Abstract:
Title: SINGLE STRAND DIMENSIONAL CONSTRUCTION OF DNA IN 3D SPACE

A process of generating a three-dimensional (3D) addressable array of biopolymer nucleic acids comprises providing a nanoscale three-dimensional shape having a surface and binding a plurality of biopolymer nucleic acids to the surface of the 3D shape in a regular pattern. The 3D biopolymer nucleic acids can be hybridized with complementary probes having reactive chemical groups to provide an enzyme active site mimic or artificial catalyst. Such mimics or catalysts can be in the biofuels and other industries.
Single Strand Dimensional Construction of DNA in 3D Space

TECHNICAL FIELD

[0001] This invention relates to a three-dimensional addressable array of biopolymeric nucleic acids and processes for manufacturing such arrays. Such arrays can be functionalized with complementary chemical reactive probes to provide catalytic enzymes.

BACKGROUND ART


DISCLOSURE OF INVENTION

[0003] The present invention is directed to a process for generating addressable arrays of biopolymer nucleic acids connected to three-dimensional (3D) substrates that can be used as nanoscale manufacturing platforms. The biopolymer nucleic acid can comprise DNA, PNA, RNA, or LNA. The biopolymer nucleic acid can be bound to the substrate using dip pen nanolithography, femtosecond laser-mediated patterning via a sacrificial layer, laser mediated lithography onto pre-formed grooves or risers on the surface, or magnetically-mediated alignment across the contoured surface. The biopolymer nucleic acids can be attached to metallic (e.g., gold), plastic, and/or polymer substrates. Diverse molecules, such as amino acids, can be connected to the 3D addressable platforms. The activated platforms can be used as catalysts or enzyme active site mimics. The platforms can be used to generate novel catalysts via combinatorial chemistry.

[0004] The invention is further directed to a process for replicating addressable arrays of biopolymer nucleic acids connected to 3D substrates using impression molding wherein the array being replicated is transferred to a flat stamp or a roller. Alternatively, the replication process can comprise using pressure molding wherein pressure is added into an elastic substance configured as a bladder or wherein an elastic material expands to interact with the array being replicated due to an environmental change (adding a reagent, temperature, etc).

BRIEF DESCRIPTION OF DRAWINGS

[0005] The accompanying drawings, which are incorporated in and form part of the specification, illustrate the present invention and, together with the description, describe the invention. In the drawings, like elements are referred to by like numbers.
FIG. 1A is a schematic illustration of cellulase progressing about a cellulose microfibril. FIG. 1B is a schematic illustration of synthetic glycoside hydrolase (E1 Endoglucanase mimetic) platform axially arrayed with ssDNA and hybridized with functionalized probes. FIG. 1B also shows a cellulose oligomer for size comparison. FIG. 2 is a schematic illustration of exemplary enzyme active site-like master shapes amenable to fabrication by nanoscale lithography, including a cylinder, acute (pointed) trough, cleft/crater, and compound helix. FIG. 3A is a schematic illustration of ssDNA arrayed in regular 2D patterns. FIG. 3B is a schematic illustration of ssDNA lithographed atop a 3D univariable radius mold. Electrical leads used for managing the electrostatic potential of the gold template unit are shown. FIG. 4 is a schematic illustration of ssDC-specific variations of lift-off production. FIG. 5 is a schematic illustration of representative molds used for NIL-based production of templates and platforms. FIG. 5A is a roller-type array of hemisphere-shaped molds. FIG. 5B is a flat stamp-type array of truncated cone-shaped molds. FIG. 6 is a schematic illustration of 3D platform generation from templates. FIG. 7 is a schematic illustration of 3D template generation from a masters using an elastic template material. FIG. 8 is a schematic illustration of 3D template generation from a master using an expansive template material. FIG. 9 is a schematic illustration of an exemplary ssDC catalyst. FIG. 10 is a schematic illustration of the assembly of universal catalytic platforms having helical and cleft geometries. FIG. 11 is a schematic illustration of an exemplary ssDC™ "Catalyst-on-a-Chip:" FIGS. 12A-C are schematic illustrations of enabling technologies for cybernetization. FIG. 13 is a schematic illustration of the twenty natural occurring amino acids, representing the vast majority of the monomer content of biological enzymes. FIG. 14A-C are schematic illustrations of catalysis within enclosures. FIG. 15 is a schematic illustration of an exemplary master shape comprising permanent ssDNA lithographed in a curvilinear pattern on gold. FIG. 16 is a schematic illustration of ssDNA iteratively anchored on DPN-spotted receptor points, and stretched linearly over a template shape to produce a cartesian pattern. FIG. 17 is a schematic illustration of ssDNA iteratively anchored on Fsec laser-depleted points, via functionalization with SA or anti-DIG, on a sacrificial layer and stretched linearly over a master shape. FIG. 18 is a schematic illustration of ssDNA iteratively anchored on Fsec laser-depleted points on a 2D surface and 3D master contour, and stretched curvilinear or linearly within pre-lithographed grooves.
FIG. 19 is a schematic illustration of stretching of one ssDNA strand from an Fsec laser-lithographed anchor point, and into a confining tunnel that transitions into a groove or rise, via linear vector magnetic field lines.

FIG. 20 is a schematic illustration of stretching of a phosphorothioate-modified ssDNA strand onto lithographed gold via unwinding and unraveling from a colloidal enclosure susceptible to translocation in a magnetic field.

MODES FOR CARRYING OUT THE INVENTION AND INDUSTRIAL APPLICABILITY

Part I. Single Strand Dimensional Construction

[0006] Single Strand Dimensional Construction (also referred to herein as "ssDC"; both "Single Strand Dimensional Construction" and "ssDC" are trademarks of Incitor LLC) utilizes single strand DNA and nanoscale forms to address physico-chemical functionality in three-dimensional (3D) space and for the development and production of nanoscale 3D structures requiring specific placement of discrete molecules, to provide catalysts and other products. Combining aspects of heterogeneous catalysis, polymer dynamics, multifacial organic synthesis, supramolecular assembly, microarrays and directed evolution / combinatorial chemistry (DE/CC), ssDC can enable the mass production of diverse products. Examples include the potential to replicate and improve upon almost any existing enzyme, and discovery of new modes of catalysis.

[0007] ssDC is based on a Single Strand Template Manufacturing process (also referred to herein as "SSTM"; "SSTM" and "Single Strand Template Manufacturing" are both trademarks of Incitor LLC), which can synthesize mimetic peptides, bio-polymers, and other molecular constructs directly from DNA in a templated fashion. With SSTM, high density arrays of DNA addresses are fixed to a two-dimensional (2D) surface with geometric precision and serve as manufacturing platforms on which synthesis is facilitated via hybridization and coupling chemistry that occurs via a stable interface between the DNA on solid phase, and monomeric components that are polymerized in the bulk, liquid phase.

[0008] Since the manufacturing platform shape in ssDC is not limited to 2D (or "flat") geometries, nor are the final products required to de-link from the template after synthesis, ssDC also presents the option of performing the above functions under conditions in which an extra dimension is advantageous. As a result, 3D platforms can also serve as the foundation for many products and processes, for example multifacial synthesis facilitated by topology, bottom-to-top assembly of supramolecular products having stereo-specifically placed components, and catalytic centers on which to perform enzyme-like functions.

[0009] The third of these example applications, the creation of platforms which mimic enzymes, or bio-inspired heterogeneous catalysts, will be described herein in detail. The techniques and methods described herein are meant to be exemplary only of the overall technology and its applications.
Examples using ssDC as a Process for Generating Catalysts

[0010] An example of the application of the present invention is to develop production templates and catalytic platforms having geometry inspired by enzymes useful for the biofuels industry. Most cellulases and other glycoside hydrolases perform their functions largely within cleft-like active sites that recognize, bind, and position substrates, stabilize and promote the formation of intermediates, and generate and release products - which are usually fermentable sugars (see G. J. Davies and B. Henrissat, "Structures and mechanisms of glycosyl hydrolases," Structure 3, 853 (1995)). The present invention enables the successful construction of nanoscale catalysts that can mimic this geometry and functionality while also utilizing more resilient materials in their construction.

[0011] FIG. 1 illustrates the concept. FIG. 1A shows a cellulase progressing about a cellulose microfibril (public domain image of the U.S. Dept. of Energy, Oak Ridge National Laboratory, ORNL Review 40(1), (2007)). FIG. 1B shows a synthetic glycoside hydrolase (E1 Endoglucanase mimetic) platform axially arrayed with ssDNA and hybridized with functionalized probes. Cellulose oligomer is shown above the platform for size comparison.

[0012] A process of generating catalysts using ssDC can comprise the following steps: a) generating shapes with nanoscale dimensions that mimic the geometry of enzyme active and/or binding sites; b) binding ssDNA to the nanoshapes in a repeatable and predictable manner; c) optionally reproducing these ssDNA impregnated nanoshapes using mass production techniques; and d) functionalizing the ssDNA impregnated nanoshapes by binding complementary ssDNA sequences bound to various molecules, such as amino-acids, to specific locations on the nanoshapes.

[0013] For the purposes of this description, the ssDNA impregnated nanoshapes emerging from steps a) and b) are termed "masters." The mirror images of these masters are known as "templates." The exact reproductions of the masters that are functionalized with ssDNA sequences bound to the various molecules of interest are known as "platforms." As will be apparent, masters can also be platforms if the master is eventually functionalized.

[0014] The term "polynucleobases" or "biopolymer nucleic acid" refers to single stranded DNA, single strands of RNA, PNA, or LNA in monomeric (homogeneous monomer), heterogeneous monomeric (i.e., a co-polymer of deoxyribonucleotides and nucleobasic beta-amino acids, which preserves the approximately 0.5 nm spacing and stacking ability of bases) or hybrid polymeric combinations. As an example of the present invention, the notation "ssDNA" will be used herein to indicate any of the above biopolymer nucleic acids modified for permanent attachment onto masters - to be used as complementary biopolymers in the lithography of templates.

Creation of Masters
The process of creating masters starts with the formation of a 3D nanoshape, either concave or convex. These nanoshapes can be generated by a number of different methods including, but not limited to, electron-beam lithography, nanoimprint lithography, and/or dip pen nanolithography. For the purpose of this example, cleft-shaped depressions that are created by 3D pattern transfer nanoimprint lithography (see Y. S. Kim et al., "Three-dimensional pattern transfer and nanolithography: modified soft molding," Appl. Phvs. Lett. 81, 1011 (2002)), or a photo- or electron-beam lithography process that can produce arrays of the correct mold shape, size, and separation distance (to +/- 10 nm) by selective removal of sacrificial layers deposited over a hard-cast material (see S. Y. Chou et al., "Sub 10 nm Imprint Lithography and Applications," J. Vac. Sci. Technol. B 15(6), 2897 (1997)) are assumed. The process can be completed without all of the steps described herein (for instance, catalysts may be made directly from functionalizing masters), and it can be reversed to start from a convex rather than a concave shape, for instance. In determining the exact shape and dimensions of a cleft, the measurements from the active and/or binding site(s) of an enzyme can be used, computer generated models of an enzyme's active site can be used, or the shape can merely be determined by what shapes are readily available. The end product version of the shape can be functionalized with physico-chemical groups for catalysis mimicry. Arrays of these identical shapes can then be fabricated onto a surface, such as Si (100) or SiO$_2$ (two of many examples) or other material, by photo- or electron-beam lithography, and by other methods, and then quality checked by atomic force microscopy (AFM) in tapping mode, scanning electron microscopy, or other microscopy of nanoscale visibility to verify topology.

FIG. 2 shows exemplary enzyme active site-like master shapes amenable to fabrication by nanoscale lithography, including a cylinder 22, an acute (pointed) trough 24, a cleft/crater 26, and a compound helix 28.

Once an array of identical shapes has been synthesized on a single surface, either one of two processes can occur depending upon the process used for functionalizing end catalysts: 1) Impressions of these master shapes can be taken via low intrinsic viscosity and small bleb size space-filling polymer (see G. H. Cross et al., "Refractometric discrimination of void-space filling and swelling during vapour sorption in polymer films," Analyst 125, 2173 (2000)). After casting, polymerization can be initiated by UV light or other methods. These hard-cast, permanent molds can be used for reverse production of additional masters, or mass production of end-user platforms; or 2) ssDNA can be added to the array of shapes, wherein this array of shapes is called a "master."

The first process is used when the creation of catalysts requires the separation of induction of form, then addition of DNA. The master without ssDNA is used to introduce shape. The master with ssDNA then adds DNA to the shape.
[0019] The second process is used when shape and DNA are simultaneously applied within catalyst manufacture. Although this second process of adding ssDNA to the master is more fully described in Part III of this description, some of the available options of adding ssDNA include: (i) dip pen lithography (where a nanoscale stylus aligns and stretches DNA via electrostatic and interfacial influence), (ii) laser-mediated pattern lithography onto pre-formed grooves or risers which lessens translocational uncertainty, and/or (iii) magnetic field patterning of stretched DNA using linear portions of fields that affect a susceptible mass attached to the terminus of the DNA. The synthetic DNA can be chemically modified to form covalent bonds with the template, and added in close proximity (10 nm spacing) in order to pattern a high density addressable array on the eventual product. The pattern created can be curvilinear or Cartesian, which facilitates predictable addressing and simplifies the dynamic modeling of physico-chemical functions and of catalysis profiles.

[0020] FIG. 3 shows examples of ssDC-specific variations on basic patterning approaches described in referenced publications. FIG. 3A shows ssDNA 31, 32, 33, and 34 arrayed in regular 2D patterns (see L. M. Demers et al., "Direct Patterning of Modified Oligonucleotides on Metals and Insulators by Dip-Pen Nanolithography." Science 296, 1836 (2002)). FIG. 3B shows ssDNA 35 lithographed atop a 3D univariable radius mold (e.g., Au 36 deposited onto titanium 37) (see C. M. Waits et al., "Microfabrication of 3D silicon MEMS structures using gray-scale lithography and deep reactive ion etching," Sensors and Actuators A 119, 245 (2005)). Electrical leads 38 and 39 can be used for managing the electrostatic potential of the gold template unit are also shown.

[0021] Once masters have been constructed, they can be used in one of three ways: 1) if they have no ssDNA on them, they can be used to reproduce the nanoshape; 2) if they have ssDNA on them, they can be used to reproduce the grid of ssDNA (and possibly the shape) on a shape produced by a non-ssDNA activated master; or 3) if they have ssDNA on them, they can have complementary probes connected to them that functionalize the master, turning it into a "platform," or a functionalized ssDC product.

[0022] There are a number of methods of using masters to generate templates and eventually platforms, with the desire to speed production and lower production cost. Examples of these methods include impression molding and pressure molding, each of which is described in detail below. These terms are not meant to imply any process that might exist outside of this description that uses such terms. The following sections describe "impression molding" and "pressure molding" techniques for generating templates and platforms.

Impression Molding

[0023] In impression molding, two different masters can be used. The first consists of a master with shape only. This master is impressed onto soft polymer material, the polymer is
hardened by UV light or other methods, and the template is removed by lift-off, generating
the original master shape at scaled quantities.

[0024] FIG. 4 shows lift-off production comprising ssDC-specific variations on basic themes
described in referenced publications (see P. W. Snyder et al., “Biocatalytic Microcontact
Printing,” J. Oro. Chem. 72, 7459 (2007); and Duke University Office of News and
Communications, Sept. 2007). A template 41 with molded shapes 42 is hybridized with
address nucleotides 43, and then impressed onto a polymer platform 44, releasing probe
ssDNA 45 within the impressions and preserving mirror-image complementary addresses.

[0025] ssDNA can be added to these shaped templates by using the master that has ssDNA
attached to it. Complementary DNA or PNA (Peptide Nucleic Acid) intended to form the
addressable array of the template can be hybridized to the master with ssDNA, “inking” the
latter for pattern transfer. A nucleobase sequence complementary to and the mirror image
of the DNA that was placed on the master can be mass produced as templates.

[0026] In most cases, the templates can be produced from roller-type templates or by
stamp-and-peel molds by re-aligning the blank templates with the master with ssDNA. FIG.
5 represents molds that can be used for NIL-based production of templates and
platforms using ssDC-specific variations on basic themes described in referenced
publications. FIG. 5A shows a roller-type array of hemisphere-shaped molds (see H. Tan et
shows a flat stamp-type array of truncated cone-shaped molds (see S. Diegoli et al.,
Aerospace Eng. 221(4), 589 (2007)).

[0027] To facilitate transfer of template ssDNA or PNA, and to preserve the re-usability of
the masters, the lithographed ssDNA can be covalently bonded to the master material, e.g.,
via phosphorothioate-on-gold bonds, or amine ssDNA backbones on siloxane functionalized
hard cast Si (100). In addition, the transfer of polymer onto the template in a manner that
preserves a mirror image pattern and addressability of the template (i.e., “nucleobases up”) can
be facilitated by the following: temperature, variations in master surface energy and
potential, UV or other polymerization methods, roll vs. lift-off templating, solvation, lubrication
(tribology), mechanical and other factors. Platforms can be made in the same way as
templates, but using templates with and without ssDNA instead of masters with and without
ssDNA.

[0028] FIG. 6 illustrates a method of ssDNA transfer for both 3D templates and platforms.
The figure shows phosphorothioate-backboned ssDNA 62 lithographed on a gold template
64, after lift-off from a platform 66, leaving complementary address DNA or PNA 68.
Pressure Molding

[0029] Another exemplary method to reproduce a master into templates and/or platforms also uses a soft polymer or other material that becomes elastic or expansive under certain conditions. The master can be placed into a container that restricts lateral movement. Complementary DNA or PNA intended to form the addressable array of the template can be hybridized to the master with ssDNA, "inking" the latter for pattern transfer.

[0030] A soft polymer or other elastic or expansive material can then be added into the container. If the material is elastic, the elastic material can be configured as a bladder. The outer surface of the bladder can be prepared to permanently bind to ssDNA. Pressure can be applied to the interior of the elastic bladder forcing the material to expand to the dimensions of the container, adhering to the shape of the master and picking up the complementary DNA previously hybridized to the master. The elastic material can then be solidified in a manner appropriate to its inherent properties, such as UV cured or other process. If the material is expansive, the same steps can be taken, save that instead of applying pressure, the conditions which cause expansion (e.g., temperature change, pH change, etc.) can be introduced to the material forcing it to adhere to the shape of the master and pick up the ssDNA.

[0031] FIGS. 7 and 8 demonstrate these methods. FIG. 7 shows 3D template generation from a master using an elastic bladder. Pressure forces the template elastic material to expand, taking on the shape of master and connecting to complementary ssDNA previously laid down on master. After the template bonds to the complimentary ssDNA, the template can be peeled off. FIG. 8 shows 3D template generation from a master using expansive material. Environmental change forces the template expansive material to expand, taking on the shape of the master and connecting to complementary ssDNA laid down on master. The template can then be peeled off.

[0032] The manufacture of platforms can proceed in the same manner as template manufacture. Templates can be used in place of the master for the construction of the platforms. These processes can be repeated as many times as necessary to generate the number of platforms desired.

Activation of Platforms into Catalysts

[0033] ssDC platforms can be considered as nanoscale 3D high density addressable arrays with the DNA or PNA patterned to nanometer-level precision that can be replicated on billions of copies. The platforms' shape make them amenable to conversion into enzyme-like catalysts, in part, because they resemble the size and shape of active sites, and the addressable DNA provides an ability to "activate" those shapes with complementary probes carrying reactive chemical groups. Upon hybridization, these groups will be localized in the platforms at positions similar to the orthogonal side chains of active site amino acids that
facilitate catalysis in the enzyme that was originally mimicked in the creation of the master shapes.

[0034] Accounting for conformational changes that occur during the catalysis process (a function provided for by linkers between the chemical groups and probes that hybridize to the platform addresses), surface energy, tribology, solvation and other factors important for catalysis, the now activated platform is an ssDC product having the ability to bind cleaned cellulosic or aligic material, stabilize and catalyze the formation of transition states, and release depolymerized sugars, just like natural cellulases (glycoside hydrolases) and alginases (trans-esterases).

[0035] An additional benefit provided by the use of nanoimprint polymer lithography, which was originally developed for the microprocessor industry, is the optional integration of electric, magnetic, optical, and other systems that facilitate enzyme active-site movement, e.g., via nanoscale actuators which translocate, vibrate and/or change the conformation of certain parts of the activated platform. Enzyme active site flexibility during the catalysis profile is a key part of substrate binding, transition state formation, product release and other activities, the absence of which prevents efficient catalysis and renders the active site a "static heterogeneous catalyst" (see E. Z. Eisenmesser et al, "Enzyme Dynamics During Catalysis," Science 295 (5559), 1520 (2002); M. Karplus and J. Kuriyan, "Molecular dynamics and protein function." Proc. Natl. Acad. Sci. 102(19). 6679 (2005); A. Kohen and J. P. Klinman, "Enzyme Catalysis: Beyond Classical Paradigms," Ace. Chem. Res. 31, 397 (1998); and C-L Tsou, "Active Site Flexibility in Enzyme Catalysis," Annal. NY Acad Sci. 864, 1 (1998)).

[0036] FIG. 9 shows an exemplary ssDC catalyst 92 that illustrates simplified activation of a master or platform shape 66 with chimaeric amino acid-DNA probes 94 that enable chemical functionality in 3D space, and simple tribology with negatively-charged phosphorothioate (P), or neutral (N) backbones 96.

Combinatorial Development via Directed and Random Evolution

[0037] Because the product DNA or PNA can be made indefinitely re-useable by permanent mounting on resilient material, DE/CC can be performed on the platforms generating billions of combinatorial variations on a given shape, and many different shapes are possible by beam lithography. For example, a simple platform shown in Table 1, comprising only eight (8) different ssDNA strands lithographed in a cross-hatch pattern, enables forty (40) unique address locations. Each of these addresses can hybridize to a small probe library of oligomers having only one of twelve (12) chemical functions each, and linked at only one location (e.g., on the 3' terminus). Thus, even this simplest attempt at Random Evolution can produce 40^{12} or 1.68E+19 different combinations, increasing the probability that some will be useful catalysts.
Table 1. Combinatorial variations presented by a 40-mer platform and one monofunctional, singly-linked 40 x 12-mer Probe Library for each address.

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Probes Library 1 for Address "N" --> one function on one location

1 -- NH2
2 -- NH-alkane
3 -- COOH
4 -- IMZ
5 -- PIP
6 -- PYR
7 -- Indole
8 -- alkane
9 -- benzyl. ring
10 -- Hydroxyl
11 -- Mercaptaly
12 -- Succinimide

[0038] The leverage provided by more "random" evolution is further shown by the fact that orthogonal chemical functions can be placed on either termini of a PNA probe, in the intervening backbone, or at multiple locations. In addition, several functions can be placed on the same probe molecule (if self-reactive, then appropriate protecting groups must be part of the PNA design), and the types of linkers used to connect orthogonal chemistries to the probes can be modified to facilitate flexibility and optimize catalysis. Even in the absence of orthogonal functions, the amide backbone of PNA and the diester vertebrate of ssDNA and RNA can be synthetically modified to provide a given charge, charge density and, thus, surface energy to that portion of the platform, providing for management of solvation, counterions, dipole moments, dynamic solvability / tribology and other key features of catalysis.

[0039] On the other hand, attempts to produce catalysts that mimic enzymes are inspired by, though not limited to, active site design and bio-catalysis profiles. Rather than relying exclusively on the power of probability, more subtle variations on the geometric and chemical themes presented by protein enzymes can be undertaken to optimize a known catalytic process, which itself is based on a certain sequence of amino acids scaffolded and dynamically translocated in 3D space (see R. Chakrabarti et al., "Sequence optimization and designability of enzyme active sites," Proc. Natl. Acad. Sci. 102(34), 12035 (2005)).

Computer modeling of catalysis profiles, from binding to release, can be varied to enable the following exemplary improvements of ssDC™ catalysts over protein enzymes: (i) increase the substrate binding affinity (through management of surface energy and, thus, "stickyness" to the acceptor region), (ii) place stronger orthogonal acid functions where it has already been determined that the amino acids Aspartate (Asp) and Glutamate (Glu) perform acid-
catalyzed hydrolysis of D-1/4-Glycosidic bonds in cellulose, and/or (iii) modify the platform shape to allow metal co-factors that contribute to hydrolysis, however not in natural cellulases, to speed-up the overall reaction. Those skilled in the art will appreciate many other options enabled by this invention.

[0040] Such efforts can be undertaken diligently and with design intent because some end products are not desired, such as, for example, those which (i) onto which substrates become recalcitrant or "stuck," (ii) are so acidic that they damage the substrate or each other, and/or (iii) become intransigently contaminated with metals. However, as mentioned, both Random Evolution in a discovery mode (where most of the products are rejected as a matter of expectation), and Directed Evolution, which initially deviates from the enzyme model only through proven paths, but then increasingly evolves more independently when useful trans-biotic catalysis paths are discovered, can be used iteratively to produce improved products. This process may develop and discover catalysts much more quickly than can be expected by the genetic engineering, production, purification, testing and qualification of protein-based enzymes that, regardless of these efforts, preserve their inherent limitations.

[0041] Directed and Random Evolution, and Combinatorial Chemistry, can also be used in concert akin to how the immune system successfully produces antibodies that bind to almost any antigen, including the myriad of substances produced by the human body as part of the "self vs. non-self" recognition function. For example, though most protein antibody molecules preserve an almost universal "Y" shape, the hypervariable regions that recognize foreign and familial substances can be induced to generate nearly mole numbers of variations \((10^{23})\) via both Directed (once a foreign antigen is encountered) and Random (if a system insult cannot be immediately identified) combinatorial shuffling of the genetic code from which the hyper variable regions of the protein are programmed. Whichever method, and at whatever point in development, proves useful, the DE/CC aspect of ssDC can be used to meet the needs of a customer in the shortest possible time.

[0042] Some parameters available with ssDC™ catalysts that are amenable to Combinatorial Variation include:

- Platform size, shape, surface modification and material
- Pattern and density of nucleobasic addresses arrayed
- Composition of high density addressable arrays: ssDNA, RNA, PNA, and mixes thereof
- Orthogonal chemical functions: amino acids, transcendent (e.g., organic) chemistries
- Presence, absence and labiality of protection groups
- Location of chemical function(s) on the probe: terminal, intervening, mixes thereof
• Single, multiple or no functions on the probes, to manage surface charge
• Length, stiffness, size, dipole moment, hydrophilicity/phobicity of linker groups
• Probe backbones: phosphodiester, phosphorothioate, amines, hydrazides, etc.
• Integration with electronic, magnetic and/or optical systems
• Aqueous, organic, interfacial catalysis at trans-biotic conditions: extremes of temperature, pH, pi, ionic salts, metal ions, high shear forces and integration into liquid, solid and colloidal phases.

De Novo Catalysis Discovery via Directed Evolution

[0043] As well as making progressively better "mimetic enzymes" for the biofuels market, the present invention enables development of platform shapes not derived from any specific biological precursor, but geometrically tailored with the potential to carry-out a wide variety of catalytic processes. Such Universal Catalytic Platforms (UCP) can be based on ssDC technology while leveraging a DE/CC library of nucleobasic probes (made from ssDNA, RNA, PNA, etc.) that have been activated with chemical functions over and above those of amino acids (see D. R. W. Hodgson and J. M. Sanderson, "The synthesis of peptides and proteins containing non-natural amino acids," Chem. Soc. Rev. 33, 422 (2004)). This combinatorial generation of large libraries of completely abiotic products can iteratively produce stronger and more efficient catalysts which operate under lower temperatures and present newer and simpler catalysis profiles than are possible with protein-based enzymes. This discovery process is referred to herein as "Physical Directed Evolution" (PDE).

[0044] An exemplary method can use conical shapes with peripheral DNA strands arrayed longitudinally and a small probe library. FIG. 10 shows the assembly of UCPs having helical and cleft geometries: (a) address ssDNA arrayed inside material stamped with a conical mold; (b) individual UCP unit prior to "activation" with probe oligomers; (c) library of oligomers (ssDNA, RNA, PNA) end-modified with active functions; (d) individual UCP unit "activated" by hybridization with end-modified oligomers; (e) two UCP units activated in different ways by hybridization with dissimilar oligomers; (f) finished activated UCP unit sliced from mold to preserve an intact helix; and (g) finished activated UCP unit sliced from mold to form a univariable radius cleft.

New Trans-Biotic Catalysis Profiles and System Integration

[0045] With PDE, multiple rounds to generate, qualify, test, reconsider, redesign and regenerate 3D catalysts (iteratively idealizing shape, DNA patterns, linker strategies, chemical functions and other parameters listed above) can provide increasingly improved products, but also can enable the discovery of heretofore unknown modes and profiles of catalysis that are not possible with protein-based enzymes. In addition, platform qualities, such as surface energy (which determines charge, solvation and tribology) and
conformations that are inducible by electromagnetic (EM) fields can be engineered in ways that are impossible with purely biological molecules.

[0046] As implied, the linkers attaching reactive chemical and other functions to probe oligomers are intended to mimic the conformational changes that occur in the enzyme active site as it reacts to the presence of substrate, and facilitates the creation of intermediates and products during the catalysis profile. A potentially superior method that ssDC provides is the enabling of conformational changes via electro-active polymer foundations that also bend and relax, open and close, extend and contract, when in dependent contact with a substrate, intermediate or product.

[0047] For example, a platform can be fabricated with conductive channels arrayed to resonant piezoelectric crystals and to polymer blocks composed of material with conformational susceptibility to defined EM frequencies. This can enable one or more portions of the mimetic active site to vibrate when it senses that contact has been made with a substrate (or vibrate constantly in a default mode assuming a constant current). This can initially be set at a frequency and duration equal to the movement of the catalytic residues and active site segments in the glycosidase being mimicked. Subsequent frequencies, amplitudes and geometries can be varied to optimize catalytic rates, and in conjunction with industrial processes that are being optimized with the intention of integrating the catalyst. Based on well-qualified methodology that produces electroactive polymer-based microprocessors, the integration of ssDC catalysts into feedback and control systems enables conformational changes and real time monitoring, and can help identify the most promising candidates during a directed evolution discovery based process.

[0048] FIG. 11 shows an exemplary ssDC™ "Catalyst-on-a-Chip." The 3D shape can be DE/CC-optimized for functionality, locations, linkers, tribology and geometry. For example, leads for electrical input-output 112, electro-active harmonic actuators under opposed residue blocks 114, and an optically-linked center 116 are shown.

activating/monitoring system for catalytic platforms (see A. M. Fennimore et al, "Rotational actuators based on carbon nanotubes," Nature 424, 408 (2003)).

[0050] After activation with functionalized probes, ssDC catalysts provide the processing advantages of heterogeneous catalysts, the combinatorial flexibility of re-usable high density addressable arrays, the strength of high impact industrial polymers, and a ready ability to be integrated into biofuels monitoring processes. Unlike biological enzymes, ssDC catalysts are not limited to the orthogonal functions of amino acids, nor are the platforms to any particular shape. They can be constructed of materials that survive conditions which would normally denature or neutralize biotic enzymes. The platforms can be readily integrated into industrial processes such as packed filters, reaction columns, sedimentation materials and mixing surfaces. In addition, the incorporation of feedback/control systems enables monitoring of the catalysis process, rapidly identifying the most promising candidates for continued evolution, overall accelerating the development and optimization of new catalysts.

[0051] This effort can be undertaken in conjunction with process optimization, enabling engineering design to develop in-synch with an evolving catalyst-based methodology. As a result, entire biofuels production processes can be optimized at each stage to perform with peak efficiency no longer hampered by the limitations imposed in using biotic enzymes. Higher or lower temperatures, further extremes of pH and ionic salt content, shear forces, the use of organic solvents and other conditions that optimize hydrolysis of cellulose and other biomass - but which are currently not utilized because they would destroy protein-based enzymes - can be performed with ssDC catalysts. Alternatively, ssDC catalysts can be developed to perform optimally on existing production processes.

[0052] As long as the addressable nature of the single strand DNA is preserved (a condition provided-for in SSTI and ssDC as the stable interface that protects the polynucleotide chains by an immiscible aqueous/organic phase, even while undertaking potentially caustic and corrosive organic reactions in the bulk phase), combinatorial efforts can be undertaken to generate progressively improved versions of any desired product, the monomeric components of which can be functionalized to single strand DNA for site-specific placement.

[0053] Once an ideal catalyst or other product has been finalized, an additional step can be undertaken prior to system and process integration. Covalent bonds that render dehybridization impossible can be created between the probes and their addresses, permanently affixing the physico-chemical functions to the polymer-based platform. If desired, this extra step can increase the tenacity and usable life of the catalyst, in particular improving the ability of active site functional blocks to conform, resonate and vibrate at the high frequencies (e.g., greater than one Gigahertz) required for dynamic catalysis to occur efficiently.
Finally, after combinatorial chemistry and evolution-based discovery are completed, the test platforms can be "washed" to revert back to simple 3D addressable platforms, ready for hybridization with new sets of complementary probes. They can then be reused for a wide-range of purposes, including synthesizing bio-mimetic polymers, discovering other catalytic modes and profiles, performing supramolecular assembly, and serving as high density addressable arrays for the construction of bio-sensors, drug delivery vehicles, microchip components and other self-assembled devices.

**Part II. Application of ssDC in the Creation of Heterogeneous Catalysts**

**Process to Develop Greatly Improved Catalysts**

[0055] The Single Strand Dimensional Construction (ssDC) technology described herein eliminates many of the problems with protein-based enzymes, such as fragility, brief shelf lives, limited catalytic abilities, and production from genetically-engineered microorganisms grown with expensive infrastructure. ssDC accomplishes this by transferring the production, development and function of biological enzymes to a nanotechnology-based platform that performs catalysis in a more robust, versatile and active manner.

[0056] Biological enzymes are made from polymerized amino acids. The twenty naturally-occurring amino acids, in different sequences, post-translational modifications and folded structures, are responsible for catalyzing thousands of biochemical reactions. Though versatile and effective - enabling life to exist in a myriad of forms - enzymes generally have slow rates of catalysis and, like all naturally-produced products, are non-recalcitrant to the environment (i.e., are bio-degradable).

[0057] In addition, the catalytic activity of enzymes derives from a small set of chemical functions, including acids, amines, amides and indoles, "mild" electrophiles, and saturated and unsaturated hydrocarbons - all with limited reactivities and, thus, catalytic strengths. This "inferiority" of the orthogonal potential of biological enzymes vs. that of trans-biotic and artificial reagents exists, in part, because proteins having too highly reactive chemistries are difficult to sequester and recycle inside cells, and are potentially toxic to both the host organism and to the environment in general.

[0058] FIG. 13 show the twenty natural occurring amino acids, representing the vast majority of the monomer content of biological enzymes (Dept. of Bacteriology, Univ. of Wisconsin-Madison).

[0059] ssDC technology can be used to manufacture artificial enzymes, develop new versions of those enzymes, and discover new modes of catalysis that natural enzymes are unable to execute. This is accomplished by using modified versions of industry-standard nanolithography processes in the construction of platforms on which the catalytic functions are carried out. The production and development methodology of ssDC is based on three-dimensional (3D) pattern transfer lithography. The versatility of this nanoscale level
technology can produce platforms in a wide range of shapes and sizes, providing the foundation to mimic almost any exiting catabolic enzyme.

Enzyme-Mediated Catalysis

[0060] The ability of enzymes to catalyze biochemical reactions depends greatly on the coordination of protein geometry with chemical functionality that translocates inside the active site during the catalytic process. This site generally contains many of the dependent functions of catalysis, including substrate binding, transition state formation and stabilization, chemical conversion and substrate release. As mentioned, combinations of polymerized amino acids can catalyze thousands of different biochemical processes. However, these reactions generally only take place within a limited range of temperature, pressure, salt content, ionicity and pH, and nearly without exception under aqueous conditions.

[0061] The key to making mimetic (i.e., improved) versions of enzymes is to replicate their catalytic functions on a more versatile, stable, and active platform. This is first accomplished by accurately modeling the active sites of enzymes as generalized closed curves, clefts, and helices, then fabricating large arrays of these shapes. The resultant enclosures can be designed to trap substrates and localize in 3D space the amino acid groups and other physico-chemical functions, e.g., electrostatic-based surface energy and dynamic conformational ability, that contribute to converting substrates into products.

[0062] The products of ssDC manufacturing are solid phase heterogeneous catalysts. However, they are the most advanced ever created. Like fluidized bed reactors, reactive filters and immobilized biological enzymes, ssDC catalysts accelerate the conversion of liquid phase substrates into products via chemical functions anchored to a solid surface. Like most other heterogeneous catalysts, they work best if the following are provided for and optimized: the substrates are well-dissolved in the liquid phase - usually salt water, the surface area of catalysts relative to total volume is maximized, the mass transport of substrates to, and products away, from the catalytic surface is enhanced - usually by mixing and/or phase partitioning, and the catalysts themselves have been designed correctly.

[0063] The "correct" design of catalysts, whether solid phase heterogeneous, like ssDC products, or the liquid/colloidal phase type - of which protein enzymes are a subset, results in an enclosed, active and highly specific chemical system that carries out the conversion of substrate to product in the shortest possible time and in the most energy efficient manner. For enzymes, this can be accomplished by a polymer of amino acids that folds into a 3D structure. For ssDC catalysts, this can be accomplished by the use of platforms that are shaped like enzyme active sites, and which contain all the physico-chemical functions necessary for conversion of substrates into products.

[0064] FIG. 14 show catalysis within enclosures comprising ssDC-specific variations on basic themes depicted in referenced publications. FIG. 14A shows a simplified space-filling
model of a cleft geometry and functions common to glycoside hydrolases: acidic and basic residues in spatial opposition with tyrosyl groups that function as "positioners" (see W. Nerinckx et al., "An elaboration on the syn-anti proton donor concept of glycoside hydrolases: electrostatic stabilization of the transition state as a general strategy," FEBS Letters 579, 302 (2005); and E. J. Taylor et al., "How Family 26 Glycoside Hydrolases Orchestrate Catalysis on Different Polysaccharides." J. Biol. Chem. 280(38), 32761 (2005)). FIG. 14B shows a zeolite designed as a platinum atom trap (public domain image of the U.S. Dept. of Energy, Lawrence Berkeley National Laboratory, Berkeley Lab Research Review, Fall 2001). FIG. 14C shows a supramolecularly-assembled 2D ring scaffold synthesized from cyclic alkene subunits (see H. H. Kung and M. C. Kung, "Heterogeneous catalysis: what lies ahead in nanotechnology," Applied Catalysis A: General 246(2), 193 (2003)).

PART III. Options for Fabrication of ssDC Templates for Catalysis Development and Production

Using ssDC to Generate Superior Catalysts

[0065] An application of ssDC is to develop templates and production platforms having geometry inspired by the catalytic centers of enzymes useful for the biofuels industry. Most cellulases and other glycosidic hydrolases perform their functions largely within cleft-like active sites that recognize, bind and position substrates, stabilize and promote the formation of intermediates, and release products (see D. E. Kosland, "Application of a Theory of Enzyme Specificity to Protein Synthesis." Proc. Natl. Acad. Sci. 44 (2), 98 (1958); A. Warshel et al., "Electrostatic basis for enzyme catalysis." Chem. Rev. 106(8), 3210 (2006); and J. B. West et al., "Enzymes as Synthetic Catalysts: Mechanistic and Active-Site Considerations of Natural and Modified Chymotrypsin." J. Am. Chem. Soc. 112, 5313 (1990)). A successful nanoscale catalyst will mimic this geometry and functionality while also utilizing more resilient materials in their construction (see L. Jiang et al., "De Novo Computational Design of Retro-Aldol Enzymes." Science 319(7), 1387 (2008)).

ssDNA Placement Guidelines

[0066] To place ssDNA onto a master shape according to the present invention, the surface of the material can be functionalized for DNA lithography. As one example, physical vapor deposition, in the presence of an electric potential between the solid and plasma phases, can deposit a thin (-50 A) gold layer backed by titanium (-250 A), for the mercaptyl-to-gold anchoring of phosphorothioate modified ssDNA. If using amine backbone-modified ssDNA, the surface of a textured array crafted from SiO₂ can be chemically converted to siloxanes amenable to covalent bond formation to amines. This latter method requires more delicate functionalization to ensure backbone-specific binding due to the presence of primary amines and guanidinium groups on adenosyl, cytosyl and guanosyl bases. Masters can then be lithographed with ssDNA, RNA or PNA. The DNA is synthetic, chemically modified to form
covalent bonds with the master, and added at high (10-25 nm spacing) density in order to serve as a complementary template for patterning a high density addressable array on the platform and/or template. The lithography can be accomplished by using variations on dip pen lithography (where a nanoscale stylus aligns and stretches DNA), laser-mediated pattern lithography onto pre-formed grooves, and/or magnetic and other linear field-mediated patterning of stretched DNA.

[0067] The ability to carry out processes that mimic enzymes requires an accurate understanding of catalysis profiles and the dynamic localization in 3D space of significant chemical and other functions (see W. Wang et al., "BIOMOLECULAR SIMULATIONS: Recent Developments in Force Fields, Simulations of Enzyme Catalysis, Protein-Ligand, Protein-Protein, and Protein-Nucleic Acid Noncovalent Interactions." Ann. Rev. Biophys. Biomolec. Struc. 30, 211 (2001)). An addressable array with a regular, geometric pattern significantly decreases the uncertainty inherent in such localization and uses less complex finite element analysis and vector-based force field calculations when modeling functional activity, dynamic conformations of key catalytic groups, and transition state profiling for optimizing catalysis.

[0068] Because of this expediency, a cartesian (x, y as in FIG. 3) or curvilinear (r, θ as in FIG. 15) pattern of polynucleobases can be permanently lithographed onto masters. This enables straight-forward addressing of physico-chemical functions on the platforms, consistent throughout an array of billions of such forms simultaneously. As mentioned above, geometrically uniform addresses also simplify the computer-based modeling of active site design, orthogonal function behavior, dynamic functional translocation and other activities (see W. Warshel and W. W. Parson WW, "Dynamics of Biochemical and Biophysical Reactions: Insight from Computer Simulations," Quart. Rev. Biophys. 34, 563 (2001)). Most of these are rendered via finite element and vectoral force field based programs which are hampered in resolving a set of input parameters when the spatial coordinates on which those elements and vectors are modeled are disambiguated by irregular coordinates (see M. Garcia-Viloca et al., "How enzymes work: analysis by modern rate theory and computer simulations." Science 303(5655), 186 (2004)).

[0069] FIG. 15 shows an exemplary master shape, comprising permanent ssDNA 151 lithographed in a curvilinear pattern on gold 152.

Guidelines for Anchoring, Stretching and Mounting ssDNA on a Surface

[0070] An important factor in maintaining ssDNA in a stretched state ex facto is the congruency between the nature of the area over which the DNA was stretched, and what is likely artificial modifications made to the DNA in order for it to serve as master material. As mentioned, phosphorothioate-backboned ssDNA on gold can be used for permanent templating (see A. Csaki et al., "DNA monolayer on gold substrates characterized by
nanoparticle labeling and scanning force microscopy,” Nucleic Acids Res. 29(16), 81 (2001). However, a strong reducing potential in the bulk solvent may be required to keep the a-S phosphate groups from forming disulfide bonds with each other prior to stretching (this also helps reduce intra- and inter-strand hydrogen bonding between nucleobases in the DNA, which if not suppressed entangle the strands and inhibit stretching). During stretching and mounting, the redox potential needs to be transitioned to a more oxidized state that promotes a-S-to-gold covalent bonds. However, this preferably should not occur too quickly or else the DNA can prematurely mount to the gold surface and become resistant to further stretching, ruining the construction of geometrically precise addresses. 

Another factor that is considered is the increasing formation of a rare surface phenomenon - as the ssDNA is stretched, a hydrophobic interface bracketed by a symmetric potential difference is formed. This is actually a “pseudo-phase,” more akin to an incompatible colloid interacting with a true solid phase, i.e., a solvated polymer with wall effects. This interface/pseudo-phase envelopes the ssDNA and is formed by the intended backbone-only mounting of natural or modified phosphodiester groups on a recepitive solid phase. As the ssDNA transitions from a coiled coil to a linear polymer, successive backbone residues ‘point down’ and successive nucleobases ‘point up.’ With each successfully stretched and mounted residue, the dipole moment of the surface is integrally increased due to the increasing net potential difference between the higher electron density phosphodiester, phosphorothioate, phosphoramidite (or other backbone) groups, and the amine-rich, generally cationic nucleobases. 

More importantly, if ssDNA or RNA is used, the strongly hydrophobic ribose groups will start to form an “oily mezzanine” due to the tendency of the cyclized sugars to align via ring stacking, water repulsion and Van der Waals interactions. This phenomenon occurs in double stranded DNA in the core of the 3D helix and is partially responsible for dsDNA’s immense strength and tensile cohesion relative to unhybridized ssDNA. When lithographed at high density on a surface, and if hybridized using physiological salts (including Mg^{2+}), the mezzanine formed by the complementary strand will be sufficiently repulsive that Watson-Crick bonds will be preserved even, for example, in the presence of high energy exchange reactions occurring in the bulk organic phase directly proximal to the DNA. Though seemingly bizarre, this advantageous phenomenon enables a wide variety of trans-biotic chemistries in immiscible solvents (e.g., dichoromethane, toluene or hexane), to be executed without damage to the addresses on the platform. 

If PNA is used to lithograph the masters, a hydrophilic pseudo-phase can be formed by stacking of nucleobase cyclic groups. However, this does not have the homogeneity of stacked deoxyribonucelotides, and a potential difference is not necessarily formed. If desired, the latter effect can be constructed by increasing the potential difference between
the nucleophilic tertiary amine backbone of the PNA (with any beta-amino acid except Proline) and, e.g., electrophilic groups like hydroxyl moieties on the solid phase.

Options for Constructing Templates from Masters

[0074] An ssDC template can have the following characteristics in order to serve as a foundation or mold for the mass production of catalytic platforms: 1) the template assumes a positive, projectional shape that fills the entire 3D negative space (the open cleft or helix) of the end product platform - both individually, for each mold unit, and consistently throughout the template array during the production of platforms; 2) the single strand DNA or PNA on the template is an exact mirror-image pattern of the addressable array on the platform, and generates this pattern on the product via complementary base pairing (i.e., A to T and G to C); 3) the template is composed of material that enables permanent integration of DNA, PNA or other nucleobase polymers via backbone covalent bonds that leave nucleobases on both the template and platform free for hybridization; and 4) the template does not warp, bend or otherwise lose integrity over its expected lifetime as a mold that produces platforms. Specifically, the pattern and base chemistry of the ssDNA/PNA is preserved on the template throughout multiple stamping cycles.

[0075] Characteristic 1, 3 and 4 are not of significant concern in the ability of ssDC to produce catalysts in the near future. The state of the art in 3D nanoimprint pattern transfer, in the process from hard cast or beam-lithographed originals to soft cast polymer hardened into a product, is such that the creation of a mold replicating the negative shape of the master (accurate to under one fourth of a cubic nanometer) is a readily available and applied technology. Materials that can survive thousands of stamp-and-lift or rolling cycles for platform production are also straightforward to acquire, apply to this process, and to shape correctly. These materials can also be surface functionalized to accept covalent bonds with artificially-modified DNA, resulting in permanent, mirror-image complementary addresses. For example, vapor deposited gold (atop titanium) can permanently bind phosphorothioate-modified DNA with strength sufficient to survive thousands of hybridization/dehybridization (i.e., inking and stamping) cycles. Another option is siloxane functionalized templates which can permanently bind amine backbone-modified DNA, backed by a similarly hard substance like aluminum that preserves shape integrity (see E. Magni and G. A. Somorjai, "Electron Irradiation Induced Chemical Vapor Deposition of Titanium Chloride on Gold and on Magnesium Chloride Thin Films. Surface Characterization by AES, XPS, and TPD," J. Phys. Chem. 100(35), 14786 (1996)).

[0076] Characteristic 2, however, represents an important and delicate aspect of ssDC, and the quality of which will determine whether or not platforms will undertake catalysis or have consistent combinatorial ability. In short, the ability to lithograph modified polynucleobases like ssDNA or PNA onto a master with sub-nanoscale geometric precision, and to have
those oligomers presented in the correct axial orientation for hybridization, is a key enabling technology for constructing templates.

[0077] The best currently understood, available, and applicable options for template construction are as follows: A) dip pen nanolithography (DPN) of ssDNA onto 3D shapes; B) femtosecond laser-mediated patterning of ssDNA via a sacrificial layer; C) laser-mediated lithography onto pre-formed grooves on a 3D surface; and D) magnetically-mediated polynucleobase alignment across a contoured surface. These options are described in more detail below.

Option A. Construction of Masters by Direct Lithography

[0078] One direct method of placing ssDNA onto 2D or 3D surfaces is by the use of dip pen nanolithography (see J. H. Wei and D. S. Ginger, "A Direct-Write Single-Step Positive Etch Resist for Dip-Pen Nanolithography," Small 3(12), 2034 (2007); and D. Bullen et al., "Parallel dip-pen nanolithography with arrays of individually addressable cantilevers," Appl. Phvs. Lett. 84(5), 789 (2004)). An atomic force microscope (AFM) stylus is used as a partially adsorbant electrostatic solid phase that attracts a charged polymer. ssDNA can be terminally modified for end-specific (i.e., 5' or 3') anchoring onto a determined starting point - which preserves ex facto identification of the DNA strand by determination of its length - then stretched therein. Several ligands are amenable to end-functionalization of ssDNA, including biotin (for high affinity binding to streptavidin), and digoxigenin (for liganture to its antibody).

[0079] After anchoring, ssDNA can then be pulled across a 2D surface based on proper management of the following physico-chemical factors: 1) The induced and intrinsic charge of the AFM tip; 2) The native charge of the polymer, a function of charge density, counter ions, and polymer dipole moment. The latter depends on the orientation of the polymer relative to the AFM tip, particularly if a moment vector exists that is orthogonal to the long axis, as in ssDNA; and 3) The electrostatic and tribological nature of the surface, which includes solvation, charge, charge density and inhomogeneous potentials (and, thus, ssDNA mounting energies) caused by "not perfectly flat" surfaces.

[0080] These factors contribute to, or inhibit, the transitioning of a strand of ssDNA from a coiled coil to a linear form that approaches its theoretical maximum length. When applied to heterogeneous 3D surfaces, other factors come into play that will be elaborated upon below. However, when using DPN on homogeneous 3D surfaces like the master shapes described above, general considerations of polymer dynamics and material qualities are usually sufficient to describe the salient factors. These include: (i) stretching the ssDNA with sufficient force to stretch it, but not break it or the bond between it and the terminal anchor; (ii) stretching the ssDNA with a (+z) vector contribution, i.e., "upwards," particularly over a 3D elevation; (iii) the change in potential vectors that such contours cause, particularly at the
transitions between flat and curved points where potentials tend to "pinch" and change depending on their magnitude and direction; and (iv) if lithographing at high strand density, the potential repulsion or attraction caused by the presence and proximity of ssDNAs already lithographed.

[0081] FIG. 16 shows ssDNA 151 iteratively anchored on DPN-spotted receptor points 163, and stretched linearly over a template shape 152 to produce a cartesian pattern.

Option B. Femtosecond laser anchoring under a Sacrificial Layer

[0082] An alternative to DPN-mediated spotting of anchor functions is the use of femtosecond (Fsec) lasers to remove areas of a sacrificial layer of material that functionalizes a 2D surface and/or the 3D contour that is intended as the master mold (see Y. Dong et al., "Femtosecond pulsed laser micromachining of single crystalline 3C-Sic structures based on a laser-induced defect-activation process," J. Micromech. Microeng. 13, 680 (2003); G. Pellegrini et al., "Technology development of 3D detectors for high-energy physics and imaging," Nucl. Instr. Methods Phvs. Res. Section A: Accelerators, Spectrometers, Detectors and Associated Equipment 487(1-2), 19 (2002); and S. H. Park et al., "Direct Fabrication of Micropatterns and Three-Dimensional Structures using Nanoreplication-Printing (nRP) Process." Sensors and Mater. 17(2), 65 (2005)). The advantages of this approach over DPN may include more precise, faster and simpler placement of smaller anchor points, thus reducing the chances of more than one strand binding to a given location, and sacrificial layers come in a wider variety of compositions than mask-based lithography materials (which underlay the sacrificial layer and form the 3D shape), enabling greater control over surface energy, solvation and tribological factors. Overall, these can improve the ability to stretch and mount ssDNA.

[0083] Given a 20-nm spacing density of 100-mer complement ssDNA, 250 billion strands can potentially be lithographed on 1 sq. cm master (500,000 on a side). Though no estimates for the speed of DPN technology to create this many spots exists, Fsec lasers can theoretically generate this many anchor points on a sacrificial layer in about an hour.

[0084] Fsec lasers are relatively common technology and widely used in the microchip and nanolithography industries. Spatial control is precise to +/- 0.1 nm, and energy deposition (time of exposure) is accurate to +/- 0.1 fsec (100 attoseconds). Targeting is usually controlled in situ by computer controlled potential differences on the diode-based lasing element which elicit quantum effects that alter the emission vector with extreme precision. This eliminates the need for mechanical re-alignment of the laser (a factor impossible to accurize to +/- 1 nm, even with nano-positioners based on standard lasers due to Brownian movements), thus there are no moving parts. Also, no liquid flow-based schemes are required. All of such shortcomings are factors in DPN-mediated spotting of anchor points.
Overall, the use of Fsec lasers facilitates removal of billions of sacrificed points, accurately and evenly, in a very short time.

[0085] FIG. 17 illustrates a similar strategy of stretching ssDNA to that shown in FIG. 16. ssDNA 151 is iteratively anchored on Fsec laser-depleted points 173, via functionalization with SA or anti-DIG 174, on a sacrificial layer 175 and stretched linearly within pre-lithographed grooves 185. Anchor points of strands 4-1 1 describe lithographed confinement grooves 184, and stretched curvilinear or linearly within pre-lithographed grooves 185. Option C. Construction of Masters by Lasers over a Grooved Surface

[0086] Whatever method of anchoring ssDNA is used, an additional factor inherent in the sacrificial or lithographed layer is the option of patterning straight (for cartesian addresses) or curved and axial (for curvilinear addresses) depressions or grooves in which stretching and alignment can be enhanced. Briefly, a linear conformation of even a charged or stretch-resistant polymer, again like ssDNA, is more likely if other conformations are suppressed by factors adequately described as "wall effects" (see J. T. Mannion et al., "Conformational Analysis of Single DNA Molecules Entropically Induced Motion in Nanochannels.", Biophys. J. 90, 4538 (2006); and C. Han et al., "Gradient nanostructures for interfacing microfluidics and nanofluidics." Appl. Phys. Lett. 81(16), 3058 (2002)). Functionalizing the walls with, for example, hydrophobic groups - sufficient to repel the charged nucleobases, but not enough to attract deoxyribose groups - will further maintain a linearized polymer conformation (see H-S Choi et al., "Photopatterning of gold and copper surfaces by using self-assembled monolayers," Curr. Appl. Phys. 7, 522 (2007); and M. J. Tarlov et al., "UV Photopatterning of Alkanethiolate Monolayers Self-Assembled on Gold and Silver," J. Am. Chem. Soc. 115, 5305 (1993)). The "floors" of the grooves can be functionalized appropriately for the type(s) of backbones on the polymer, for example, a net cationic charge for electrostatic binding of plain, anionic phosphodiester backboned ssDNA; gold for [a-S-to-Au bonds] for phosphorothioate-modified ssDNA; or coulomb buffered siloxane for amine-modified ssDNA.

[0087] An important contribution of grooves is that more complex patterns can be produced by photo- or electron-beam lithography. This concept can incorporate Fsec laser-based spotting of anchor points for the iterative addition of homogeneously end-functionalized DNA in a strategy of "sacrifice, functionalize, add DNA, repeat ... stretch." For example, curvilinear grooves can be cut into each of billions of master units in an array. FIG. 18 shows ssDNA 151 iteratively anchored on Fsec laser-depleted points 173 on a 2D surface and 3D master contour 184, and stretched curvilinear or linearly within pre-lithographed grooves 185. Anchor points of strands 4-1 1 describe lithographed confinement grooves.
which facilitate a conformational change of ssDNA from a coiled coil to an extended form prior to placement atop a 3D mold contour. This illustration also implies a method of stretching ssDNA that is based on translocating the other end of the polymer, via a magnetic or weighted polymer bead, inside grooves and across radii, that is independent of stretching via DPN. This will be elaborated upon in Option D below.

[0088] Of note, surfaces can also be patterned with extensions or rises, rather than grooves. Current art of mask-based lithography can incorporate deposition of, e.g., gold atop titanium in lines or other patterns, or conversely use electro-magnetic energy to remove all surface gold except for a desired cartesian or curvilinear pattern for the mounting of phosphorothioate ssDNA.

[0089] Stretching of ssDNA from the functionalized anchor to a terminal point can be facilitated by a paramagnetic bead of approximately 25 nm in diameter, or a latex bead of the same size. Electromagnetic and/or optical fields (the latter via laser "tweezers") can be used to move the bead about the grooved or raised patterns. Field arrays of an electro-optical nature that can accomplish this stretching are described in Option D.

Option D. Electromagnetic Alignment over a Contoured Surface

[0090] Lastly, whatever method of anchoring ssDNA is utilized and irrespective of the nature (or absence) of a sacrificial layer and contours therein, the method of stretching of ssDNA across and over, or about, a 3D surface can be of importance in the construction of masters.

[0091] Considering the lithographed ssDNA, two methods of stretching the polymer can be used: a linear field pulling of anchored ssDNA strands into a progressively confining tunnel that transitions into narrower grooved depressions carved into the 3D shape, as shown in FIG. 18; and/or a varying vector field that pulls a bead around and inside a groove. Regarding the latter, a magnetic or optical field can be rotated around a stationary master, or vice-versa - for example, the master may be secured on a table which articulates about a linear electro-optical field.

[0092] Appropriate magnetic fields can be produced by permanent magnets localized at a distance where the field geometry is linear, or by electromagnets that can be programmed to produce linearly homogeneous fields. The latter can also be dynamic, facilitating translocation of a susceptible bead for the pulling of ssDNA.

[0093] FIG. 19 shows stretching of one ssDNA strand from an Fsec laser-lithographed anchor point 173, and into a confining tunnel that transitions into a 25-40 nm wide groove or rise 185, via linear vector magnetic field lines 196. Removal of a sacrificial layer by multiple Fsec laser pulses can reveal an underlayer which can then be functionalized with digoxigenin, for example. In this example, anchoring of only one DNA strand can be accomplished by addressing of a pre-made construct comprising a (1:1:1:1) molar content ratio of the following components (5'-to-3' orientation): 50 nm anti-digoxigenin-coated latex
bead 197; 5'-DIG—(ssDNA sequence)—3'-Biotin 191; and a 25 nm superparamagnetic streptavidin-coated bead 198.

[0094] Again, grooves can be replaced with risers - which does not necessitate a change in the manner of stretching. However, when not presented with the advantages of confining walls that help maintain ssDNA in a linearized conformation, it is prudent to perform the stretching protocol more diligently with risers. This strategy actually produces a master more amenable to platform generation due to the ease of stamping a positive shape into soft-cast polymer, rather than stamping a negative into which polymer is expected, though not guaranteed, to flow. The latter is akin to the process of Intaglio printing (with the somewhat obvious uncertainties inherent therein when performed at the nanoscale).

[0095] An option for decreasing the chances of modified DNA backbones from forming covalent bonds to the solid phase before the particular residue has been stretched is to confine the unwound ssDNA in a liposome or other temporary enclosure which "spools" the charged polymer and prevents premature surface attachment. This approach has the added advantage of facilitating translocation about a confined space since a relatively "smooth" colloidal enclosure, with intended surface properties, rather than a potentially "sticky" coiled mass of polymer, is being moved about a contoured surface. However, an enclosure has the disadvantage of being limited to fairly short ssDNA segments (250-mer or less - however, this is sufficient for most small active site mimetics), because longer strands can require larger liposomes or other enclosures. This will potentially clog the groove.

Obviously, this is not a problem if stretching is performed onto lithographed raised beams, as long as the fatty acid or other colloidal content of the enclosure does not adversely react to the risers, or detach from the enclosures. Gold is not particularly susceptible to contamination with anionic or cationic fatty acids, and the problem is partially ameliorated if the enclosure presents a surface that is unreactive to gold or the intervening surface, i.e., is composed of a micelle-like structure covered by an immiscible scaffold.

[0096] Colloids having strong, inducible dipole moments, super paramagnetic and some ferromagnetic properties (though the latter are mostly polymers functionalized to magnetic beads) are available and can be induced to form a uniform vector magnetic moment. After cross-linking in a heterogeneous mix of other colloids, or polymerization, exposure to an approximately one Tesla linear magnetic field will align the magnetic moments of the monomers and produce a structure that is susceptible to translocation by another magnetic field that is used for stretching. Alternatively, enclosures can be decorated with, or attached to, a super paramagnetic bead for more simplified manipulation, translocation and stretching of the DNA.

[0097] Besides alignment of magnetic moments, there is another important need for the partial polymerization of the "magnetosome." As ssDNA is pulled out of the enclosure by
stretching and mounting, it is necessary for solvent molecules to enter the interior from the 
bulk phase in order to equalize hydrostatic pressure on the enclosure. Fatty acids and other 
long chain molecules that form nearest-neighbor covalent bonds in the presence of UV light 
can be used to form the cage-like spherical lattice with porosity that allows DNA to escape, 
and water molecules and salts to enter. The overall result is ideally a sphere of enclosed 
ssDNA within a reducing aqueous environment, shielded by a hydrophobic core abutted by a 
non-reactive aqueous periphery, the entire complex of which can be translocated by an 
induced magnetic field for pulling inside a groove, or atop a lithographed rise. 

[0098] FIG. 20 shows stretching of a phosphorothioate-modified ssDNA strand 201 onto 
lithographed gold 202 (with contour 203), via unwinding and unraveling from a colloidal 
enclosure susceptible to translocation in a magnetic field 196. The bead 207 can comprise a 
liposome fabricated with a hydrophobic core 204 and hydrophilic periphery 205 scaffolded 
with latticed and superparamagnetic polymers doped into the lipid mix. A unidirectional 
magnetic moment of the scaffold (cartesian pattern) can be induced by an approximately 
one Tesla or greater field after polymerization in UV light to form a hollow shell. 
Alternatively, a larger (e.g., 250 nm diameter) paramagnetic bead can be functionalization 
with the same liposome enclosure. Lattice work, in this case, can serve to mechanically 
maintain the enclosure. 

[0099] The present invention has been described as a method of single strand dimensional 
construction. It will be understood that the above description is merely illustrative of the 
applications of the principles of the present invention, the scope of which is to be determined 
by the claims viewed in light of the specification. Other variants and modifications of the 
invention will be apparent to those of skill in the art.
CLAIMS

What is claimed is:

1) A process of generating a three-dimensional addressable array of biopolymer nucleic acids, comprising:
   a. providing a nanoscale three-dimensional master shape having a surface, and
   b. binding a plurality of master biopolymer nucleic acids to the surface of the master shape in a regular pattern.

2) The process of claim 1, wherein the binding step b) comprises binding the master biopolymer nucleic acids to the surface of the master shape using dip pen lithography.

3) The process of claim 1, wherein the binding step b) comprises binding the master biopolymer nucleic acids to the surface of the master shape using laser-mediated pattern lithography.

4) The process of claim 3, wherein the surface comprises a plurality of grooves or risers and wherein each of the master biopolymer nucleic acids is bound to a groove or riser.

5) The process of claim 1, wherein a susceptible mass is attached to a terminus of each master biopolymer nucleic acid and binding step b) comprises binding each master biopolymer nucleic acid to the surface of the master shape using magnetic field patterning.

6) The process of claim 1, wherein the nanoscale three-dimensional master shape comprises a cylinder, trough, cleft, or compound helix.

7) The process of claim 1, wherein the master biopolymer nucleic acid comprises DNA, PNA, RNA, or LNA.

8) The process of claim 1, further comprising hybridizing a plurality of complementary template biopolymer nucleic acids to the plurality of master biopolymer nucleic acids on the master shape.

9) The process of claim 8, further comprising binding a surface of a template shape to the plurality of complementary template biopolymer nucleic acids and removing the template shape with the bound complementary template biopolymer nucleic acids from the master shape.

10) The process of claim 9, wherein the template binding comprises impression molding.

11) The process of claim 10, wherein the template array being replicated is transferred to a roller or a flat stamp.

12) The process of claim 9, wherein the template binding comprises pressure molding.

13) The process of claim 12, wherein the pressure molding comprises adding pressure to an elastic substance configured as a bladder.
14) The process of claim 9, further comprising hybridizing a plurality of complementary platform biopolymer nucleic acids to the plurality of template biopolymer nucleic acids on the template shape.

15) The process of claim 9, further comprising binding a surface of a platform shape to the plurality of complementary platform biopolymer nucleic acids and removing the platform shape with the bound complementary platform biopolymer nucleic acids from the template shape.

16) The process of claim 1 or 15, further comprising hybridizing the master or platform biopolymer nucleic acids with complimentary probes comprising reactive chemical groups.

17) The process of claim 16, wherein the reactive chemical groups comprise amino acids.

18) The process of claim 16, wherein the reactive chemical groups mimic an enzyme active site.

19) The process of claim 16, wherein the reactive chemical groups provide a catalyst.

20) A three-dimensional (3D) addressable array comprising a plurality of biopolymer nucleic acids bound to the surface of a 3D shape in a regular pattern.

21) The 3D addressable array of claim 20, wherein the surface comprises metal, plastic, or polymer.

22) The 3D addressable array of claim 20, wherein the metal comprises gold.

23) The 3D addressable array of claim 20, further comprising a complimentary probe hybridized to each of the plurality of biopolymer nucleic acids, each of the complimentary probes comprising a reactive chemical group.

24) The 3D addressable array of claim 23, wherein the reactive chemical groups comprise amino acids.

25) The 3D addressable array of claim 23, wherein the reactive chemical groups mimic an enzyme active site.

26) The 3D addressable array of claim 23, wherein the reactive chemical groups provide a catalyst.
FIG. 7
# International Search Report

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<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<td>X</td>
<td>US 2005/0088980 A1 (Cubicciotti) 28 April 2005 (28 04 2005) para [0021], [0022], [0078], [0195], [0265], [0281], [0463], [0530]</td>
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Further documents are listed in the continuation of Box C.

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