors. In addition, methods for the specific coating of a tip with enzyme or effector molecules are provided, which can particularly serve for the application in scanning probe microscopy. The described methods, surfaces, supports, devices, tips and scanning probe microscopes can be used for the storage and/or analysis of data, in cell biology or for conducting measurement and examination methods, like e.g. in forensics, environmental and chemical analytics, molecular biology or pharmaceutical diagnostics.
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Enzyme-assisted nanolithography

Field of the invention
The present invention generally relates to an enzyme-assisted method for nanolithography and devices for carrying out such methods. In particular, the present invention relates to a lithographic method for structuring a surface of a support, wherein this surface is chemically or physically modified by a locally restricted enzymatic activity in the immediate vicinity of the surface, preferably by positioning of an enzyme or effector molecule immobilized to a tip in the immediate vicinity of the surface. The present invention further relates to a support with surfaces obtainable by the method according to the present invention and to devices containing such supports, like chips, microarrays and biosensors, particularly DNA and protein chips. Furthermore, the present invention relates to methods for the specific coating of a tip with enzyme or effector molecules, so that the tip comprises enzymes or effector molecules, respectively, preferably in an area of less than 100 nm, preferably less than 50 nm and particularly preferred in an area of 10 nm or less.

The present invention further relates to tips obtainable by the method according to the present invention and particularly scanning probe microscopes containing tips according to the present invention. The methods, surfaces, supports, devices, tips and scanning probe microscopes according to the present invention can be used for the manufacturing of e.g. nanochips and microarrays and for the storage and/or analysis of data, for the application in cell biology or for conducting measuring and examination methods, like e.g. in forensics, and in chemical or biological analytics.

In the text of the present application, various publications are referred to with specification of author and date. Detailed bibliographical references of these publications can also be found subsequent to this description, immediately before the claims. The disclosures of these publications, in their entirety, are hereby incorporated by reference in this application in order to describe more comprehensively the state of the art known to the person skilled in the art at the time of the invention described and claimed herein.

Background of the invention
Conventional lithography methods, like photolithography, are used to manufacture structures of a size of several hundred nanometers, even under production line conditions as in semiconductor industry. There is a constant need for increasing the resolution of these
methods. Possible routes are using shorter wavelengths (UV light) or other media like in electron lithography, see also the International Technology Roadmap for Semiconductors at http://public.itrs.net. However, it is not clear how structure sizes of 10ths of nanometers or only a few nanometers can be manufactured routinely by mere optimization of currently common technologies.

A possible alternative technique, which has been proven successful on a lab basis, is surface modification by scanning probe techniques. E.g. soft polymeric films can be modified by mechanical interaction of an atomic force microscope tip (Wendel, Lorenz et al. 1995). The dip pen technology (Piner, Zhu et al. 1999), where a suitable ink is adsorbed on an AFM tip and then locally released on a sample, has also attracted a lot of interest.

In the future there will not only be a need for structuring surfaces at a very small (nanometer) scale as driven by semiconductor industry, but there will also be the need to chemically modify surfaces for instance in biotechnological applications. The miniaturization of assays, such as DNA chips or protein chips will require smaller chemically well defined structures. A survey of the best-known biochip systems is given by Bowtell in Nature Genetics Supplement 21 (1999), 25-32.

There is also a strong need to miniaturize diagnostic devices, biosensors or to create chemical "nanoreactors" (local environments capable of performing certain chemical tasks). Even if it is conceivable that conventional techniques like UV-lithography will achieve resolutions in the order of 10ths of nanometers, it is not clear, however, how the need for chemical modifications will be solved.

Enzymes were also described for chemical modification of surfaces, e.g. by applying them to a suitable surface by means of a nanofountain pen, where a substrate, which can be digested by the enzyme, was fixed (Ionescu, Marks et al., 2003). Here, however, surface changes caused by changes of the substrate, which occurred all by themselves, were already observed when merely the preconditions required for conducting the method were given. Furthermore, the enzyme was used more in the sense of “etching”, i.e. the classical etching of the surface, while the enzymatic activity played a subordinate part.

In another application, phospholipase present in the medium was used to modify a lipid film. Here local disturbances in the order of the lipid film caused by the mechanical interaction of the AFM tip were used to modify the sample locally (Grandbois, Clausen-Schaumann et al. 1998).
In documents US 6,001,587 and WO97/06468 A2, further methods for modifying a surface by the use of enzymes are disclosed. However, methods for the specific enzyme-assisted structuring of a surface fulfilling the requirements of e.g. chip industry and nanobiotechnology are not available up to now.

Document US 5,824,470 discloses a method for coating a tip with enzyme, wherein the tip is brought into contact with a support and wherein the enzyme is bound by a polyvalent binding partner via a spacer to the tip.

The technical problem underlying the present invention is to provide surfaces and methods for their manufacturing fulfilling the above-mentioned requirements. This technical problem is solved by means of the embodiments according to the present invention, which are characterized in the claims and explained in the subsequent description.

The present invention

The present invention relates to a method for structuring a surface of a support, wherein the surface is chemically or physically modified by a locally restricted enzymatic activity in the immediate vicinity of the surface.

According to the present invention, the essential feature of the local enzymatic activity at the surface to be structured can be achieved by specifically switching on and switching off the enzymatic activity in the immediate vicinity of the surface and/or by controlled coating of a tip with few or even only one enzyme or effector molecule.

The invention is based on the surprising finding that a method for the specific local, chemical or physical modification of a suitable surface can be obtained with the aid of biological or biologically derived enzymes. The enzyme can preferably be fixed on the tip of a scanning probe microscope, which facilitates the chemical or physical modification with a very high resolution, eventually on the basis of individual molecules.

The method described herein can be applied to a wide range of enzyme molecules and therefore enables a technology platform that can be used for different applications. Enzymes are common tools in diagnostics and biotechnology. The probably best-known applications are PCRs (polymerase chain reactions) or ELISAs (enzyme linked immunosorbent assays). They have even found use in household products of daily use, e.g. in form of detergent
additives. According to the present invention, the vast knowledge on enzymes from these fields can be used in future applications and available enzymes and suitable substrates can be relied upon.

The present invention further relates to supports with surfaces that are obtainable by the method according to the present invention and to devices containing such supports, like chips, microarrays and biosensors, particularly DNA and protein chips.

Furthermore, the present invention relates to methods for the specific coating of a tip with enzyme or effector molecules, comprising bringing the tip in contact with a flat support, on which enzymes or effector molecules are bound by means of a polyvalent binding partner A, like streptavidin, to a binding partner B, like biotin, which is bound on the support, and wherein at least a part of the tip comprises substantially the same binding partners B, in this case biotin.

The wording "substantially the same binding partners B" means that both binding partners B need not be exactly the same binding partners but that both binding partners B have the property to bind to binding partner A. Furthermore, the wording "tip" in the present application also comprises beads, probes, micro or nanoparticles and the like. Particularly, the wording tip refers to the tip of an atomic force microscope.

The present invention further relates to tips obtainable by the method according to the present invention and particularly scanning probe microscopes containing tips according to the present invention.

Finally, the present invention relates to uses of the methods described herein, surfaces, supports, devices, tips and scanning probe microscopes for the manufacturing of e.g. nanochips and microarrays and for the storage and/or analysis of data, for application in cell biology or for conducting measuring and examination methods, like e.g. in forensics, and in chemical or biological analytics.

The figures show:

**Fig. 1:A.** An atomic force microscope tip (scanning electron micrograph, width of the pyramidal tip about 4 µm) is coated at its apex with an enzyme molecule (yellow and
light gray, respectively) by means of interaction of the groups attached to the enzyme (green and medium gray, respectively, in this case streptavidin, depicted as plug-in knob) with the groups attached at the tip surface (red and dark gray, respectively, in this case biotin, depicted doubly concave).

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B. The protein, i.e. the enzyme is linked to a streptavidin molecule (complex depicted doubly concave) having four binding sites (green or edges, respectively) for the ligand biotin (depicted as plug-in knob). The enzymes are fixed on a biotinilated support. The likewise biotinilated atomic force microscope tip is brought in contact with the enzyme-coated sample.

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Enzyme molecules are then bound to the tip and to the support. After separation of the tip from the support, about 50% of the doubly bound enzyme molecules will remain at the tip. This procedure allows the coating of only the very apex of the atomic force microscope tip.

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Fig. 2: Possible reaction schemes. The illustrations show by way of example several reaction schemes. By inserting additional effector molecules, like co-enzymes or other molecules, arbitrarily complicated reaction schemes can, of course, be obtained.

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A. The substrate (S) is immobilized on the surface and becomes soluble due to the enzymatic activity. S is immobilized on the sample and the enzyme cleaves S, so that product P becomes soluble and the remainders of S, which are still on the surface of the sample, show (chemical or physical) characteristics different from those of S.

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B. The substrate (S) is present in the medium (bulk phase) and becomes insoluble due to the activity of the enzyme. S is dissolved in the medium and product P is immobilized (adsorbed) on the surface by chemical or physical interaction.

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C. The substrate is present in the medium. The soluble product P then chemically modifies the molecules present at the surface.
D. The substrate is present in the medium. The soluble product P reacts with a co-factor B to form a complex PB. The complex PB can now physically modify the surface, for instance by accumulating on the surface (analogous to scheme A).

E. The substrate is present in the medium. The soluble product P reacts with a co-factor B to form a complex PB. The complex PB can now chemically modify the surface (analogous to scheme A).

F. The substrate is present in the medium. The soluble product P reacts with a co-factor B, so that a modification of the co-factor to form B* occurs. The modified co-factor B* can now chemically or physically modify the surface.

Fig. 3: The enzyme alkaline phosphatase was immobilized to an atomic force microscope tip. The substrate BCIP was present in the surrounding medium.

A. The product of the enzymatic reaction together with NBT forms a non-water-soluble complex precipitating on the surface.

B. Arbitrary features can be generated on the surface by slowly approaching the atomic force microscope tip to direct contact with the surface.

Detailed description of the invention

The present invention relates to a lithographic method for structuring a surface of a support, wherein this surface is chemically or physically modified by a locally restricted enzymatic activity in the immediate vicinity of the surface.

As mentioned before and explained in the examples, a method for the modification of a suitable surface with the help of biological or biologically derived enzyme molecules is provided. The enzyme molecules are brought into the immediate vicinity of the sample and modify the sample by means of material deposition, material removal or modification of sample molecules. The essential features of the invention are the following:

- a suitable surface is modified directly or indirectly by the chemical activity of an enzyme and
the enzyme is positioned, relatively to the sample, in such a way that the modification only occurs locally, see e.g. Figure 3.

The main advantage of this idea lies in the utilization of the chemical activity of one enzyme out of the multiplicity of available enzymes, whose properties the method according to the present invention is taking advantage of. This facilitates the creation of a technology platform for many different applications.

The method according to the present invention is preferably characterized in that a resolution of less than about 200 nm, preferably less than 100 nm, more preferably less than 50 nm and particularly preferred less than about 10 nm can be obtained.

Materials common in array and chip technology, like glass, gold and plastic membranes, which can also represent the surface itself, can be used as supports and surfaces. Particularly suitable are metals, e.g. copper, titanium, chromium, gold and especially platinum. Usually the support surface is derivatized. Glass surfaces, for example, can be treated with silane reagents, like aminopropyltriethoxysilane (APTES), 3-mercaptopropyltrimethoxysilane (MPTS), glycidoxypropyltrimethoxysilane (GPTS), bis(hydroxyethyl)aminopropyltriethoxysilane (HE-APTS), hydroxybutyramid-propyltriethoxysilane (HBPTES) and (perfluoroocctyloxy)propyltriethoxysilane (POPTS). Preferably gold, Ni-NTA, avidin or biotin-coated supports made of glass, silicon, silicon oxide or silicon nitride are used. Gold-coated glass supports are particularly preferred. The gold surface can e.g. be coated by differently functionalized alkanethiol-monolayers (SAMs = self assembled monolayers) like biotin, N-oxysuccinimide ester, epoxy groups, maleimide groups, amino groups and oligo(ethylene glycol).

For the manufacturing or modification, respectively, of protein chips particularly soft supports like PVDF, nitrocellulose and polystyrene can be used. As a multiplicity of different molecule types can be fixed on glass surfaces, glass will preferably be used in the methods according to the present invention. Possible surfaces are particularly also surfaces that are already loaded with biomolecules, like DNA-chip formats, which are also commercially available, e.g. by the Affymetrix company, who offers DNA-microarrays. The manufacturing of arrays of immobilized biomolecules, which can also be modified in the sense of the present invention, are described e.g. in the German patent application DE 100 41 809 A1.
From the above explanation it follows that a support can simultaneously also represent the surface. Accordingly, the terms "support" and "surface" can be used as synonyms, where appropriate.

Supports in the sense of the present invention are usually materials with rigid or semi-rigid surface and therefore to be designated as solid. Planar surfaces are standard. The form of a support can be manifold and can vary according to the kind of its application. Possible are e.g. object carriers, chips, dipsticks, stamps, caps, particles, spherical bodies, like pellets or small beads, membranes, sheets, discs, foils or plates. Wafer formats can also be used as supports and can be separated, if desired. The supports can furthermore have additional useful components, e.g. encapsulated chips.

According to the present invention, the term "modification" particularly comprises the embodiments described herein, like deposition, removal or actual modification of the substrate. The modification of the surface can be performed with a very high resolution, wherein eventually one enzyme molecule performs only one molecular modification in only one place. The modification of the surface can occur directly due to the activity of the enzyme or indirectly, so that the enzymatic products themselves modify the surface.

If not stated otherwise, the term "enzyme" is used in its conventional sense. An enzyme is, among other things, a chemically active molecule modifying a substrate molecule and thereby producing one or more product molecules. An enzyme can chemically modify the substrate molecule, can cleave it into several sub-molecules or perform a combination of both. The chemical modification of the substrate molecule will lead directly or indirectly to a chemical or physical modification of the sample. As this modification only occurs locally, this process is — according to the present invention — called enzyme-assisted nanolithography.

Effector molecules are usually understood to be substances that can both activate and inhibit the enzyme reaction. Effectors are usually not involved in the reaction itself, but can trigger it, according to the understanding of the present invention. According to their effect, they can be referred to as activators or inhibitors. According to the present invention, the term "effector molecule" can also designate co-factors like NADPH as well as, in general, molecules that are capable of triggering an enzymatic reaction, e.g. by binding to the enzyme, thereby activating
it, i.e. making it enzymatically active. Accordingly, the effector molecule can be a co-factor, a substrate, a catalyst or also an enzyme. Co-factors are also called co-enzymes.

In this context, vitamins, being components of co-enzymes, can also be effector molecules, e.g. thiamin (vitamin B1) or thiamindiphosphate as co-enzyme of the oxidative decarboxylation, riboflavine (vitamin B2), forming the B2-complex with nicotinic acid, nicotinamide, folic acid and pantothenic acid, pyridoxine (vitamin B6), which is needed in transaminations and decarboxylations, cobalamine (vitamin B12), important with isomerases, ascorbic acid (vitamin C) as H-donor in oxidoreductases, and biotin (vitamin H) as CO-group transmitter.

In the case of application of effector molecules immobilized to a tip, preferably allosteric enzymes are used according to the present invention, e.g. at the surface of the medium, which in addition to their catalytic center also have binding sites, where the effector molecule can bind reversibly.

Some points are essential for performing the method according to the present invention. Enzyme and substrate, for instance, must be brought in close vicinity to the surface (distance in the range of nm or 10ths of nm) and be positioned relatively towards each other. This can be achieved by immobilizing the enzyme or an effector molecule to a tip.

The enzyme or effector molecule can be immobilized chemically to a bead (glass, latex or other materials) or to the tip of a scanning probe microscope (scanning tunnel microscope, atomic force microscope, SNOM or others). The bead or the tip can be positioned by external forces, e.g. electric, magnetic or optical controlling forces (magnetic or optical traps) or others, or by means of a micropositioning device (Piezo transmitter, microtechnologically manufactured electromechanical transducers and so on) relatively to the sample. Immobilization of the enzyme or the effector molecule can be achieved by covalent attachment, chemical attachment or physical adsorption.

Covalent bonds can e.g. be achieved with silane or thiol chemistry, chemical attachments could employ specific biological interactions, like the ligand-receptor interactions (e.g. biotin-streptavidin, antibody-antigen and the like). Physical immobilization can apply electrostatic forces, Van-der-Waals forces, hydrophobic forces and the like. Immobilization can also be achieved by combining the above-mentioned methods.
Accordingly, in one embodiment of the method of the present invention, the tip is a micro or nanoparticle and preferably comprises latex, glass, polystyrene or silica beads. Such beads can e.g. be coated with biotin or streptavidin. These beads can be locally positioned by means of so-called optical tweezers. This technique is based on so-called optical traps; see e.g. Ashkin 1970, 1997. A newer type of a 3-D trap (single-beam gradient force trap) was described in Ashkin, 1986.

Optical tweezers are based on the principle that small transparent dielectric objects (with nobject > nenvironment) are transported by a force into the focus of a focused laser beam; see e.g. Hegner, 2002. Dielectric objects can be, as mentioned above, polystyrene microparticles, glass or silica beads, which are in turn supports for biomolecules, i.e. can be enzymes or effector molecules according to the present invention.

The make-up of a single-beam gradient force trap as well as of mechanical accessories like a liquid cell and a mechanically adjustable table are known to the person skilled in the art. The same applies to control programs, e.g. in LabView, to be able to move the Piezo table relatively to the optical trap and to calibrate the optical trap; see e.g. the dissertation "Structure of optical tweezers" (Aufbau einer optischen Pinzette) by Andy Sischka, Physics Faculty, University of Bielefeld, Germany (June 2002).

In another preferred embodiment of the method according to the present invention, the tip is the apex of a scanning probe microscope.

All scanning probe microscopes have in common the need for precise controlling of the distance between tip and sample surface and of the lateral screen movement with a resolution in nanometer range and considerably higher. This precision of movement can be realized by Piezo elements. These consist of Piezo ceramics having the feature of contracting or elongating when voltage is applied.

Scanning probe microscopes are used in many cases for local modification of surfaces. The probably best-known cases are the scratching or hammering of holes in a soft polymeric film by means of atomic force microscopy (Wendel, Lorenz et al., 1995), dip pen technology (Piner, Zhu et al., 1999), where an ink adsorbed to a tip is slowly released on a sample, or the local electrooxidation of a conductive sample by the current of electrons in a scanning tunnel microscope.
In a more recent publication experiments were described, where an enzyme fixed to an atomic force microscope tip was used for the modification of a suitable surface. Here the specific binding of the substrate molecule to the enzyme molecule was used for removing the substrate from the sample (Takeda, Nakamura et al., 2003).

However, in contrast to the present invention, in these experiments only the specific enzyme-substrate binding was taken advantage of and the substrate (peptide) was brushed off the surface, so to speak. In contrast to this, the method according to the present invention is based on the utilization of the chemical, i.e. enzymatic activity of the enzyme. In other words, the product of the chemical reaction, which the enzyme accelerates, is utilized to modify the target surface.

Another significant difference to the present invention lies in the fact that with the force microscope used by Takeda et al. no specific coating of the apex surface with enzyme occurred and no sufficient local restriction could be achieved due to the multiplicity of attached enzyme molecules. Furthermore, the authors did neither point out the necessity of a local restriction of the enzymatic activity, nor did they recommend any measures as to how to achieve it.

Scanning probe microscopes which can be used for the method according to the present invention comprise the scanning tunnel microscope (STM), the scanning nearfield optical microscope (SNOM) and particularly preferred the atomic force microscope (AFM), which was used for the experiments described in the examples. With the AFM, a tip scans the atomic ridges of the sample object to be examined. The probe consists of a tip formed by few atoms (in the ideal case there is only one atom at the tip), which is mounted to a movable thin "beam". If this fine tip is moved towards the surface, at first a Lennard-Jones potential is at work between the foremost atom of the tip and the surface atom, i.e. dependent on the distance, at a larger distance at first an attractive force, then a repulsive force acts on the tip. The force of the tip is held constant while scanning the surface. The thus changed inflection of the "beam" is registered with a sensor. An atomic force microscope can be used as sensor, though usually a deflected laser beam will be applied. Scanning probe microscopes are known to the person skilled in the art. Recent developments in this field are e.g. described in the European patent application EP 0 896 201 A1.
As shown in the illustrations of Figure 2, the substrate molecule chemically modified by the enzyme can be present at the surface or in the surrounding medium. In one embodiment of the method according to the present invention the substrate of the enzyme is immobilized on the surface. In an alternative embodiment the substrate is present in a medium on the surface. However, it is also possible to combine both embodiments; with varying priority factors, if necessary.

Usually the substrate molecule is present in the surrounding medium to guarantee for a constant amount of substrate available. For the removal of material from the surface, the substrate is preferably presented on the sample and the enzyme can cleave off parts of the substrate molecule (e.g. lysozyme can cleave off parts of a fixed polysaccharide). Other embodiments are also acceptable, however. For example glucose oxidase can locally produce hydrogen peroxide, which then chemically modifies a suitable surface.

As mentioned above, the enzyme in the method according to the present invention can be present in a medium on the surface, particularly when the effector molecule is bound on the tip.

In a preferred embodiment of the method according to the present invention, the enzyme is attached to the apex of the tip, see also the examples.

As already mentioned above, it is important to restrict the enzymatic activity locally and, if necessary, temporally. The activity of the enzyme molecule can e.g. be switched on and off by an external control. In this embodiment it is also possible, even though less preferred, that the entire tip, e.g. the entire atomic force microscope tip, is coated with enzyme or effector molecules, wherein the activity of the enzyme at the surface is controlled by an external signal. A suitable external signal can be mediated by e.g. an electric or magnetic field, surface charge, pH changes, ion gradients or an optical signal; see also the above explanations concerning optical tweezers.

Therefore, any signal is suitable which facilitates that only enzyme molecules in the immediate vicinity of the sample to be modified are active. A possible approach is the creation of these activating conditions only in the vicinity of the surface. For example, a photoactivatable enzyme molecule can be activated by the evanescent field of a total internal light reflection at the sample/medium border. In another embodiment, a surface pH value
different from the pH value in the medium can be created by local surface charges (or surface potential). As most enzymes have a pH-dependent activity, this method can be adapted in such a way that the enzyme is only active in the vicinity of the sample surface.

An easy way to achieve switching on and off the activity of the enzyme is e.g. dependent on the availability and non-availability of the substrate. This can easily be achieved by a substrate solved in the medium, which can be substituted by a medium without substrate. However, the activity of the enzyme can also be modified by external stimuli (electric or magnetic fields, irradiation with light or electromagnetic waves, temperature, pH value).

The same applies to the activity of the substrate or to molecular groups, which are to be modified on the surface. The use of temporarily inactive compounds that are activated by irradiation with light (e.g. "caged compounds" like caged ATP, caged CA etc.) is a standardized procedure to make the substrate available after an external stimulus.

Alternatively or additionally, the local activity of the enzyme in the vicinity of the surface can be achieved by coating the tip in only one particular place, in case of the atomic force microscope tip only at the foremost end, with active enzyme or effector molecules; see e.g. Figure 1. Accordingly, it is preferred that only the outer edge of the apex of the tip is equipped with enzyme or effector molecules. Particularly preferred, the apex of the tip carries only one enzyme or effector molecule.

As already mentioned above, the structuring according to the present invention, i.e. modification of a surface, is based on the enzymatic activity of an enzyme, i.e. an enzyme molecule transforms a substrate molecule S to form a product molecule P. In order to render this chemical reaction for surface modification usable, several concepts are conceivable; see the illustrations in Figure 2, by way of example.

In one embodiment of the method according to the present invention, the modification of the surface results from the precipitation of a product of the enzymatic reaction, while in another embodiment the surface modification results from the modification of the substrate on or within the surface.

It is apparent that further reaction schemes can be applied according to the present invention. The common basic idea is that a local enzymatic reaction modifies in its vicinity a sample
surface by inserting molecules or by removing or modifying molecules on the sample surface. This can be performed directly by the enzyme or indirectly by the product of the enzymatic reaction. The enzyme or the active product may need additional co-enzymes or auxiliary molecules for the completion of this task.

Basically, all types of enzymes, like oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases can be used for the method according to the present invention. In one embodiment of the method according to the present invention, the enzyme is selected from the group consisting of oxidoreductases, glucoseoxidases, transferases, kinases, hydrolases, carboxylic acid ester hydrolases, lipases, ribonucleases, hyaluronidases, invertases, amylases, beta-galactosidases, proteinases and polymerases. Particularly preferred, the enzyme alkaline phosphatase, glucoseoxidase, DNA or RNA-polymerase, or a catalytic antibody is used.

The substrates and, under certain circumstances, necessary co-factors of enzymes are known to the person skilled in the art and e.g. comprise nucleotides, amino acids or precursors of dyes, especially in the case of metabolizing enzymes, i.e. product-"building" enzymes. In the case of anabolic reactions, the substrate can comprise e.g. nucleic acids, (poly)peptides, sugars or dyes.

By way of example, the following enzymatic reactions shall be mentioned:

The surface is coated with Phospholipid. The enzyme phospholipase cleaves the phospholipid into two separate molecules that become soluble and are removed from the surface.

The enzyme alkaline phosphatase dephosphorylates p-nitrophenylphosphates and the product binds to a charged surface due to electrostatic interactions.

The enzyme glucoseoxidase oxidizes glucose and produces hydrogen peroxide. The hydrogen peroxide oxidizes suitable molecules on the surface.

The enzyme alkaline phosphatase dephosphorylates the substrate BCIP/NBT, the product becomes insoluble and precipitates on a suitable surface; see also the examples.

As described above, the enzyme molecule can be bound chemically by the toolkits available in biotechnology. For instance, in the following example a phosphatase molecule was used, to which a streptavidin molecule was covalently attached. Streptavidin has four binding sites for biotin. The atomic force microscope tip was covalently coated with biotinilated silane. After a method according to the present invention, the dipping of the atomic force microscope tip into
a streptavidin-enzyme-complex solution led to the coating of the atomic force microscope tip at only its apex with enzyme molecules; see Example 1 and Figure 1.

The present invention also relates to supports that are obtainable according to one of the above-described methods. As described above and shown in the examples, the method according to the present invention for the first time enables the manufacturing of supports and, respectively, which are exactly structured in a size range of nanometers.

Furthermore, the present invention relates to devices containing the supports according to the present invention. Corresponding devices comprise chips, microarrays, diagnostic devices, biosensors or nanoreactors. The supports according to the present invention or their surfaces, respectively, can of course be further modified depending on the intended application, e.g. installed into casings and/or loaded with molecules, e.g. biomolecules, by means of conventional methods.

In a preferred embodiment, the device comprises a DNA or protein chip. So-called gene chips can e.g. be used for forensic applications. Furthermore, it is possible with the method according to the present invention e.g. to manufacture so-called PISAs (protein-in-situ arrays), which can nowadays only be used in the conventional size range of multiwells.

As already described above, a particularly preferred embodiment of the method according to the present invention makes use of a tip, which has only a small controlled area, e.g. only the foremost end of a atomic force microscope tip or the tangential area of a bead, coated with enzyme or effector molecules in order to achieve a resolution in the range of nanometers. This cannot be achieved by standard techniques like pipetting or masking and the like.

According to the present invention, a new method was found to collect enzymes or effector molecules off a flat support by bringing a tip in contact with this support; see also Example 1. In a second step the tip is then moved towards the sample surface to be modified.

In a further aspect, the present invention therefore relates to a method for the specific coating of a tip with enzyme or effector molecules, comprising bringing the tip in contact with a flat support, on which enzymes or effector molecules, by means of a polyvalent binding partner
A, are bound to a binding partner B on the support and wherein at least a part of the tip has substantially the same binding partners B. Basically, any tip or probe like already described above can be used.

It is particularly preferred in the method according to the present invention that only the distal end of the apex of the tip is coated. Here it is of course especially preferred that the tip is covered with only one enzyme or effector molecule.

Usually binding partners A with at least two free binding sites each for binding partner B are used in the method according to the present invention. Typical binding partners are streptavidin and biotin or antibodies or fragments of antibodies as well as corresponding antigens.

The present invention also relates to tips that are obtainable according to one of the above-described methods, preferably tips of scanning probe microscopes. Accordingly, the present invention also relates to scanning probe microscopes containing a tip according to the present invention. Tips according to the present invention, in particular tips having only one enzyme or effector molecule, can for instance also be applied in cell biology, e.g. for examining the activity of enzymes or other molecules on the membrane of cells.

As already evident from the prior art, a multiplicity of possible applications emerge for the methods, supports or surfaces, respectively, devices, tips and scanning probe microscopes according to the present invention, e.g. for the manufacturing of nanochips or microarrays, to mention only two.

Diagnostics, choice and control of therapy for tumor and metabolic diseases; examination of the genetic predisposition (SNPs), particularly the detection of mutations, also in the forensic field; gene expression control; sequencing, particularly sequencing by hybridizing; microbiological applications like in bacteriology and virology, e.g. for the differentiation of different strains; ELISA applications with immobilized antibodies, antigens, receptors and ligands in clinical chemistry, food chemistry and environmental analytics; allergy diagnostics with immobilized allergens; high-throughput screening of substance libraries; information and communication technology and the like.
The methods, supports or surfaces, respectively, devices, tips and scanning probe microscopes according to the present invention can particularly be used for storage and/or analysis of data, cell manipulation or for performing measuring or assay methods.

Furthermore, most recent techniques are applied, like bioMEMS (see e.g. "Fundamentals of Microfabrication: The Science of Miniaturization", Second Edition, Marc J Madou, University of California, Irvine, California, USA, (2002) ISBN: 0849308267), μTAS (see e.g. the "μTAS Virtual Journal", which is available in electronic form exclusively and deals with micro-total-analysis systems (μTAS) and can be visited on the internet page of the ELSEVIER publishing house).

The disclosure content of the above-cited prior art documents is hereby incorporated in this application by reference, particularly relating to the sources concerning scanning probe microscopy and sensor systems. These and other embodiments are apparent and obvious to the person skilled in the art and comprised by the description of the present invention.

Further literature on one of the above-mentioned materials and devices, which can be used in the sense of the present invention, can be taken from the prior art, e.g. from public libraries using e.g. electronic tools. Furthermore, there are public databases, like "Medline", which are accessible on the internet.

The present invention is illustrated in the following examples. The examples and figures are meant to help understanding the invention; however, they are not meant to restrict the invention, as it is recited in the following claims, in any way and must not be understood in any such way.

**EXAMPLES**

**Example 1: Specific coating of a tip of an atomic force microscope.**

For the exclusive coating of only the apex end of the atomic force microscope tip we have prepared a flat sample, which was likewise coated with biotinilated silane. We added enzyme to this sample, so that the sample was coated. When bringing the biotinilated atomic force microscope tip in contact with this sample, the biotin groups at the apex of the atomic force microscope tip can also bind to streptavidin molecules due to the polyvalence of streptavidin.
On account of geometrical restrictions it is actually conceivable that streptavidin binds to maximally two biotin groups at the surface and consequently the two other binding sites must be directed towards the atomic force microscope tip. The immobilization of streptavidinized proteins at the apex of a scanning probe tip and the preparation of a suitable surface can e.g. be performed as follows:

Cleaning
1. Cleaning of the surface of a piece of an oxidized Silicon Wafer (CrysTec S3012) in a mixture of concentrated sulfuric acid (Riedel de Haen – C8029) and 30 % hydrogen peroxide solution (Merck 8.22287.1000) (ratio 3:1) in an ultrasonic bath for 15 min.
2. Rinsing of the wafer with deionized water (MilliPore quality).
3. Irradiation of an atomic force microscope tip (Veeco Nanoprobe NP-STT) with UV light for the cracking of organic dirt on the surface.

Silanization
4. Preparation of a mixture of:
   - 9 ml methanol p.A. (Riedel de Haen – 32213)
   - 80 μl (microliters) concentrated acetic acid (Fluka 45731)
   - 370 μl deionized water
   - 370 μl N-2-aminoethyl-3-aminopropyltrimethoxysilane (Merck 8.19172.0100)
5. Insertion of the wafer and the AFM tip into this mixture for 30 min.
6. Withdrawal and double washing of the wafer and the AFM tip in methanol.
7. Drying of the wafer and the AFM tip with nitrogen gas.
8. Heating of the wafer and the AFM tip in a heater at 120 °C for 3 min.

Applying on the biotin linker
9. Preparation of a solution of:
   - 4 ml dimethyl sulfoxide (DMSO) (Fluka 41640)
   - 1 μg (microgrammes) biotin-N-hydroxysuccinimide ester (NHS-biotin) (Sigma H1759)
10. Insertion of the wafer and the AFM tip into this mixture for 2 h.
11. Withdrawal and double washing of the wafer and the AFM tip in ethanol (Riedel de Haen 32205)
12. Drying of the wafer and the AFM tip with nitrogen gas.

**Immobilization of streptavidinized alkaline phosphatases of the wafer surface**

13. The biotinilated wafer is incubated in a 0.2 nM solution of alkaline phosphatase (Sigma S2890) for 10 min.
14. Rinsing with deionized water.
15. Incubation in a 5 mM solution of p-nitrophenylphosphate (pNPP) (Sigma N4665) in 40 mM TRIS buffer p.A. (Roth 4855.2) and 1 mM magnesium chloride (Sigma M2670) for 10 min on a stirring table.
16. Rinsing with deionized water.

**Immobilization of streptavidinized alkaline phosphatases on the AFM tip**

17. Installation of the biotinillized AFM tip and the prepared wafer in an atomic force microscope.
18. Approaching of the AFM tip to the surface of the wafer.
19. Slow movement of the AFM tip across the surface (~100 nm/s) over a distance of ~50 μm. Hereby single streptavidinized alkaline phosphatases bind to the AFM tip.

Since the same streptavidin molecule is now attached to the support and the tip, it is probable that when withdrawing the tip streptavidin will remain attached to the tip. Since the enzyme is covalently attached to streptavidin, we will thus collect active enzyme on our atomic force microscope tip. Due to the size of the molecules and the spaces involved in this scheme it is possible to coat only the very apex of the atomic force microscope tip. We can estimate that maximally the foremost 10 nm of the atomic force microscope tip might be coated.

**Example 2: Structuring of a surface with the atomic force microscope tip of Example 1.**

For the generation of the structures shown in Figures 3B and 3C the AFM tip functionalized according to the above-described procedure was installed in an AFM (Asylum Research MFP 3D). Mica (Plano GmbH, Wetzlar) was used as surface to be structured.
40 mM TRIS buffer (Roth 4855.2) with 1 mM magnesium chloride (Sigma M2670), which is mixed in a 1:1 ratio with the substrate BCIP/NBT (Sigma 6404) was used as surrounding medium.

The alkaline phosphatase (Sigma S2890) immobilized at the tip herein converts the solved substrate in a way to render it insoluble. For the generation of the spot structure (Fig. 3C) the AFM tip was brought in contact for each spot with the glimmer surface for 20 s. After "writing" the same area was depicted in tapping mode.

For the generation of the coherent L-shaped structure (Fig. 3B) the AFM tip was brought in contact and moved at a speed of 10 nm/s. This area was subsequently also depicted in tapping mode.
Literature


Claims

1. Lithographic method for the structuring of a surface of a support, wherein the surface is chemically or physically modified due to a locally restricted enzymatic activity in the immediate vicinity of the surface.

2. Method according to claim 1, wherein an enzyme or effector molecule immobilized to a tip is positioned in the immediate vicinity of the surface.

3. Method according to claim 2, wherein the tip is a micro or nanoparticle.

4. Method according to claim 3, wherein the micro or nanoparticle comprises latex, glass or silica beads.

5. Method according to claim 2, wherein the tip is the apex of a scanning probe microscope.

6. Method according to claim 5, wherein the scanning probe microscope is a scanning tunnel microscope (STM) or an atomic force microscope (AFM).

7. Method according to any one of claims 1 to 6, wherein the substrate of the enzyme is immobilized on the surface.

8. Method according to any one of claims 1 to 6, wherein the substrate is present in a medium on the surface.

9. Method according to any one of claims 1 to 8, wherein the enzyme is present in a medium on the surface.

10. Method according to any one of claims 1 to 8, wherein the enzyme is attached on the apex of the tip.

11. Method according to any one of claims 1 to 9, wherein the effector molecule is a cofactor, a substrate, an enzyme or a catalyst.
12. Method according to any one of claims 1 to 11, wherein the activity of the enzyme at the surface is controlled by an external signal.

13. Method according to claim 12, wherein the external signal is mediated by an electric or magnetic field, surface charge, pH changes, ion gradients or an optical signal.

14. Method according to any one of claims 1 to 13, wherein only the outermost end of the apex of the tip is equipped with enzyme or effector molecules.

15. Method according to claim 14, wherein the apex of the tip carries only one enzyme or effector molecule.

16. Method according to any one of claims 1 to 15, wherein the modification of the surface results from the precipitation of a product of the enzymatic reaction.

17. Method according to any one of claims 1 to 15, wherein the modification of the surface results from the modification of the substrate on or within the surface.

18. Method according to any one of claims 1 to 17, wherein the enzyme is selected from the group consisting of oxidoreductases, glucoseoxidases, transferases, kinases, hydrolases, carboxylic acid ester hydrolases, lipases, ribonucleases, hyaluronidases, invertases, amylases, beta-galactosidases, proteinases and polymerases.

19. Method according to any one of claims 1 to 18, wherein the enzyme is alkaline phosphatase, glucoseoxidase, DNA or RNA-polymerase or a catalytic antibody.

20. Method according to any one of claims 1 to 19, wherein the substrate comprises nucleotides, amino acids or preliminary stages of dyes.

21. Method according to any one of claims 1 to 19, wherein the substrate comprises nucleic acids, (poly)peptides, sugars or dyes.

22. Support, obtainable according to a method of claims 1 to 21.
23. Device containing a support of claim 22.

24. Device according to claim 23, which is a chip, microarray, diagnostic device, biosensor or nanoreactor.

25. Chip according to claim 24, which is a DNA or protein chip.

26. Method for the specific coating of a tip with enzyme or effector molecules, wherein the tip is brought into contact with a flat support, on which enzymes or effector molecules are bound by means of a polyvalent binding partner A to a binding partner B on the support and wherein at least a part of the tip has substantially the same binding partners B.

27. Method according to claim 26, wherein the tip is a tip as in any one of claims 3 to 6.

28. Method according to claim 26 or 27, wherein only the distal end of the apex of the tip is coated.

29. Method according to any one of claims 26 to 28, wherein each binding partner A has at least two free binding sites for binding partner B.

30. Method according to any one of claims 26 to 29, wherein binding partner A is streptavidin and binding partner B is biotin.

31. Method according to any one of claims 26 to 30, wherein the tip is equipped with only one enzyme or effector molecule.

32. Tip, particularly probe, obtainable by a method according to any one of claims 26 to 31.

33. Scanning probe microscope comprising a tip according to claim 32.

34. Use of a method according to any one of claims 1 to 21, of a support according to claim 22, of a device according to any one of claims 23 to 25, of a tip according to claim 32 or
of a scanning probe microscope according to claim 33 for the manufacturing of nanochips or microarrays.

35. Use of a method according to any one of claims 1 to 21, of a support according to claim 22, of a device according to any one of claims 23 to 25, of a tip according to claim 32 or of a scanning probe microscope according to claim 33 for the storage and/or analysis of data, cell manipulation or for the conduction of measuring of examination methods.
Fig. 1
Fig. 2
Fig. 2 (continued)
Fig. 2 (continued)
A

BCIP + Phosphatase $\rightarrow$ Indigo Dye

NBT $\rightarrow$ Formazan

B

Fig. 3
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

| IPC | 7 | B82B3/00 | G12B21/02 | G03F7/20 |

According to International Patent Classification (IPC) or to both national classification and IPC.

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols):

| IPC | 7 | B82B | G12B | G03F |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched:

Electronic data base consulted during the international search (name of data base and, where practical, search terms used):

EPO-Internal, WPI Data, PAJ, INSPEC, COMPENDEX, BIOSIS, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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**X** Further documents are listed in the continuation of box C.

**X** Patent family members are listed in annex.

* Special categories of cited documents:

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  *S* document member of the same patent family

**Date of the actual completion of the international search**

4 May 2005

**Date of mailing of the international search report**

17/05/2005

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