The advances in biotechnology and nanotechnology are spawning a new and exciting manufacturing tool – bionanofabrication – which enables revolutionary ways of building complex bionanostructures on surfaces with nanometer precision. Here, we highlight the opportunities and challenges in the development of this emerging technology and discuss its use in genomics, proteomics, nanostructures, nanomaterials, drug discovery, and synthetic biology. To provide useful insights into the future directions of bionanofabrication, current developments in characterization techniques, fabrication methods, and their integration and control in manufacturing processes are discussed.

The excitement generated by nanotechnology derives from the promise of manipulating matter atom-by-atom and molecule-by-molecule to create devices with performances and functionalities that are orders-of-magnitude better than those provided by current manufacturing technologies. The rapid development of nanoscale patterning and lithography technologies plays a substantial role in realizing this dream. The invention of synthetic photosensitive compounds, e.g. poly(vinyl cinnamate)-based negative photoresists and diazoquinone-based positive photoresist, in the 1930s laid the foundation for many modern-day lithography and etching techniques used in microfabrication. For years, devices such as microprocessors and microelectromechanical systems (MEMS) have been machined out of crystalline Si by lithography (e.g. photolithography, X-ray lithography, and electron- and ion-beam lithography), as well as by bulk micromachining processes such as wet and dry chemical etching, SiO₂ growth, and vapor deposition.

While engineers and scientists race to shrink the size of transistors and MEMS components through nanofabrication to create the next generation of high-performance electronic devices, biologists and life scientists have just begun to employ micropatterning and, to a more limited extent, nanopatterning techniques to build high-throughput detection systems for genomic and proteomic studies.
Bionanofabrication lies at the intersection of nanotechnology and biotechnology, and in many cases can trace its origins back to technologies that are at least a century old. Many strategies employed in bionanofabrication, especially those for nanolithography and nanopatterning of biomolecules (top-down nanofabrication), can trace their origins to analogous strategies in conventional micro- or nanofabrication. For example, soft lithography, used to deposit biomolecules over a large area in a parallel fashion\textsuperscript{10}, is an analog of photolithography used to create transistors on a Si substrate, and arguably owes much to methods developed in the printing industry. Similarly, dip-pen nanolithography (DPN) directly places biomolecules on surfaces at the nanoscale using micromachined cantilevers of an atomic force microscope (AFM)\textsuperscript{11} and is the nanoscale analog of writing with quills.

The early development of bionanofabrication has largely focused on the creation of biologically active structures for biosensing and diagnostics by fairly straightforward modifications and, in some cases, the extension of conventional nanofabrication techniques. More recently, there is a growing interest in using biomolecules as active components in nanofabrication processes and the development of new biomolecule-based materials systems. For example, enzymes can be deposited to synthesize and excavate nanostructures of nucleic acids and proteins at the nanoscale\textsuperscript{12,13}. In addition, the use of biomolecules for nanoscale assembly has become a promising and effective solution to difficult positioning problems in the fabrication of nanostructures (bottom-up nanofabrication).

The capability of bionanofabrication to manipulate biomolecules with nanometer precision on surfaces in a massively parallel fashion is potentially revolutionary because it would enable the creation of novel devices with performance metrics that go beyond those currently envisioned. However, the direct application of nanofabrication techniques and processes to realize the potential of bionanofabrication remains a difficult, though, in our opinion, not insurmountable task. In this review, we highlight the opportunities and challenges that researchers and scientists face in developing bionanofabrication processes. We provide specific examples of the uses of bionanofabrication in the areas of biosensing, nanomaterials, drug discovery, and synthetic biology. Next, we discuss current developments in bionanofabrication techniques and processes. Finally, we outline potential future directions, with particular emphasis on fabrication processes and their control, integration, and characterization.

**Opportunities and challenges**

The incorporation of ‘soft-wet’ biological components into conventional nanofabrication platforms designed and built for ‘hard-dry’ semiconductors, conductors, and dielectrics brings both new opportunities and challenges since biomolecules possess some unique properties:

- A wide range of biomolecules, including nucleic acids, proteins, lipids, and oligosaccharides, react with other biological components by molecular recognition\textsuperscript{14,15}, which is important for bottom-up nanofabrication based on self-assembly;
- Enzymes can catalyze the synthesis and removal of both biological and synthetic molecules\textsuperscript{16,17};
- Biomolecular reactions (biotransformations) are highly selective and site-specific\textsuperscript{17}. There are many enzymes that cleave DNA at particular sites (restriction enzymes) and link two pieces of DNA together (ligases)\textsuperscript{17}. Proteases that digest proteins at specific sites\textsuperscript{16,17} and enzymes that add functional motifs to proteins\textsuperscript{18} are just a few examples of protein-modifying enzymes;
- Biomolecular reactions are often highly efficient under physiological conditions, so the yields of biotransformations are substantially higher than those by chemical syntheses\textsuperscript{19,20}; and
- The use of biomolecules and biological processes is often environmentally friendly, so treatment of the waste products can be minimal and the by-products pose little health risk compared to the reagents used in semiconductor processing.

On the other hand, there are also significant constraints associated with the use of biomolecules in bionanofabrication. These are:

- The ultrahigh-vacuum (UHV) conditions used in conventional nanofabrication approaches are incompatible with biomolecules, whose function and structural integrity in most cases are destroyed in a high-vacuum environment;
- Even in an aqueous environment, many proteins can easily lose their native, active conformation after deposition because they can unfold and bind nonspecifically to a surface; and
• Biological reactions, with some notable exceptions\textsuperscript{21,22}, must take place in an aqueous solution or buffer, which limits their uses in many electronic applications.

**Current applications and potential uses**

The unique challenges posed by the use of biomolecules in nanofabrication are substantial, but surmountable. To fabricate efficiently with biomolecules and make use of their unique properties, a radical shift away from the high-energy, high-vacuum processing paradigm of conventional micro- and nanofabrication is needed. Although the field of bionanofabrication is still in its infancy, its impact and promise go far beyond biological applications where creative uses of biomolecules remotely resemble those envisioned by biologists who originally studied and isolated them.

**Genomics and proteomics**

Early implementation of bionanofabrication targets genomics, proteomics, and high-throughput biosensing and diagnostic applications. Bionanofabrication strategies are used to reduce the spot size of the deposited biomolecules, to increase the spot density of arrays, and to improve the throughput of deposition processes\textsuperscript{23-25}. Through miniaturization of diagnostic devices and equipment, the cost efficiency, speed, and accuracy of micro- or nanoarray-based detection technology can be improved substantially by reducing the amounts of reagents required, the reaction volumes, and detection errors\textsuperscript{26}.

**Nanostructures and nanomaterials**

As bionanofabrication becomes increasingly mature and sophisticated, more and more research efforts will turn to exploit the intrinsic functionality of biomolecules to create new nanostructures, processes, and nanomaterials systems. For example, bacterial S-layer proteins\textsuperscript{27,28} and DNA tiles (Fig. 1)\textsuperscript{29} self-assemble into crystalline two-dimensional templates with highly controllable and predictable periodic features for patterning metal nanostructures. The assemblies have potential applications in DNA computing and nanoelectronics\textsuperscript{30-32}.

Recently, the direct deposition of biomolecules has gained considerable attention, because it allows the precise placement of biomolecules at the nanoscale. A variety of biomolecules such as collagen\textsuperscript{33}, oligonucleotides\textsuperscript{34}, biotin-streptavidin nanostructures\textsuperscript{35}, elastin-like polypeptides (ELPs)\textsuperscript{36}, and DNase I\textsuperscript{12} can be deposited to create bionanostructures by DPN for biosensing and nanoscale surface modifications. In addition, the roles of novel biomolecules in miniaturized fluidic systems, and potentially nanofluidics, are exemplified by the recent use of thermally or pH-responsive proteins as biological actuators in microfluidic systems\textsuperscript{37}.

**Drug discovery and synthetic biology**

More recently, new and exciting opportunities for bionanofabrication have emerged in the fields of synthetic biology and recombinant protein engineering. The firm integration of recombinant protein engineering techniques such as phage display\textsuperscript{38}, directed evolution\textsuperscript{39,40}, and combinatorial libraries\textsuperscript{41,42}, as well as the discovery and isolation of novel proteins from extremophiles (microbes that thrive in extreme environments)\textsuperscript{43,44}, would further strengthen bionanofabrication approaches by providing

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*Fig. 1 DNA-based bionanofabrication by self-assembly of DNA molecules. (A) DNA strands, which have complementary sticky-end overhangs, self-assemble into a branched junction. (B) These branched junctions can further self-assemble into DNA nanogrids owing to the orientation of the complementary sticky ends, as shown in the AFM images of DNA lattices. (Reprinted with permission from\textsuperscript{102. © 2003 AAAS.})*

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proteins with improved thermal stability, binding specificity, and solvent compatibility.

This integrated approach, albeit at the microscale, is exemplified by a recent report that micropatterned DNA arrays could be used as templates for high-throughput gene synthesis and protein expression (Fig. 2)\(^{45}\). In this specific example, the use of patterned oligonucleotide sequences is extended to drug discovery and lead validation, also providing a valuable tool for systems biology. This technology can easily be applied and adapted to DNA nanoarrays, significantly improving their throughput and processing capability.

Another emerging trend is the use of recombinant nanostructured biocompartments such as vesicles, viruses, and phages, which allow predefined functional biological components to self-assemble into a sophisticated self-contained system. These biocompartments are ideal for bionanofabrication. They not only serve as nanoscale reaction chambers for mineralization and metallization\(^{46,47}\), but also provide a means to introduce docking sites to spatially direct the assembly of biological, metallic, and semiconducting nanostructures (Fig. 3)\(^{48}\).

Finally, another major opportunity for bionanofabrication lies in harnessing the amazing biotransformation capabilities of enzymes to potentially synthesize semiconducting materials. For example, silicatein filaments and subunits have been isolated in a marine sponge and were shown to catalyze polymerization of silica and Si at low-temperatures\(^{19,20}\). This discovery heralds the future development of Si deposition at both micro- and nanoscales with novel enzymes under ambient conditions, and blurs the distinction between biological and nonbiological components used in bionanofabrication.

**Current developments in techniques and processes**

**Taking soft lithography down to the nanoscale**

Soft lithography was first developed in the Whitesides laboratory at Harvard in 1993\(^{49}\). Initially, the process was termed microcontact printing (µCP), in which polydimethylsiloxane (PDMS) stamps with micron features were cast against negative masters generated by photolithographic processes\(^{50}\). These stamps were coated in alkanethiol inks and used to create self-assembled monolayers (SAMs) on substrates through conformal contact in a massively parallel fashion. The technique has spawned many other related techniques and variations\(^{10,51}\).

Most notably, in the context of this review, soft lithography was first used to pattern proteins in a direct print fashion in 1998\(^{52}\); chicken immunoglobulins (IgGs) were directly patterned onto glass and polystyrene surfaces at the
nanoscale for protein recognition, and on polymers using the biotin-streptavidin adapter system. In addition to PDMS, other materials are being studied for their applicability to pattern biomolecules. By increasing the strength of the stamps used for printing, feature sizes of µCP can be reduced to less than 100 nm (nanocontact printing, nCP) by avoiding the complication of stamp collapse. For example, stamps made out of polyolefin plastomers (POPs) exhibit better mechanical stability than PDMS and are able to print proteins, particularly fibrinogen, down to 100 nm feature sizes. The development of protein patterning was further extended to the printing of multiple proteins using microchannels and single proteins (various antibodies) on a surface (line widths <100 nm) (Fig. 4). These studies highlight the potential impact and current limits of soft lithography for construction with proteins at the nanoscale.

Soft lithography has also been used to directly pattern DNA. For example, Xiao and coworkers used µCP with precise alignment to fabricate and synthesize oligonucleotide arrays on surfaces in a step-wise, single-nucleotide fashion for DNA hybridization studies. Another study demonstrated the direct printing of DNA-surfactant molecules on the microscale. More recently, patterning of 20 bp oligonucleotides and 500 bp and 1600 bp PCR fragments was accomplished with a feature size of about 800 nm. These studies show how soft lithography can be used to fabricate DNA arrays and point toward the simultaneous printing of multiple oligonucleotides.

**Scanning probe lithography for bionanofabrication**

DPN is a versatile scanning probe-based technique for fabricating both organic and biomolecular nanostructures. In general, DPN involves the coating of an atomic force microscope (AFM) cantilever tip with the desired molecule to be transferred. Once the tip is brought into contact with the target substrate, the molecules assemble on the surface because of their specific or nonspecific affinity. When the tip is translated across the surface, the molecules are deposited in the wake. Originally, this technique was used to directly deposit organic molecules on surfaces at the nanoscale, e.g. an alkanethiol onto Au, an organosilane onto Si, and a positive onto a negative polyelectrolyte, or vice versa. This approach was made more generic in later work by nanopatterning a SAM of 16-mercaptohexadecanoic acid (MHA) on Au surfaces to serve as a template for nanopatterning biomolecules. The advantage of this approach is that MHA can be reproducibly nanopatterned by DPN and is easily functionalized with biomolecules by reaction with amine groups, therefore the DPN process does not have to be optimized empirically for each combination of substrate and ink. For example, immunoglobulin G, lysozyme, biotin-streptavidin, ELP (Fig. 5), alkylamine-modified DNA, and cowpea mosaic virus could be nanopatterned with feature sizes on the order of 100 nm by this approach.

Direct-write DPN, though more difficult, has also been developed for the deposition of biomolecules onto surfaces. For example, a hexanethiol-modified oligonucleotide ‘ink’ was deposited on Au and oxidized Si substrates, with feature sizes from 200 nm to microns. Similarly, this strategy was applied to DNA labeled with fluorescent molecules of different colors for direct optical detection. This direct-write approach is also applicable to proteins, demonstrated by the successful deposition of thiolated collagen and collagen-like peptides onto Au substrates, generating 30 nm wide line patterns for cell binding assays. This approach has been extended to catalyze biotransformations with nanoscale spatial precision; in the first example of this approach, DNase I molecules were deposited locally by DPN to digest an oligonucleotide SAM with nanoscale precision (Fig. 6). Conversely, this strategy should also be applicable to create DNA nanostructures enzymatically, for example, by terminal
deoxynucleotidyl transferase, which repeatedly adds mononucleotides to the 3’ end of oligonucleotide initiators (Fig. 7)\textsuperscript{68}.

In a complementary bionanofabrication approach that was demonstrated on peptides, a \textit{Staphylococcal serine V8} protease-functionalized AFM probe was used to digest carboxylic acid groups of peptides coated on a surface substrate\textsuperscript{69}. In another study, streptavidin-conjugated enzymes were attached to the tip of an AFM cantilever coated with biotinylated groups, by scanning on surface substrates coated with an enzyme of choice. Using this technique, a probe functionalized with alkaline phosphatase was prepared and used to locally generate nanostructures of a water-insoluble complex in the presence of its substrate and cofactor (Fig. 8)\textsuperscript{13}. These prototypical bionanofabrication approaches demonstrate the capability of DPN for both creating and excavating bionanostructures.

One of the limitations of DPN is the requirement of reinking for patterning biomolecules on a large area. A technique called anodization lithography, in which an electric potential is applied between a conducting substrate and an AFM cantilever to oxidize the area beneath the tip\textsuperscript{70}, has been developed. An attractive feature of anodization lithography is the elimination of an ink from the process, so that nanopatterned templates can be generated with high-spatial resolution in a single uninterrupted scan. This approach is particularly powerful for bionanofabrication if the anodization reaction is carried out on a protein-resistant material. It generates nanoscale patterns of reactive groups...
to which protein molecules can be conjugated, while ensuring minimal nonspecific protein adsorption in the background. Bovine serum albumin (BSA) dot patterns with a feature size as small as 26 nm have been fabricated with this strategy.

Along a similar line, nanopatterns of biomolecular nanostructures can be generated on resist-thiol-precoated Au substrates by mechanical removal of resist molecules (nanoshaving) followed by subsequent chemical modifications, or replacement of resist molecules by biomolecules in the solution in which nanoshaving is performed (nanografting). The nanografting strategy is applicable to a variety of proteins as demonstrated by the fabrication of 40 × 40 nm² to 200 × 250 nm² square patterns of BSA, lysozyme, and rabbit IgG using this technique. In addition, nanografting of DNA-derivatized Au nanoparticles on Au surfaces at below 100 nm lateral resolution has also been demonstrated. However, nanografting is often limited by its slow patterning speed. This problem can be overcome by maintaining a small droplet of the desired solution between an AFM tip and a hydrophilic surface (instead of submerging the AFM tip in solution) while nanopatterning is performed. This technique is known as meniscus force nanografting, which is capable of generating nanopatterns with a lateral resolution as low as 60 nm on Au surfaces.

**Nanopipettes for bionanofabrication**

Nanopipettes are micromachined pipettes that typically have inner diameters down to 3 nm. Pioneering work with nanopipettes as force sensors in scanning probe microscopy led to their use to transfer a liquid to a surface in a controlled fashion. For example, this technique was used to deliver an etchant onto a Cr layer to produce trenches with line-widths down to 100 nm. Bruckbauer and coworkers were the first to report the deposition of biotinylated single-stranded DNA onto a streptavidin-functionalized surface and of multiple biological entities onto unmodified surfaces by applying a voltage bias between the surrounding aqueous medium and the solution inside the nanopipette, based on scanning ion conductance microscopy (SICM).

In later work, nanopipettes were used in an ambient environment for the direct printing of protein G at a linewidth of 500 nm on an aldehyde-functionalized surface and green fluorescent protein (GFP) onto a BSA-coated surface at ~250 nm. In a different study, a protease, trypsin, was delivered through a nanopipette to locally digest adsorbed BSA, resulting in holes that were 340 nm in width and 660 nm in depth. To better control the channel size of nanopipettes, a focused ion beam was used to create a nanochannel inside an AFM tip to serve as a reservoir that acted like a nanopipette, as demonstrated by the deposition of polystyrene nanoparticles in attoliter volumes to both hydrophobic and hydrophilic surfaces. This approach should also be applicable to the deposition of biomolecules on a surface.

**Future directions**

Although the field of bionanofabrication has undergone impressive advances, it is still in its infancy and requires significant research and development to fully realize its potential. Below, we outline what we believe to be key areas that will see significant advances over the next few years.

**Fabrication processes**

Current research initiatives are focused primarily on adapting existing nanolithography techniques, which are often slow and serial, to deposit biomolecules. The majority of the
research efforts are engaged in prototyping the deposition and excavation of biomolecules through single-step biotransformation processes, while little attention is currently paid to parallel, multiple-step processing. The next challenge in bionanomanufacturing will be the integration of these individual processes into an overall process strategy that allows the fabrication of devices and systems designed for real-world applications.

For soft-lithography and scanning-probe-based bionanofabrication techniques, the improvement of feature resolution is still one of the most important challenges, as it limits the patterning of biomolecules with nanometer resolution. Empirical optimization of these methods, while useful, must be supplemented by fundamental studies that combine the physical chemistry and mechanics of these processes. For scanning-probe-based approaches, transport-related factors such as surface diffusion, scan speed, probe-ink interaction, substrate-ink interaction, and ambient humidity must be quantified and controlled to maximize resolution, fidelity, and process reliability. For soft-lithography approaches, the aspect ratio of the stamp features, printing contact time, and mechanical properties of the stamp and substrate need to be evaluated as they are the predominant factors controlling resolution.

**Process control**

Another major issue in bionanofabrication is the need to radically increase process throughput. While scanning-probe-based approaches are inherently serial, great strides are being made toward the implementation of massively parallel cantilever arrays that work either passively or actively in modifying surfaces to improve throughput. However, substantial challenges remain in controlling individual cantilevers in these arrays. These massively parallel approaches also require more sophisticated process control systems that aid in locating and aligning nanostructures repeatedly and reliably. Since positioning accuracy depends largely on the performance of sensing elements, there is an urgent need for better nanoscale metrology technologies.

Bionanofabrication approaches that are based on the sequential deposition and/or removal of biomolecules require continuous position confirmation and realignment. One approach to achieving this is by fast AFM imaging (<1 s), which allows for a quick alignment registration between fabrication steps. Alternatively, AFM can be used in conjunction with confocal microscopy to locate patterned areas optically. For the registration of multiple-step stamping processes with μCP or nCP, an innovative solution employs a lock-and-key type alignment after each fabrication step.

An area that deserves further research is the repeated inking of the tip or stamp with multiple biomolecules, which is crucial to increasing process throughput. One approach to deal with this problem is to create a PDMS stamp with positive features with differential heights, so that various biomolecules can be patterned when the applied force is varied. Another approach focuses on the development of instruments and techniques that use micro- or nanofluidic networks to deliver multiple inks in the proximity of the patterned area.

**Process integration**

A significant bottleneck in bionanofabrication is the lack of seamless integration among fabrication and assembly steps that operate at length scales ranging from the molecular to the millimeter scale. Research on bionanofabrication remains fragmented with most endeavors focused on individual processes, although integration among different manufacturing platforms and control systems is much needed.
needed. A vision of a hierarchical, multiscale bionanofabrication process involving several (bio)nanofabrication steps is shown in Fig. 10. One can imagine that bionanostructures could be created through step-wise biotransformations using fast scanning or optical detection for alignment, together with sophisticated control algorithms. These nanoscale transformations could be observed and controlled by relaying optical, chemical, or electrical signals to and from the macroscopic world. However, the fusion of the nanoscale world with the macroscale world is far from trivial, and remains a grand challenge in bionanomanufacturing.

Characterization and analysis

Current implementations of bionanofabrication processes are severely limited by the lack of ability to thoroughly characterize and analyze the bionanostructures that are created by these processes. The structural complexity of biomolecules, their fragility, and their need to remain bathed in water to retain their function often preclude the use of techniques routinely employed in the characterization of metallic and semiconducting materials. The small amounts of analytes and their confinement to substrate surfaces further restrict the choice of analytical techniques and render many commonly used molecular biology techniques impractical or useless. Therefore, much of the current characterization for bionanostructures is based on physical attributes such as height, width, friction, or mechanical strength. The advent of in situ analytical techniques such as environmental scanning electron microscopy (eSEM)\textsuperscript{99}, cryo-transmission electron microscopy (cryo-TEM)\textsuperscript{100}, and near-field scanning optical microscopy (NSOM)-based techniques\textsuperscript{101}, which do not require elaborate sample preparations and modifications, would potentially allow more meaningful characterization of bionanostructures.

Summary

Building on the technological foundation of nano- and microfabrication, bionanofabrication offers us enabling technologies that will undoubtedly transform the current state of biosensing, nanomaterial development, drug discovery, and synthetic biology. To this end, we must tackle the daunting task of overcoming all the challenges in dealing with biomolecules, as well as in resolving the problems that impede fabrication processes, their process control and integration, and characterization of the resulting nanoscale structures and devices. Attempting these tasks, however, is clearly worthwhile because of the enormous benefits that can be gained by harnessing the unique properties biomolecules offer, enabling the creation of nanotools and devices with functionalities and properties that rival those built with their nonbiological counterparts. \textit{NT}

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