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(54) **NANOARRAYS OF BIOLOGICAL PARTICLES, METHODS FOR THE FABRICATION THEREOF**

NANOARRAYS AUS BIOLOGISCHEN TEILCHEN SOWIE VERFAHREN ZU IHRER HERSTELLUNG

NANORÉSEAUX DE PARTICULES BIOLOGIQUES, PROCÉDÉ POUR LA FABRICATION DESDITS NANORÉSEAUX

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(73) Proprietor: **Northwestern University**
Evanston, IL 60208 (US)

(72) Inventors:
- **MIRKIN, Chad, A.**
  Wilmette, IL 60901 (US)
- **VEGA, Rafael, A.**
  Evanston, IL 60201 (US)
- **MASPOCH, Daniel**
  Evanston, IL 60201 (US)
- **SALAITA, Khalid**
  Evanston, IL 60201 (US)

(74) Representative: **Lahrtz, Fritz**
Isenbruck Bösl Hörschler LLP
Patentanwälte
Postfach 86 06 80
81635 München (DE)

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- **VEGA RAFAEL A ET AL: "Functional antibody arrays through metal ion-affinity templates" CHEMBIOCHEM, vol. 7, no. 11, 8 August 2006 (2006-08-08), pages 1653-1657,1632, XP002470256 ISSN: 1439-4227**
- **SALAITA KHALID ET AL: "Sub-100 nm, centimeter-scale, parallel dip-pen nanolithography." SMALL (WEINHEIM AN DER BERGSTRASSE, GERMANY) OCT 2005, vol. 1, no. 10, 1 August 2005 (2005-08-01), pages 940-945, XP002470257 ISSN: 1613-6829**

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Description

BACKGROUND

[0001] Microarray technology has led to significant advances in many areas of medical and biological research, [1] opening up avenues for the combinatorial screening and identification of single nucleotide polymorphisms (SNPs),[2] high sensitivity expression profiling of proteins,[3,4] and high throughput analysis of protein function.[5] However, current microarraying technologies, such as spotting with pin arrays, inkjet printing or methods derived from photolithography, are limited in their practical resolution - from hundreds to tens of microns, depending on the technique. The density of the fabricated arrays and therefore the number of distinct deposited biological entities (which include but are not limited to proteins, nucleic acids, carbohydrates, lipids, and especially complex or assemblies thereof, such as viruses or cell components) are therefore limited. There is therefore a need for a method capable of few-microns and sub-micron-scale arraying of said biological entities.

[0002] In addition, the dimensions of each site in currently fabricated microarrays are typically much larger than the size of individual biological molecules or assemblies being deposited. Therefore, large numbers of said entities are present in each site and only the statistical, collective behavior of these ensembles can be studied. Isolated biological particles have been deposited at random on solid substrates by e.g. contacting a very dilute solution of said biological particles with a solid substrate with carefully selected surface chemistry during a carefully selected amount of time, optionally followed by rinsing steps. This method offers little if any control over the density and placement of said particles. There is therefore a need for a miniaturization method - down to the nanometer length scale - with the potential to "site-isolate" nano- and microscale biological entities at the single-particle level with precise positioning. With such method, new opportunities will be available to the biochemical and biomedical research communities to begin to study such entities individually rather than collectively. Site-isolation is of commercial interest, e.g. in pharmaceutical R&D during drug discovery - especially to rapidly elucidate the fundamental mechanism of interaction between a drug candidate and its target - without need for difficult and time-consuming techniques like crystallization and X-ray analysis. Other commercially relevant single particle biology experiments include, for example, studying (a) the effect of the relative orientation between biological entities on their interactions; (b) the cooperative behavior of a selected number of pathogens infecting simultaneously e.g. a cell; (c) the binding of a single antibody with an antigen or conversely the cooperative behavior of multiple antibodies towards an antigen; (d) variations in the interaction of a target drug with individual forms of a polymorphic protein or that of various members of the same protein family; and (e) the effect of genetic variations among viruses in an array on their interaction with another biological item.

[0003] Prior to this invention, advances have been made in immobilizing virus particles on templates created by DPN and micro-contact printing.[11,12] However, a need exists to provide the ability to chemically control the position of the immobilized virus structures at the single-particle level. This is in part because of limited resolution (vide supra), the size of the particles interrogated, and especially the chemistry used to immobilize them. Indeed, prior efforts have focused on the genetic modification of a virus particle to present unnatural surface binding functionality to the patterned interface.[11,12] For reasons of cost and scalability, it is preferable to avoid manipulation of said viruses. There is therefore a need for a method to chemically control the position of the immobilized virus structures at the single-particle level without need for chemical or genetic modification of said viruses.

[0004] Vega et al. "Nanoarrays of single virus particles", Angewandte Chemie. International Edition, VCH Verlag, Weinheim, DE, vol. 44, no.37, 22 August 2005, pages 6013-6015, describes an approach for the immobilization of TMV virus particles on surfaces. Through the use of DPN and small features, the virus particles can be isolated and the orientation thereof be controlled.

[0005] US 2003/0068446 A1 describes the use of DPN to construct high density peptide and protein nanoarrays. The method can be carried out with a variety of peptide and protein structures, including enzymes and antibodies.

[0006] Rozhok et al. "Methods for Fabricating Microarrays of Motile Bacteria", small, Wiley-VCH Verlag GmbH & Co KGaA, Weinheim, DE, April 2005, pages 445-451 describes fabrication of single cell microarrays where single cells are attached to predesigned line or dot features.

[0007] No admission is made that any of the references cited in this Background section or thereafter are prior art.

SUMMARY OF THE INVENTION

[0008] The invention generally relates to arrays of biological entities and in particular arrays of site-isolated biological entities. It further relates to (a) the use of direct-write nanolithographic printing for the fabrication thereof; and (b) methods of use of these arrays.

[0009] The present invention in some embodiments provides arrays of biological particles, in which at least one of the lateral dimensions of the sites is in the few-microns or sub-micron range (including nanoarrays). The invention also discloses organized, engineered arrays of site-isolated biological particles and especially arrays of site-isolated virus particles.

[0010] The invention discloses arrays and methods for the fabrication of said arrays.

[0011] A versatile coordination, metal-ion based chemistry based approach is described for immobilizing TMV virus particles on surfaces and it is shown that

through the use of DPN and small features, it is possible to isolate and control the orientation of these virus particles. Many virus particles have Zn^{2+} and other metal-binding groups in their protein coats.[18] Therefore, this approach can be generalized for manipulating many classes of virus structures at the single-particle level. Such capabilities will expand the scope of application for virus structures in fields ranging from biology to molecular electronics,[19] where such control opens new opportunities for research that cannot be addressed with microarrays or bulk systems.

BRIEF DESCRIPTION OF DRAWINGS

[0012]

Figure 1. Schematic diagram describing the selective immobilization of single virus particles on DPN-generated MHA nanotemplates treated with a solution of $Zn(NO_3)_2 \cdot 6H_2O$. The diagram is not to scale.

Figure 2. Schematic diagram describing (a) the Tobacco Mosaic Virus (TMV) external surface, which presents a high number of carboxylic/carboxylate groups from glutamate and aspartate amino acids. TMV is deprotonated at pH 3.5; (b) the sandwich formed by the carboxylate groups of the mercaptohexadecanoic acid self-assembled monolayer pattern, on one hand, the Zinc cations and the carboxylate groups present on the TMV virus, on the other hand. Without wishing to be bound by theory, it is believed that the electrostatic bridge formed by the positively charged zinc ions and the negatively charged carboxylates is responsible for the immobilization of said TMV virus on said MHA pattern. The diagram is not to scale.

Figure 3. AFM tapping mode (using a silicon cantilever, spring constant = ~40 N/m) images and height profiles of TMV nanoarrays. (a) Three-dimensional topographical images of pairs of virus particles within larger arrays: (left) a parallel array, (middle) a perpendicular array, and (right) dot arrays. (b) Topography images and height profiles of a perpendicular array of single virus particles (40 x 40 μm). (c) Topography image and height profiles of a TMV nanoarray (20 x 20 μm) formed on an array of 350 nm MHA dot features pretreated with $Zn(NO_3)_2.6H_2O$. All images were taken at a scan rate of 0.5 Hz.

Figure 4. (a) PM-IRRAS spectra of a monolayer of MHA on Au (bottom spectrum), after treatment with $Zn(NO_3)_2 \cdot 6H_2O$ (middle spectrum), and then incubation with TMV (top spectrum). (b) Topography image and height profile of an MHA array treated with the antiserum against TMV. The antibodies are electrostatically attached to the MHA features. Topography images and height profiles of a pair of parallel single virus particles before (c) and after (d) treatment with a PBS solution containing the antiserum against TMV. All AFM images were taken at a 0.5 Hz scan rate in tapping mode.

Figure 5. AFM tapping mode (silicon cantilever, spring constant = about 40 N/m) 2D and 3D image of a TMV nanoarray formed on an MHA template consisting of 600 nm x 200 nm rectangular features. More than one oriented virus is observed on each feature. The image was taken at a scan rate of 0.5 Hz.

Figure 6. AFM tapping mode (silicon cantilever, spring constant = about 40 N/m) image and height profile of a bulk gold thin film sample (7.5 cm x 2.5 cm) containing a monolayer of TMV on a Zn^{2+}-MHA modified surface. This image was taken at a scan rate of 0.5 Hz.

Figure 7. Topography images from a tapping mode AFM (Vecco) of site-isolated TMV before and after (a, b respectively) incubation with a solution of antiserum for 30 min. at 37°C. The observed increase in height shows that antibodies have bound to the TMV and therefore that they are biologically active after immobilization.

DETAILED DESCRIPTIONS OF THE PREFERRED EMBODIMENTS

INTRODUCTION

[0013] References will be made in detail to the preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings. While the invention will be described in conjunction with the preferred embodiments, it will be understood that they are not intended to limit the invention to those embodiments.
[0014] Priority provisional application serial no. 60/712,432 filed August 31, 2005 to Mirkin et al. is cited.
[0015] The following reference is made: "Nanoarrays of Single Virus Particles", Rafael A. Vega, Daniel Maspoch, Khalid Salaita, and Chad A. Mirkin, Angew. Chem. Int. Ed. 2005, 44, 2-4, 6013-6015.

ARRAYS

[0016] The present invention discloses arrays of biological particles (such as cells and cellular components, bacterial elements, spores, viruses, and other assemblies and complex of proteins, including antibodies, nucleic acids, lipids and carbohydrates), in which at least one of the lateral dimensions of the sites is in the few-microns or sub-micron range (including nanoarrays). In a preferred embodiment, the invention discloses organized, engineered arrays of site-isolated biological particles and especially arrays of site-isolated virus particles. The arrays may comprise 100 or more, or preferably 1000

or more or preferably 10,000 or more biological entities or preferably 100,000 or more biological entities. Said biological entities may be identical or not.

VIRUS

[0017] Virus arrays are a preferred embodiment and working examples are shown thereafter that demonstrate TMV arrays. However, the invention is not limited to this or any other plant virus. Animal and human virus may also be utilized, including HIV, respiratory track and flu viruses.

[0018] Viruses can be used which are generally known for those of ordinary skill in the art, see e.g. Cann, A.J., Principles of Modern Virology, Academic Press, 1993; Chiu, W., Burnett, R.M., and Garcea, R.L. (Eds), StructuralBiology of Viruses, Oxford University Press, 1997. See also R.C. Bohinski, Modern Concepts in BioChemistry, 4th Ed., 1983.

[0019] For example, a virus used herein can be a particle that can infect a cell of a biological organism. An individual virus, or a virus particle, also can be called a virion, can comprise one or more nucleic acid molecules, so called viral genome, surrounded by a protective protein coat known as a-capsid. Unlike cellular organisms, in which the nucleic acid molecules are generally made up of DNA, the viral nucleic acid molecule may comprise either DNA or RNA. In some cases, viral nuclear acid molecules comprise both DNA and RNA. Viral DNA is usually double-stranded, either a circular or a linear arrangement, while viral RNA is usually single-stranded. However, examples of single stranded viral DNA and double-stranded viral RNA are also known. Viral RNA may be either segmented (with different genes on different RNA molecules) or nonsegmented (with all genes on a single piece of RNA). The size of the viral genome can vary significantly in size. Both DNA and RNA viruses can be used herein.

[0020] In viruses used herein, the viral capsid can comprise repeating units of one or a few different proteins coded by the viral genome. These units are called protomers or capsomers. These proteins that make up the virus particle can be called structural proteins. A number of structural proteins in the viral particle can vary from one (e.g, Satellite tobacco Necrosis Virus) to 377 (Paramecium bursaria Chlorella virus).

[0021] Viruses used herein can have a variety of shapes. For example, the viral capsid can be helical (spiral-shaped) or icosahedral. One example of a virus with a helical viral capsid is tomato mosaic virus, while examples of viruses isosahedral viral capsids include Tomato Bushy Stunt Virus and Simian Virus 40. Some more complex viruses can have a capsid that is neither purely helical, nor purely isosahedral. Some more complex viruses may possess extra structures such as protein tails or a complex outer wall. For example, some bacteriophages, i.e. viruses that can infect bacterial cells, may have a capsid comprising isosohedral head bound to a helical tail, which may also have a hexagonal base plate with many protruding protein fibres.

[0022] Viral capsid and the viral genome contained therein can be together referred to as nucleocapsid. Some virus particles comprise nucleocapsids, while others contain additional structures. For example, some viruses can be enclosed in a lipid envelope acquired when the virus buds through host-cell membranes. One or more glycoproteins that bind virus particles to susceptible host cells can be inserted into this envelope. The glycoproteins can be coded by both the viral genome and the host cell genome, while the envelope's lipids and any carbohydrates present are entirely host cell's genome coded.

[0023] Viruses can vary in size, as used herein. For example, a diameter of the viral capsid can be from about 10 nm to about 400 nm, usually about from about 10 nm to about 300 nm. Some virusal capsides can have a significant length to diameter ratio. For example, capsids of some filoviruses can have a length up to 1400 nm and a diameter of only 80 nm.

[0024] Viruses can be classified according to their type of genetic material, their strategy of replication, and their structure. According to their genome and strategy of replication, viruses can be classified as follows:

A) DNA viruses:

Group I: viruses comrising double-stranded DNA. Examples include such virus families as Herpesviridae (herpes viruses) and Poxviridae (chickenpox and smallpox), many tailed bacteriophages, and a virus with the largest known viral genome, mimivirus.

Group II: viruses comprising single-stranded DNA. Examples include such virus families as Parvoviridae and bacteriophage M13.

B) RNA viruses:

Group III: viruses comprising double-stranded RNA genomes. These genomes are segmented.

Group IV: viruses comprising positive-sense single-stranded RNA genomes. Examples include SARS virus, hepatitis C virus, yellow fever virus and rubella virus.

Group V: viruses compring negative-sense single-stranded RNA genomes. Examples include filoviruses such as Ebola and Marburg viruses along with measles, mumps and rabies.

C) Reverse transcribing viruses:

Group VI: viruses that comprise single-stranded RNA genomes and replicate using reverse transcriptase. Examples include retroviruses such HIV virus.

Group VII: viruses that comprise double-stranded DNA genomes and replicate using reverse transcriptase. Example includes hepatitis B virus.

[0025] The virus can have for example an anisotropic shape. For example, the virus can have a tubular shape.

[0026] The virus can comprise an additional moiety bound to the virus.

[0027] Genetically engineered viruses and synthetically modified viruses can be used. See for example Genetically Engineered Viruses, Ed. CJA Ring, E.D. Blair; 2001.

[0028] In many cases, the virus can be used as is or as a wild type without modification.

[0029] In addition to viruses, other pathogens, including cellular parasites like malaria, are possible. Cell organelles (including but not limited to ribosomes, cellular nuclei, and other vesicles and cellular apparatuses) are also possible.

SUBSTRATE SURFACE

[0030] The substrate surface is not particularly limited but can be for example substantially flat. Substrates such as glass or metals can be used. Microfabrication methods can be used to prepare substrates and build up layers and appropriate surfaces. The virus binding sites and the sites which do not bind virus can comprise for example a monolayer. A thin layer of organic compound can form a monolayer over an inorganic substrate.

BINDING SITES

[0031] The binding sites can comprises regions with a plurality of molecular binding sites. The virus binding sites can comprise ionic binding sites. These can be for example multivalent metallic ionic binding sites including di- or tri- or tetravalent binding sites. Metals, metal ions, and metal ion binding is generally described in for example, Advanced Inorganic Chemistry, 4th Ed., Cotton and Wilkinson, 1980.

[0032] An exemplary approach used herein relies on the ability of ions, such as metal ions and divalent metal cations (e.g. Zn^{2+} which is preferred; other ions are possible) to bridge a surface patterned with features that carry an opposite charge, for example the terminal groups of a self-assembled monolayer (e.g. 16-mercaptohexadecanoic acid, MHA) or the external surface of a charged biological entity, such as a carboxylate-rich virus envelope. Other molecules that bind to said substrate and present a suitable functional group (e.g. omega-functionalized alkanethiols or organosilanes) are possible.

[0033] Metal ion binding can be carried out with metals including for example Zn(II), Cu(II), Ni(II), and Co(II). The metal ion is not particularly limited but can be based on for example ionic forms of ruthenium, cobalt, rhodium, rubidium, vanadium, cesium, magnesium, calcium, chromium, molybdenum, aluminum, iridium, nickel, palladium, platinum, iron, copper, titanium, tungsten, silver, gold, zinc, zirconium, cadmium, indium, and tin. Divalent metal cations include Cd^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, and Ni^{2+}.

[0034] Protein and peptide nanoarrays and binding sites are also described in for example US Patent Publication 2003/0068446 to Mirkin et al., which is hereby incorporated by reference.

[0035] In designing metal-based binding sites, principles of IMAC (Immobilized Metal Affinity Chromatography) can be used. See for example US Patent Nos. 5,932,102 and 6,942,802.

[0036] In some embodiments, binding sites are adapted so that the virus maintains its shape despite binding and does not substantially deform.

SITES WHICH DO NOT BIND VIRUS

[0037] Additional parts of the surface can be passivated to prevent and substantially prevent binding of virus. As a result, the sites which do not bind virus can be substantially free of virus. Passivation is generally known as described in for example US Patent Publication 2003/0068446 to Mirkin et al. A monolayer can be coated onto the substrate to provide passivation such as for example an alkane thiol or a linear alkane thiol, or an alkane thiol comprising a terminal group, as well as alkane thiols including a poly- or oligo-alkyleneglycol thiol.

SIZE AND SHAPE OF BINDING SITES FOR SINGLE SITE BINDING

[0038] The substrate binding site and sites can be adapted so that only one virus particle is disposed on each binding site. In an array, an occasional binding site can have more than one virus particle so that for example at least 70%, or at least 80%, or at least 90% of the binding sites have one virus particle. The invention further provides a method to engineer at known locations micro- to nanometer-scale sites, which geometry and dimensions approach that of the targeted biological particles, so that only a selected number of particles may be deposited on each site. Preferably, only about one particle may adsorb on each site. Furthermore, many such sites may be produced in parallel very rapidly, and precise positioning, orientation and inter-particle spacing can be guaranteed.

[0039] For example, the longest lateral dimension of each site may be between 5 microns and 1 μm or between 1 μm and 500 nm or between 500 nm and 300 nm or between 300 nm and 100 nm or inferior to 100 nm or smaller than 50 nm.

[0040] The site may be, for example, substantially a circle, a linear shape, a curvilinear shape, a square, or a rectangle. It can be for example rectangular, square or disk-like.

[0041] Rectangular sites may be between 600 nm x 200 nm and 500 nm x 180 nm, or between 500 nm x 180 nm and 400 nm x 150 nm, or between 400 nm x 150 nm

and 350 nm x 110 nm, in length and width. The shape can be for example a rectangle having a length of about 300 nm to about 600 nm, and a width of about 100 nm to about 200 nm.

[0042] Dot-shaped sites may be between 1000 nm and 500 nm diameter or preferably between 500 nm and 350 nm diameter, or preferably between 350 nm and 100 nm in diameter or preferably smaller than 100 nm in diameter.

[0043] The separation between sites may be between 15 μm and 5 μm, or between 5 μm and 1 μm or between 1 μm and 500 nm or between 500 nm and 100 nm or inferior to 100 nm or smaller than 50 nm.

[0044] The average size of the virus binding sites can provide for example a surface area of less than about 100,000 square nm, or less than about 50,000 square nm, or about 30,000 square nm to about 100,000 square nm, for each site.

LITHOGRAPHY

[0045] Methods of patterning, printing, drawing, writing, microlithography, and nanolithography are known in the art and include direct write lithography and direct-write nanolithography. See for example Direct-Write Technologies for Rapid Prototyping Applications,(Ed. Alberto Pique and D.B. Chrisey), Academic, 2002.

[0046] Examples of lithography and direct write lithography with respect to bioarrays include for example AFM-tip based lithography, DPN printing, microcontact printing, robotic spotting, and the like.

[0047] In one embodiment, the invention improves high resolution direct-write lithographic methods such as Dip Pen Nanolithography (DPN)[6] for miniaturizing biological entity structures to the nanometer length scale [7-9] (DPN™, Dip Pen Nanolithography™, NanoInk™, DPNWrite™, NScriptor™ are trademarks or registered trademarks of NanoInk, Inc., Chicago, IL). See also for example Ginger et al., Angew. Chem. Int. Ed. Engl., 2004, 43, 30-45 as well as US Patent Nos. 6,635,311 to Mirkin et al.; 6,827,979 to Mirkin et al, and 6,887,443 to Liu et al. for additional embodiments for tip based nanolithography including instrumentation, software, inks, and processes, which are hereby incorporated by reference in their entirety. Such massive miniaturization provides the advantages of larger, denser libraries for screening complex chemical and biological systems.

[0048] In a preferred embodiment, the ion-trapping sites may be fabricated using direct-write nanolithographic printing such as DPN printing.

[0049] If desired, ink mixtures comprising multiple inks or dual phase ink systems can be used as described in for example US serial no. 11/480,557 filed July 5, 2006 to Mirkin et al.

METHOD OF MANUFACTURE AND USE

[0050] Additionally, the invention describes a novel technology that utilizes DPN printing in combination with metal ions or coordination chemistry to precisely immobilize and position biological particles in the context of large arrays. In a second embodiment, the method to precisely immobilize at least one biological particle on a substrate without unnatural genetic or chemical modification of said biological particle prior to immobilization comprises the steps of (a) patterning a substrate, forming an array of sites capable of trapping ions, for example metallic cations such as those of Zinc (e.g. Zn^{2+}) or copper^{2+} or nickel^{2+}; (b) optionally passivating the areas of the substrate that were not patterned with at least one compound that prevents ion and biological entity adsorption; (c) selectively trapping said ions on top of said sites e.g., by immersion of said substrate in a solution of said ion; (d) selectively immobilizing said biological particle on said sites, e.g. by immersion of said substrate in a solution of said biological particle.

[0051] In another embodiment, the invention provides a method for immobilizing about a single biological particle per site of the nanometer-scale array, resulting in the production of site-isolated biological entities at known locations, wherein said isolation of biological entities is not the result of random adsorption from a very dilute solution.

[0052] In another embodiment, the invention provides a method for the fabrication of arrays of biological particles, wherein most of the biological particles assume a specific orientation or shape. Preferably, said orientation or shaping is provided by chemical means rather than physical means, e.g. by the interplay of the geometry and surface chemistry of said site and these of the external surface of said biological entity, rather than by the application of an external electrical, magnetic field or use of fluid flow.

PROPERTIES OF ARRAY

[0053] Preferably, the method allows the deposition of said biological particles in such manner that they remain biologically active. Biological activity may be verified, for example, by the binding of antibodies specific to said biological particles onto said particles or by the formation of Watson-Crick pairs with complementary nucleic acid strands present in solution. Properties can be examined by scanning probe methods.

ADDITIONAL EMBODIMENTS

[0054] The invention further discloses methods, kits and instruments for the fabrication and use of said arrays:

[0055] The invention also provides a kit for the fabrication of arrays of site-isolated biological entities that comprises a substrate, at least one probe, a chemical composition, a passivating solution, an ionic solution, and a biological entity solution, wherein the chemical composition is adapted to coat the at least one probe and being deposited on the substrate to form at least one pattern, wherein the passivating solution can passivate unpat-

terned areas of the substrate, wherein immersing the patterned and passivated substrate in the ionic solution afford ions selectively bound to patterned regions, wherein the biological entities may adsorb to ion-treated patterned regions.

[0056] The invention also provides an instrument capable of the fabrication of arrays of site-isolated biological entities, the instrument comprising at least one probe and fluid dispensing means for delivering the solutions of the aforementioned kit in sequence. Multiprobe AFM based lithographic methods can be used. Activated probes can be used. The instrument may be for example a modified atomic force microscope capable of direct-write nanolithographic printing and the probe may be an AFM cantilever with or without a tip or an SPM probe.

[0057] The invention also provides an instrument and a method for the observation and characterization of the interaction of an isolated biological particle with at least one second biological entity of at least one type.

WORKING EXAMPLES

[0058] Non-limiting working examples are provided.

Working Example: Site-Isolated TMV Arrays:

[0059] In this example, virus particles were site-isolated, positioned and oriented on Zn^{2+}-MHA nanotemplates generated by dip pen nanolithography. Viral immobilization was characterized using antibody-virus recognition as well as infrared spectroscopy.

[0060] The approach used in this working example relied on the ability of metal ions (Zn^{2+}) to bridge a surface patterned with features made of 16-mercaptohexadecanoic acid (MHA) and the TMV with its carboxylate-rich surface.

[0061] Tobacco Mosaic Virus (TMV) was chosen because of its anisotropic tubular structure (about 300 nm long, 18 nm diameter), size, stability, and well-characterized carboxylate-rich surface.[10] It serves as an excellent demonstrative system to evaluate how one can use DPN to control the positioning and orientation of nanoscale virus particles within an extended array.

[0062] Virus nanoarrays were fabricated by initially generating chemical templates of MHA on a gold thin film using DPN (Figure 1). The regions surrounding these features were passivated with a monolayer of 11-mercaptoundecyl-penta(ethyleneglycol) (PEG-SH) by immersing the substrate in an alkanethiol solution (5 mM in ethanol) for 30 minutes followed by copious rinsing with ethanol. The passivation layer minimizes nonspecific binding of the virus particles to the unpatterned areas. The carboxylic acid groups of MHA were coordinated to Zn^{2+} ions by exposing the substrate to an ethanol solution of $Zn(NO_3)_2 \cdot 6H_2O$ (5 mM) for one hour followed by rinsing with ethanol to remove any unbound metal ions from the surface (see also, Figure 2). The metallated substrate was then exposed to a 0.15 M NaCl 10 mM phosphate buffer pH 7 (PBS) solution containing TMV (from American Type Culture Collections) (100 microg/mL) for 24 hours at room temperature in an air-tight humidity chamber. Excess virus particles were removed by washing the substrates with NANOpure water. The cleaned substrates were then dried under a stream of N_2. All virus arrays were characterized by tapping mode AFM (TMAFM), and the chemical identity of the surface-immobilized virus particles was confirmed by treatment with a highly specific antiserum (from American Type Culture Collections) against TMV, which upon binding, increases the height of each virus particle (vide infra).

[0063] A series of DPN patterned linear nanostructures of MHA, which vary in dimensions (length and width: 600 nm x 200 nm, 500 nm x 180 nm, 400 nm x 150 nm and 350 nm x 110 nm) were systematically studied to determine the optimum feature size for single virus particle attachment. Under the conditions studied, MHA templates with feature dimensions of 350 nm x 110 nm, spaced one micrometer apart, proved to be ideal for individual particle assembly. The tendency of each virus to occupy the largest number of coordination sites results in near-perfect alignment of all the virus particles along the long axis of each rectangular template (Figure 3a and 3b). The average height of each feature on the template was 16 $\pm$ 1 nm. Furthermore, each virus particle on the lines was 45 $\pm$ 2 nm wide and 320 $\pm$ 40 nm long, parameters consistent with the presence of only one TMV particle on each MHA feature.[13]

[0064] The dimensions of the features within the array are critical for virus particle site isolation. For example, rectangular templates greater than 500 nm long or 200 nm wide yield multiple yet oriented viruses at each site, thus preventing the formation of a single virus particle array (see Figure 5). Features significantly smaller (less than 300 nm x 100 nm) do not result in uniform assembly of the virus particles with numerous sites remaining unoccupied.

[0065] The chemical templates also can be used to control the assembly of the flexible virus into unnatural conformations such as circles and other curved architectures. For example, 350 nm diameter dot templates can capture multiple virus particles, many of which adhere to the rim of the dot and adopt a curved architecture (Figure 3c). Curved TMV structures have been made before but through mechanical manipulation of the virus.[14] This approach to virus bending is in contrast with the templated approach described in these studies.

[0066] To demonstrate that the virus orientation is not a result of external variables such as washing, drying via flowing N_2 or capillary effects, independent organization of each virus particle was tested by immobilization along two different directions within one array. Indeed, when an MHA template consisting of 350 nm x 110 nm features perpendicular to one another was used, an array of site-isolated single TMV viruses perpendicular to each other was obtained (Figure 3b).

[0067] Polarization modulation-infrared reflection-ad-

sorption spectroscopy (PM-IRRAS) was used to characterize bulk gold thin film substrates modified with TMV using the same metal ion or coordination chemistry approach used to generate the TMV arrays (Figure 4a and Figure 6). The MHA monolayer exhibits two main bands in the high-frequency-CH_2-stretching region at 2856 and 2930 cm^{-1} and two in the C=O stretching region at 1741 and 1718 cm^{-1}, which are attributed to the presence of free and hydrogen-bonded carboxylic groups,[15] respectively. After the substrate was immersed in an ethanolic $Zn(NO_3)2{\cdot}6H_2O$ solution (5mM) for 1 h, the coordination of MHA carboxylic groups to Zn^{2+} metal ions was confirmed by ACO band shifts to lower energy (1602/1556 and 1453 cm-1). The C=O stretching region changes again after exposing the MHA-Zn surface to the TMV solution. Three main bands are detected in this spectral region that can be identified as the amide I band centered at 1661 cm^{-1}, which is characteristic of proteins on TMV,[16] the amide II and asymmetric COO-bands centered at 1546 cm^{-1}, and symmetric COO- band at 1458 cm^{-1}. Also the presence of CH_3 groups, attributed to proteins with methyl groups is confirmed by growth of a new band at 2967 cm^{-1} after incubation with TMV.

[0068] In this working example, the coordination chemistry including the Zn^{2+} coordination chemistry is believed important for the virus particle assembly process. Consistent with this conclusion, in control experiments, TMV will not assemble on MHA coated or patterned substrates (1 μm dot diameter), even after exposure of the template to a PBS solution of the virus for 48 hours.

[0069] To provide further evidence for the chemical identity of the tubular virus structures imaged by AFM, we treated the single virus arrays with a PBS solution of an antiserum against TMV (200 microg/mL, pH 7) at 37°C for 30 minutes, rinsed the substrates with 10 mM PBS solution and then dried them under a stream of N_2. A comparison of the AFM images of the substrate before and after incubation with antibody shows a height increase of approximately 9 nm (Figure 4c and 4d; see also Figure 7). This increase is consistent with the height of the antibody (Figure 4b) and therefore, the presence of TMV particles on the arrays. Antibody arrays were generated by first using DPN to pattern rectangular lines of MHA with feature dimensions of 350 nm x 110 nm. The area around these features was passivated with PEG-SH for 30 minutes, followed by copious rinsing with ethanol to inhibit non-specific binding. Finally the antiserum to TMV was incubated with the MHA passivated substrate at 4°C for 24 hours. For more details see reference [8].

[0070] Note that the expected height increase upon antibody binding was independently modeled and measured using direct adsorption of the antibody onto an MHA array (Figure 4b). This approach has been used to study protein binding events in the context of other protein immobilization experiments. [8]

[0071] In some cases, not all of the binding sites will have a single virus, but substantially all binding sites will have a single virus. In some cases, at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90% of the binding sites will have a single virus. Remaining sites can have more than one virus particle or in some cases no virus particle.

Experimental Section:

[0072] All DPN patterning was done with a ThermoMicroscopes CP AFM interfaced with commercialized lithographic software (DPNwrite™, NanoInk Inc., Chicago, IL) and conventional Si_3N_4 cantilevers (Thermo Microscopes sharpened Microcantilever A, force constant of 0.05 N/m). Tapping mode images were taken with a Nanoscope IIIa and MultiMode microscope from Digital Instruments. Unless noted otherwise, all DPN patterning experiments were conducted at 35% relative humidity and 24°C with a tip-substrate contact force of 0.5 nN. DPN was used to pattern MHA on gold substrate (50 nm Au and 10 nm Cr on a silicon wafer, Silicon Sense, Inc.). PM-IRRAS spectra of 2048 scans at 4 cm-1 resolution were obtained with a Thermo Nicolet, Nexus 870 with Tabletop optics module (TOM). The PM-IRRAS differential reflectance (% $\Delta R/R$) values were converted to absorbance units for comparison with conventional IRRAS data.

[0073] The following references are useful to practice the invention:

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Claims

1. An array comprising: a substrate surface, wherein the substrate surface comprises binding sites for a biological particle and also sites which do not bind the biological particle, **characterized in that** said binding sites comprise metal ion binding sites, or divalent cation metal binding sites, wherein the binding sites on the substrate surface each have a shape and a size; and one biological particle disposed on each of the binding sites, **characterized in that** the biological particle is a protein, an antibody, or a cell.

2. The array according to claim 1, wherein the biological particle assumes a specific orientation.

3. A method of making an array comprising the combination of steps: providing a substrate surface, modifying the substrate surface to provide biological particle binding sites and also sites which do not bind any biological particle, wherein said binding sites comprise metal ion binding sites, or divalent cation metal binding sites, binding the biological particle to the biological particle binding sites so that substantially only one biological particle binds to each site, **characterized in that** the biological particle is a protein, an antibody, or a cell.

4. The method according to claim 3, wherein the biological particle binding sites have a lateral dimension of between 5 μm and 1 μm, or of between 1 μm and 500 nm, or of between 500 nm and 300 nm, or of between 300 nm and 100 nm, or inferior to 100 nm, preferably smaller than 50 nm, or less than about one μm, or provide a surface area of less than about 100,000 square nm for each site.

5. The method according to claim 4, wherein the biological particle binding sites have the shape of a circle, a square, or a rectangle, or wherein the binding sites have a linear or a curvilinear shape.

6. The method according to claim 5, wherein the rectangular sites are between 600 nm x 200 nm and 500 nm x 180 nm, or between 500 nm x 180 nm and 400 nm x 150 nm, or between 400 nm x 150 nm and 350 nm x 110 nm in length and wide.

7. The method according to claim 5, wherein the dot-shaped sites are between 1000 nm and 500 nm in diameter, preferably between 500 nm and 350 nm or between 350 nm and 100 nm in diameter, more preferably smaller than 100 nm in diameter.

Patentansprüche

1. Anordnung, umfassend: eine Substratoberfläche, wobei die Substratoberfläche Bindungsstellen für einen biologischen Partikel umfasst und ebenfalls Stellen, die den biologischen Partikel nicht binden, charakterisiert **dadurch**, dass die Bindungsstellen Metallionen-Bindungsstellen oder Bindungsstellen für zweiwertige Metallkationen umfassen, wobei die Bindungsstellen auf der Substratoberfläche jeweils eine Form und eine Größe besitzen und ein biologischer Partikel auf jeder der Bindungsstellen angeordnet ist, charakterisiert **dadurch**, dass der biologische Partikel ein Protein, ein Antikörper oder eine Zelle ist.

2. Anordnung gemäß Anspruch 1, wobei der biologische Partikel eine spezifische Orientierung einnimmt.

3. Verfahren zum.Herstellen einer Anordnung umfassend die Kombination der Schritte: Bereitstellen einer Substratoberfläche, Modifizieren der Substratoberfläche, um Bindungsstellen für biologische Partikel und ebenfalls Stellen, die keine biologischen Partikel binden, bereitzustellen, wobei die Bindungsstellen Metallionen-Bindungsstellen oder Bindungsstellen für zweiwertige Metallkationen umfassen, Binden des biologischen Partikels an die Bindungsstellen für biologische Partikel, so dass im Wesentlichen nur ein biologischer Partikel an jeder Stelle bindet, charakterisiert **dadurch**, dass der biologische Partikel ein Protein, ein Antikörper oder eine Zelle ist.

4. Verfahren gemäß Anspruch 3, wobei die Bindungsstellen für den biologischen Partikel eine seitliche

Ausdehnung von zwischen 5 μm bis 1 μm oder zwischen 1 μm und 500 nm oder zwischen 500 nm und 300 nm oder zwischen 300 nm und 100 nm oder weniger als 100 nm, vorzugsweise schmaler als 50 nm oder weniger als etwa 1 μm haben oder ein Oberflächenareal von weniger als 100.000 nm^2 für jede Stelle bereitstellen.

5. Verfahren gemäß Anspruch 4, wobei die Bindungsstellen für den biologischen Partikel die Form eines Kreises, eines Quadrates oder eines Rechtecks haben, oder wobei die Bindungsstellen eine lineare oder eine kurvenförmige Form haben.

6. Verfahren gemäß Anspruch 5, wobei die Seiten des Rechtecks zwischen 600 nm x 200 nm und 500 nm x 180 nm oder zwischen 500 nm x 180 nm und 400 nm x 150 nm oder zwischen 400 nm x 150 nm und 350 nm x 150 nm in Länge und Weite sind.

7. Verfahren gemäß Anspruch 5, wobei die punktförmigen Seiten zwischen 1000 nm und 500 nm im Durchmesser, vorzugsweise zwischen 500 nm und 350 nm oder zwischen 350 nm und 100 nm im Durchmesser, noch bevorzugter kleiner als 100 nm im Durchmesser sind.

Revendications

1. Une puce comprenant : un substrat de surface, dans lequel le substrat de surface comprend des sites de liaison pour une particule biologique et également des sites auxquels ne se lie pas la particule biologique, **caractérisé en ce que** lesdits sites de liaison comprennent des sites de liaison d'ion métallique ou des sites de liaison de cation métallique divalent, dans lequel les sites de liaison du substrat de surface ont chacun une forme et une taille ; et une particule biologique déposée sur chacun des sites de liaison, **caractérisée en ce que** la particule biologique est une protéine, un anticorps ou une cellule.

2. La puce selon la revendication 1, dans laquelle la particule biologique prend une orientation spécifique.

3. Une méthode de fabrication d'une puce comprenant la combinaison d'étapes : fournir un substrat de surface, modifier le substrat de surface pour fournir des sites de liaison à une particule biologique et également des sites auxquels ne se lie aucune particule biologique, où lesdits sites de liaison comprennent des sites de liaison d'ion métallique ou des sites de liaison de cation métallique divalent, lier la particule biologique aux sites de liaison de la particule biologique de sorte que substantiellement seulement une particule biologique se lie à chaque site, **caractérisée en ce que** la particule biologique est une protéine, un anticorps ou une cellule.

4. La méthode selon le revendication 3, dans laquelle les sites de liaison à la particule biologique ont une dimension latérale entre 5 μm et 1 μm, ou entre 1 μm et 500 nm, ou entre 500 nm et 300 nm, ou entre 300 nm et 100 nm, ou inférieure à 100 nm, de préférence plus petite que 50 nm ou moins de environ 1 μm ou fournissent une aire de surface de moins de environ 100 000 nm carré pour chaque site.

5. La méthode selon la revendication 4, dans laquelle les sites de liaison à la particule biologique ont une forme de cercle, de carré ou de rectangle, ou dans laquelle les sites de liaison ont une forme linéaire ou curvilinéaire.

6. La méthode selon la revendication 5, dans laquelle les sites rectangulaires ont entre 600 nm x 200 nm et 500 nm x 180 nm, ou entre 500 nm x 180 nm et 400 nm x 150 nm, ou entre 400 nm x 150 nm et 350 nm x 110 nm en longueur et largeur.

7. La méthode selon la revendication 5, dans laquelle les sites en forme de point ont entre 1000 et 500 nm de diamètre, de préférence entre 500 nm et 350 nm, ou entre 350 nm et 100 nm de diamètre, plus préférentiellement un diamètre plus petit que 100 nm.

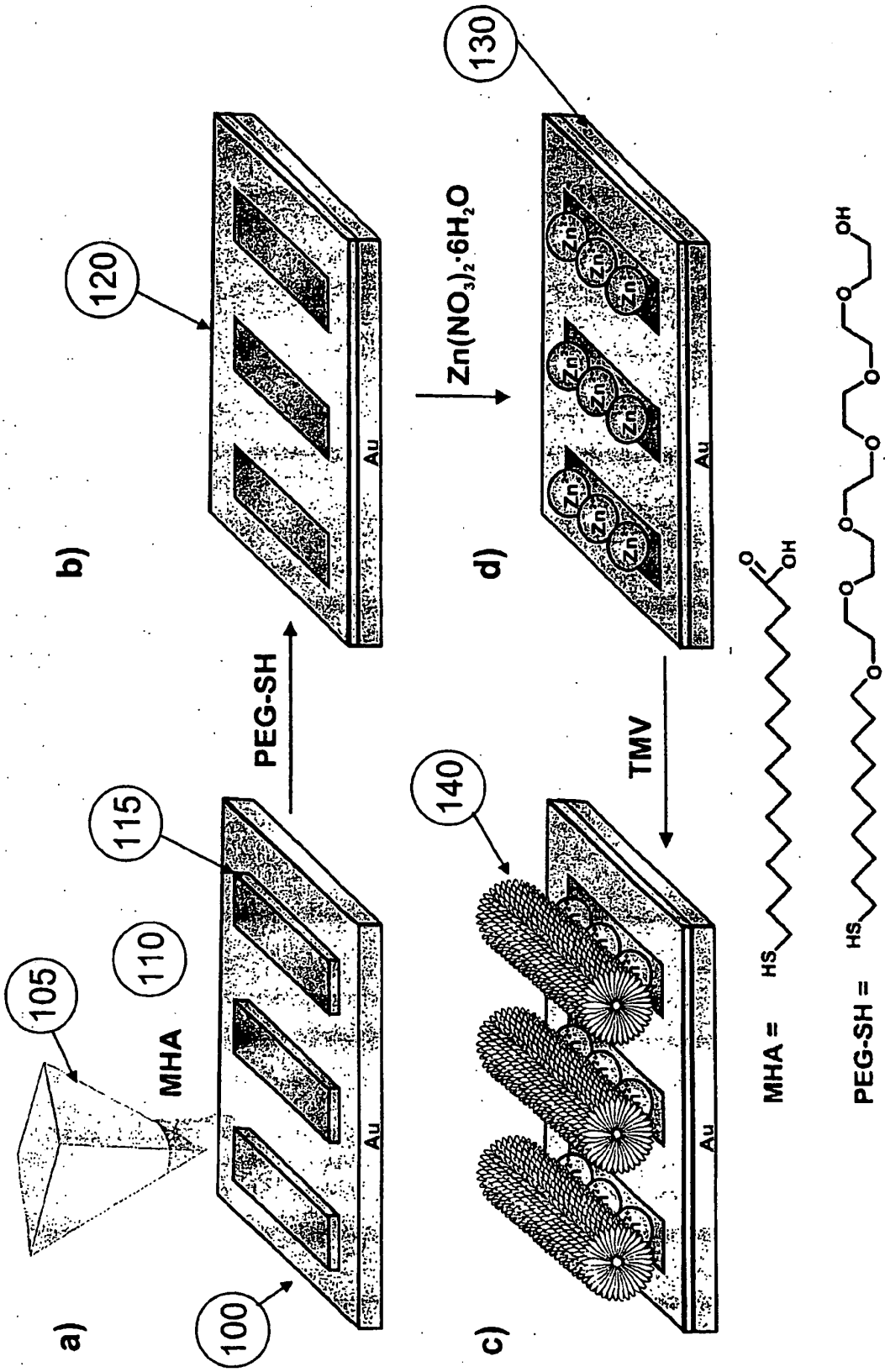
a)
b)
c)
d)
105
110
115
120
130
140
100
MHA
PEG-SH
TMV
Au
Zn
$Zn(NO_3)_2 \cdot 6H_2O$
MHA =
PEG-SH =
HS
OH

Figure 1

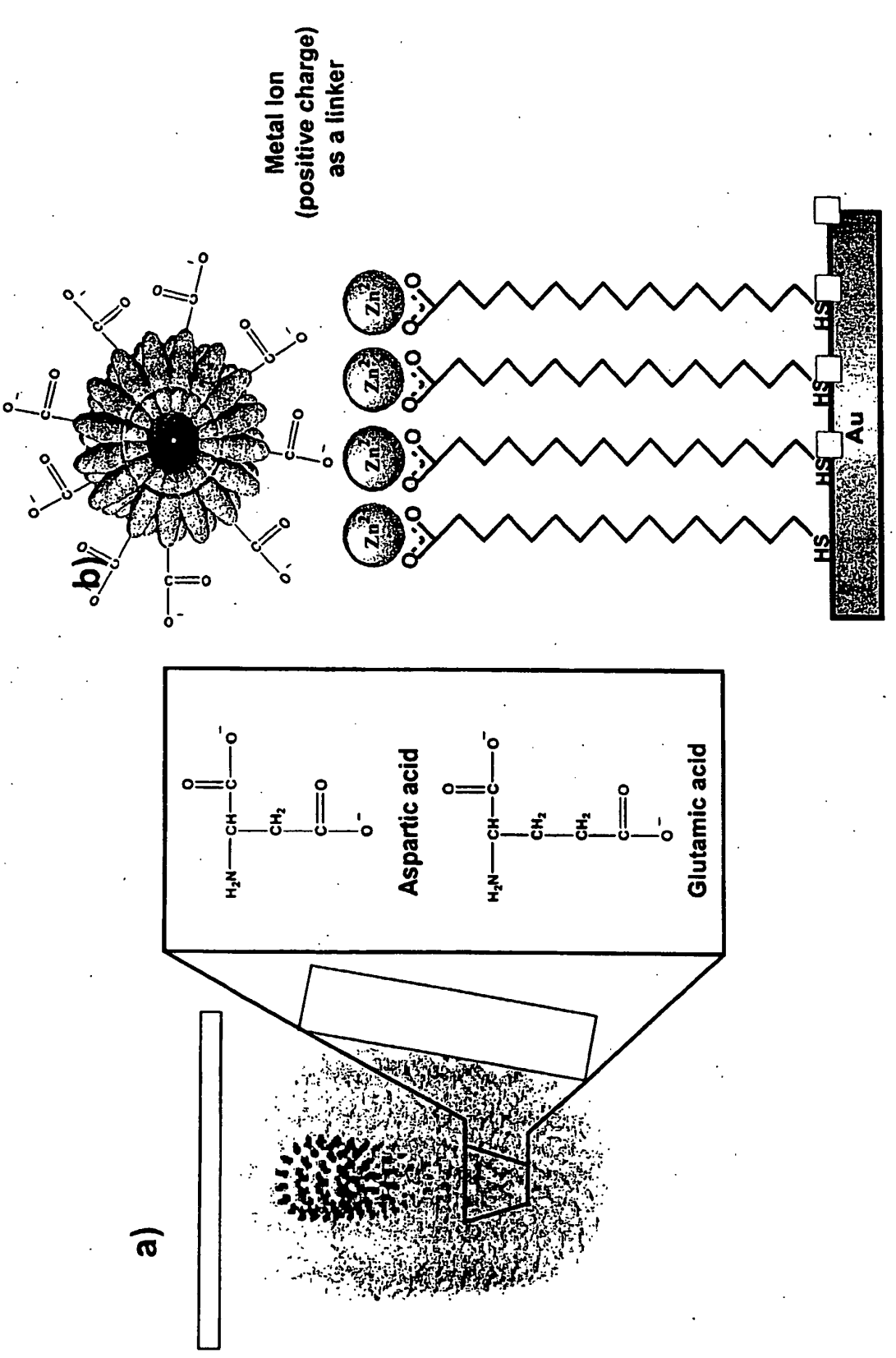
Metal Ion
(positive charge)
as a linker
b)
Zn
HS
Au
a)
Aspartic acid
Glutamic acid

Figure 2

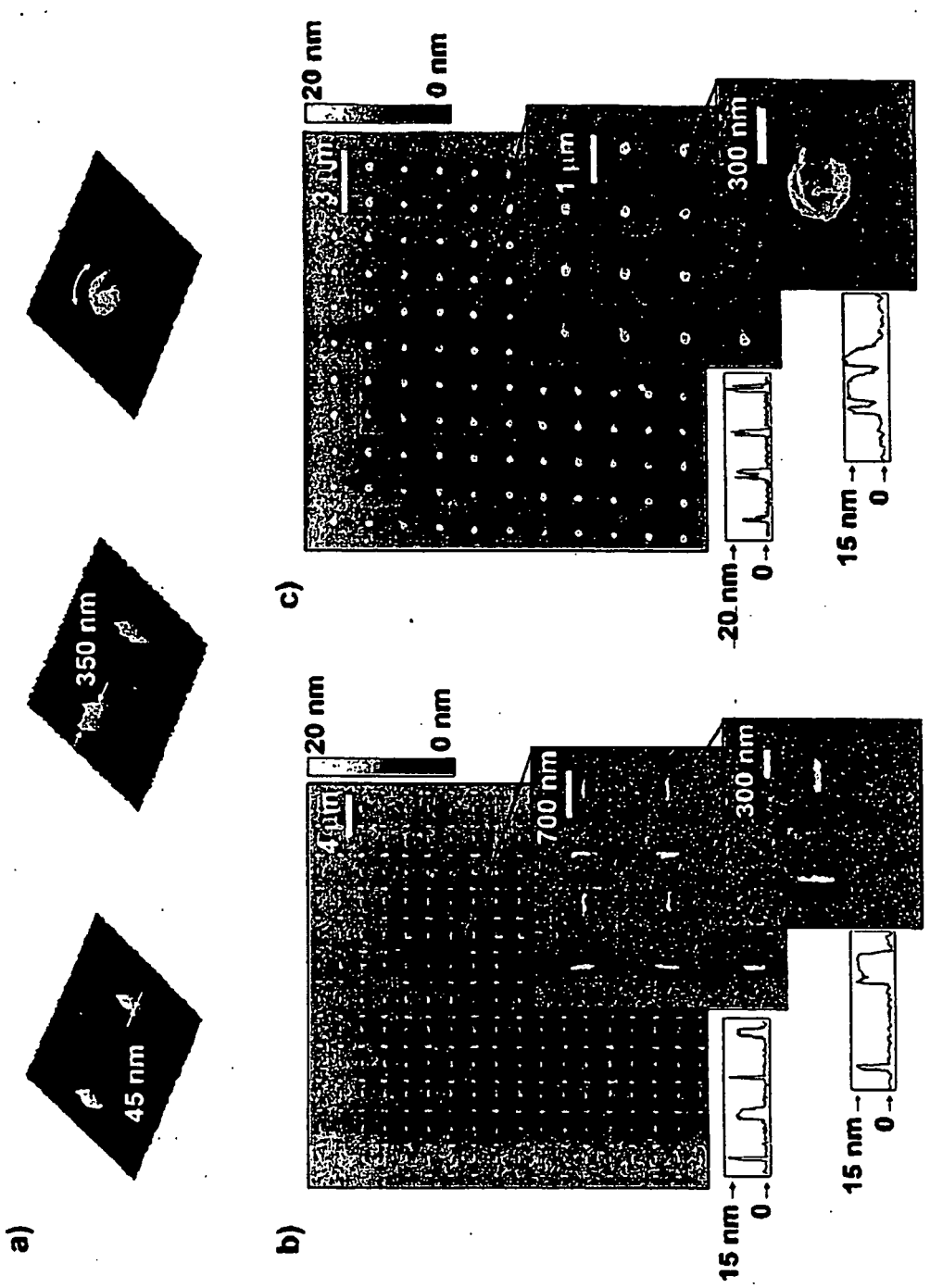
a)
45 nm
350 nm
b)
20 nm
0 nm
4 µm
700 nm
300 nm
15 nm
0
15 nm
0
c)
20 nm
0 nm
3 µm
1 µm
300 nm
-20 nm
0
15 nm
0

Figure 3

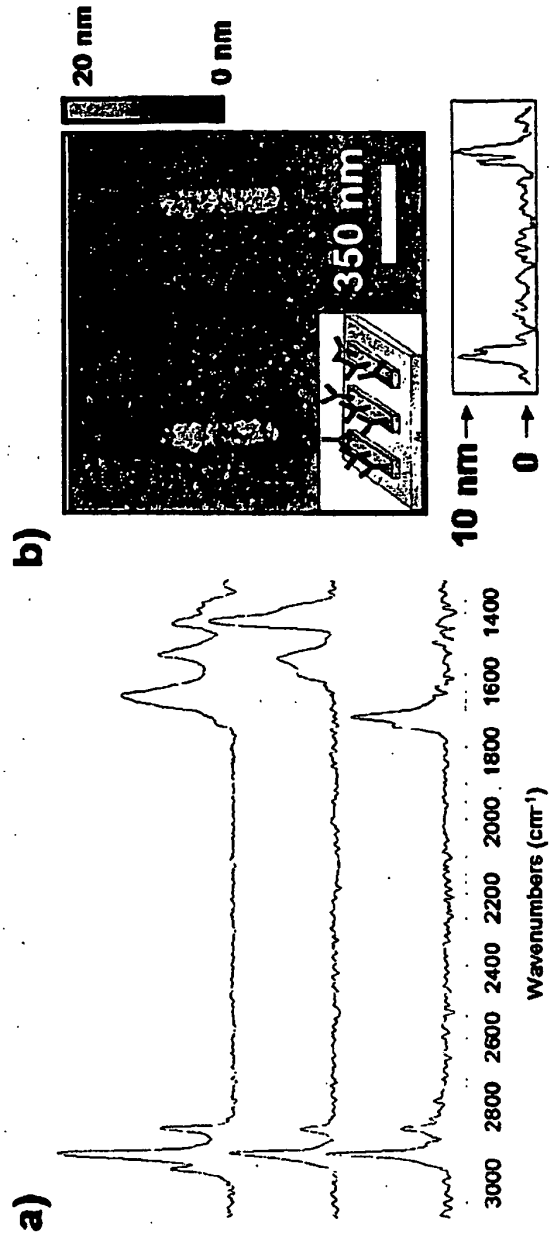
a)
3000
2800
2600
2400
2200
2000
1800
1600
1400
Wavenumbers (cm⁻¹)
b)
20 nm
0 nm
350 nm
10 nm
0
c)
20 nm
0 nm
350 nm
16 nm
0
d)
30 nm
0 nm
350 nm
25 nm
0

Figure 4

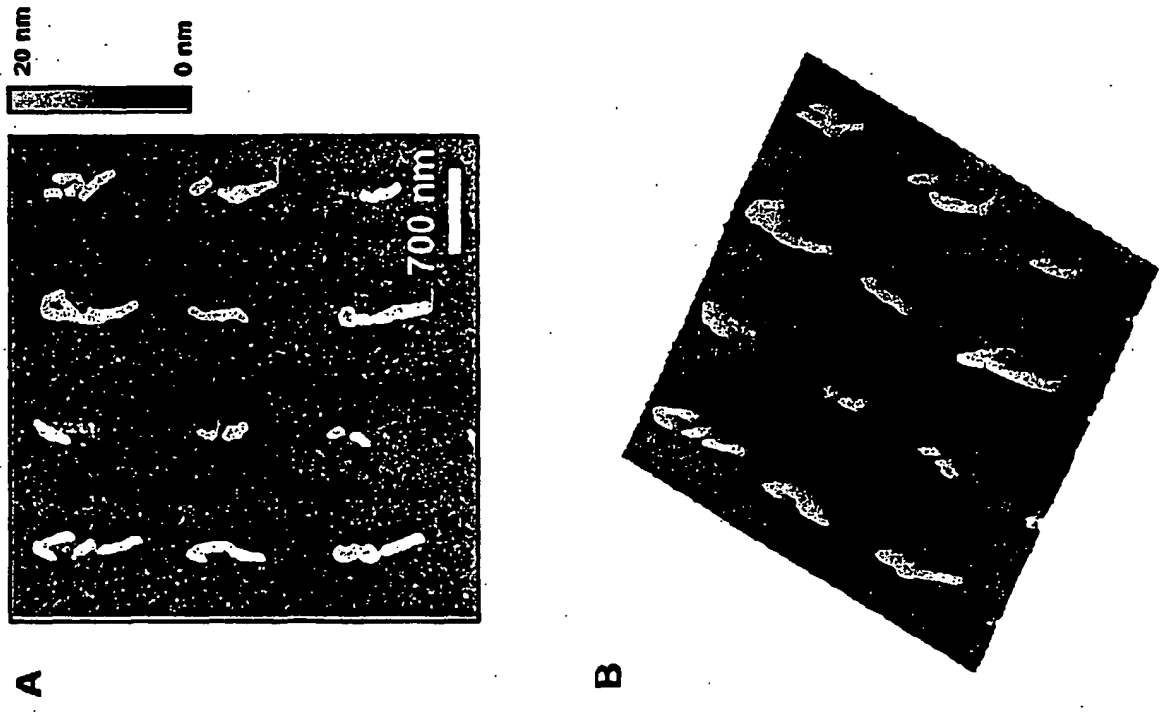

Figure 5

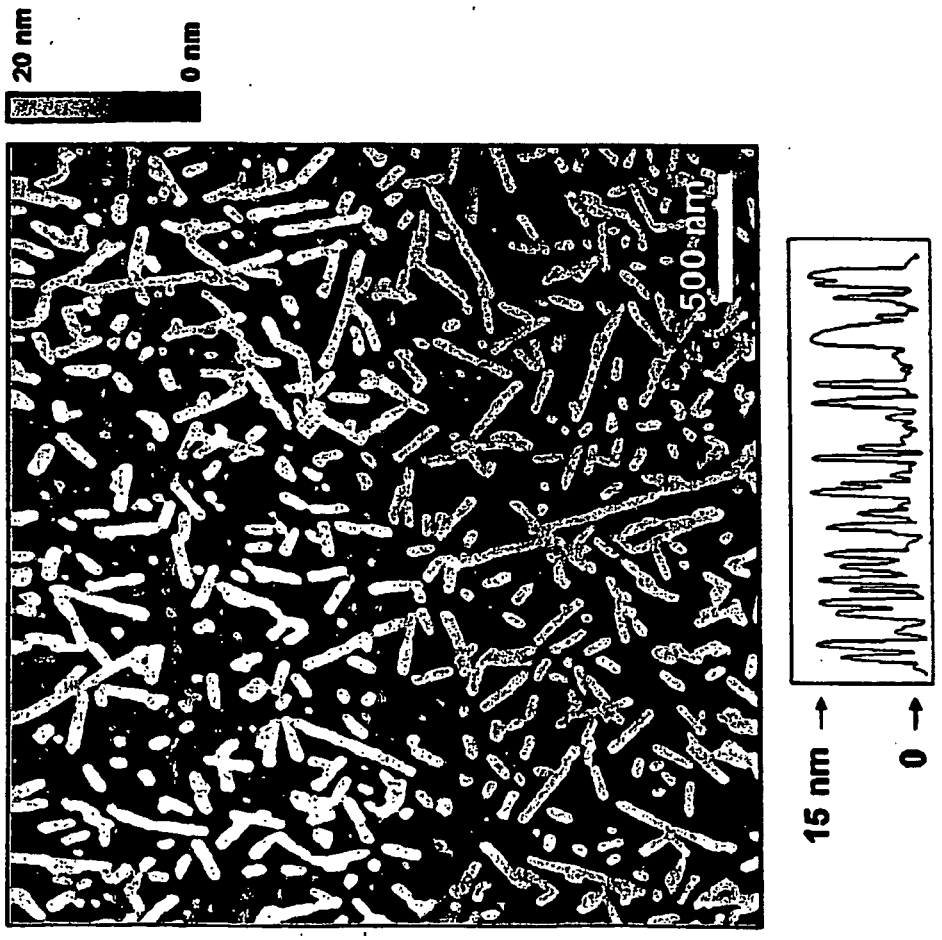

Figure 6

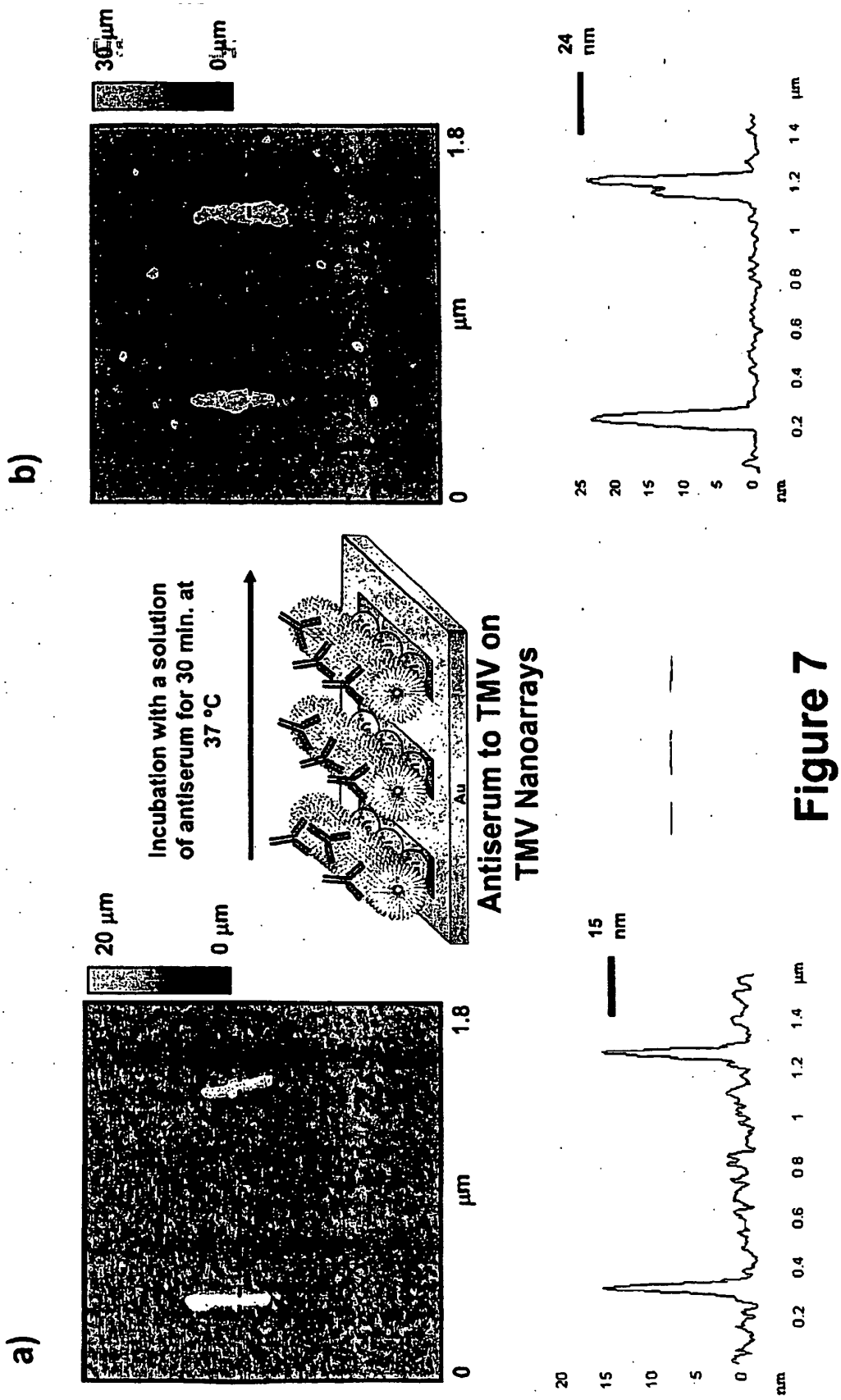
a)
20 µm
0 µm
0
1.8
µm
15
nm
0.2
0.4
0.6
0.8
1
1.2
1.4
µm
20
15
10
5
0
nm
Incubation with a solution
of antiserum for 30 min. at
37 °C
Au
Antiserum to TMV on
TMV Nanoarrays
b)
30 µm
0 µm
0
1.8
µm
24
nm
25
20
15
10
5
0
nm
0.2
0.4
0.6
0.8
1
1.2
1.4
µm

Figure 7

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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