Biofabrication: using biological materials and biocatalysts to construct nanostructured assemblies

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Emerging opportunities are placing greater demands on device fabrication: next-generation microelectronics will need minimum features of less than 100 nm, high-throughput drug screening will require facile methods to incorporate sensitive biological components into microelectromechanical systems (MEMS), and implantable devices will need to be built from biocompatible materials. Increasingly, these emerging demands are being addressed by combining traditional microfabrication methods with ‘biofabrication’: namely, the use of biologically derived raw materials and biocatalysts. Recent fabrication techniques are using biological construction materials as process aids or structural components, and enzymes are being considered for their potential to fabricate devices with high selectivity under mild conditions. If incompatibilities between biology and microfabrication can be eliminated, then biofabrication will be poised to emerge as the standard for nanoscale construction.

The marriage between biology and microfabrication has led to the integration of biologically active components (enzymes, antibodies, nucleic acids and cells) into simple devices. This marriage has revolutionized biosensing, genome and proteome analysis, and drug discovery. In the past, microfabrication has been viewed as the stronger partner: it has been responsible for the relentless increases in computing power observed in the past half-century, and methods (although sometimes cumbersome) are emerging to accommodate sensitive biological components. But traditional microfabrication, which is built on photolithography, might be approaching its limits. Although photolithography continues to achieve reductions in feature sizes (which are currently smaller than 100 nm), this reduction comes at great expense.

Questions are being raised about whether conventional ‘top-down’ fabrication will be able to construct at the nanoscale, and even whether it’s time for a paradigm shift. The search for the next standard often leads to biology for inspiration and, increasingly, for the raw materials. Thus, the marriage of biology and microfabrication is now entering a new phase in which biology is contributing more than just the sensing components and instead is beginning to assume a larger role in solving the challenges associated with fabrication at the nanoscale [1,2].

In this review, we discuss recent trends in which fabrication is using biological construction materials as process aids or structural components. We then consider opportunities for exploiting enzymes to fabricate with high selectivity under mild conditions. Finally, we suggest the potential of eliminating existing incompatibilities between biology and microfabrication.

Overview: what can biology contribute to fabrication?
Microelectronic devices and MEMS are traditionally fabricated from inorganic materials, such as silicon and metals, and in some cases from organics. The development of biosensors requires the integration of biologically active components. As shown in Figure 1, there is a growing list of examples in which biology is contributing more than simply the activities for recognition and detection. Biologically derived raw materials, such as lipids and biomacromolecules, are beginning to be exploited for construction. This trend towards biological construction materials is driven, in part, by the observation that biology assembles these materials with nanoscale precision; for example, lipid bilayers have a thickness of 4–5 nm, whereas the diameters of DNA and microtubules are 2 and 25 nm, respectively.

Another key driver for using biological construction materials in microfabrication is the fact that they allow access to a broader range of assembly options. These options, as shown in Figure 1, can augment or replace the traditional microfabrication options of photolithography and organic or inorganic chemistry. Because biological materials have various precisely controlled physicochemical properties, such as charge and hydrophobicity, their assembly can be directed through a range of stimuli. Rather than limiting assembly to light-based stimuli, biological components can be directed to assemble through various fields (e.g. electric) and gradients (e.g. pH). In addition, biological materials are endowed with capabilities for self-assembly that facilitate ‘bottom-up’ self-fabrication [3–5]. This self-assembly can result from ‘simple’ thermodynamics, such as in lipid bilayers, or

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can be guided by molecular recognition, for example, by viral coat proteins. Finally, biological components can be acted on by biocatalysts, which can facilitate assembly through highly selective enzymatic reactions that can be performed in water under mild, physiological conditions.

The integration of biological components should assist in the construction of a diversity of micro- and nanostructured products. Figure 1 provides three examples of the use of biological components to construct end products containing different amounts of biologically derived materials. First, biological components can be exploited as processing aids to allow the bottom-up fabrication of next-generation microelectronics. These products might ultimately contain no biological components; for example, biological components have been used in the creation of field effect transistors based on carbon nanotubes [6] and nanoscale semiconductor wires [7]. Second, a small amount of biologically active components can be assembled within a ‘traditional’ device to exploit the high-throughput and massively parallel capabilities of MEMS (e.g. for drug screening). Last, microscale factories can provide the localized stimuli necessary to coordinate the assembly of ‘soft’ nanostructured products that consist primarily (or completely) of biological components for implantation in the body (e.g. artificial organs) [8,9].

Above we have provided an overview of the use of biological materials in microfabrication, but to understand the potential, it is necessary to consider the details. In the following section, we cite a series of examples in which biological construction materials have been used to facilitate assembly at the micro- and nanoscale.

Exploiting biologically derived construction materials

Biology creates molecules and macromolecules with an unparalleled level of structural control. For example, proteins and nucleic acids are biosynthesized with a precise control of sequence, linkage and molecular weight—a level of control that is unachievable through chemical synthesis. The precise structure of natural materials endows them with impressive functionality, such as selective recognition and catalysis, whereas the ability to manipulate structure controllably (i.e. to alter sequence) allows properties to be engineered for specific functions.

Table 1 lists three broad characteristics of biological materials that can be exploited for fabrication: their precisely controlled structures, their physicochemical properties, and their capabilities for self-assembly based on molecular recognition.

Precisely controlled structure and size

DNA is a long, linear polymer that can be stretched to lengths on the order of micrometers and sometimes even millimeters. Because of its high aspect ratio, DNA has been exploited as a template for the assembly of metal ions into conducting wires that are 12 μm long and 100 nm wide [10]. Extensions of this work have enabled metal templating to be controlled along the length of DNA through molecular lithography [11]. Furthermore, a field effect transistor comprising carbon nanotubes has been created by combining DNA templating with assembly [6].

The ability to create fusion tails provides a powerful means by which to engineer proteins to facilitate their subsequent assembly. The familiar example is the hexahistidine sequence that directs the assembly of fusion proteins onto surfaces containing immobilized metals,
such as affinity columns. Alternative fusion tails exploit cysteine residues for thiol-based assembly onto metal surfaces [12], and fusion tails can exploit electrostatic [13] or biospecific (e.g. avidin [14] and cellulose binding [15]) assembly mechanisms. Finally, it is important to note recent efforts to evolve peptides that can bind to semiconductor materials [16].

**Physicochemical properties**

Biological materials offer a range of physicochemical properties: lipids are amphiphilic, DNA is anionic, and proteins show a range of hydrophobicity and charge depending on their amino acid sequence. In some cases, these physicochemical properties promote self-assembly; in others, they allow manipulation by external stimuli to guide transport and to direct assembly.

Numerous investigations have focused on exploiting the capabilities of lipids to self-assemble into bilayers (vesicles and membranes). Some of the goals are to use lipid or lipid-like amphiphiles to create addressable surface regions [17], to immobilize enzymes [18] and ligands [19], and to generate nanopores [20,21]. In addition, the self-assembling capabilities of lipids are being examined to facilitate self-organization over a hierarchy of length scales [22].

The polyelectrolyte properties of DNA are routinely used to guide their migration in response to electric fields. These anionic properties also allow DNA to interact with cationic species. Interactions between DNA and polypeptide are exploited in microarray printing, whereas interactions between DNA and low molecular weight polynucleotides allow the layer-by-layer assembly of salt-sensitive microcapsules [23]. There are even greater opportunities to exploit electrostatic interactions for protein-based materials because of the ability to engineer the charge on the polypeptide. For example, layer-by-layer assembly with polyglutamic acid and polypeptide has been used to entrap enzymes [24], and polypeptide has been used to guide the assembly of quantum dots [25].

Proteins have been also engineered to assemble in response to other stimuli [26,27]. For example, small increases in temperature trigger elastin-like polypeptides (ELPs) to undergo transitions from hydrophilic, water-soluble random coils to insoluble, collapsed hydrophobic globules that aggregate. This stimuli-responsive aggregation has been exploited for the reversible assembly of fusion proteins with ELP tails to surfaces that have been patterned with ELPs [28]. Notably, the transition temperature can be altered by adjusting conditions (i.e. ionic strength) and the amino acid sequence of the polypeptide [29].

**Self-assembly based on molecular recognition**

The bottom row in Table 1 lists the ability of biological macromolecules to exploit molecular recognition for self-assembly. Molecular recognition is integral to several biological processes including nucleic acid hybridization, antigen–antibody binding and integrin-mediated cell adhesion. Biology also exploits molecular recognition to self-assemble a diversity of nanometer-scale structures that range from intracellular filaments such as actin to virus particles. Thus, there is increasing interest in engineering molecular-recognized self-assembling systems for bottom-up self-fabrication.

The base-pairing mechanism responsible for nucleic acid hybridization is well understood, and it is relatively easy to create complementary strands that hybridize under controlled conditions to generate supramolecular assemblies. Importantly, the predictability of these complementary interactions permits structural control to the 1-nm level, whereas novel DNA motifs allow assemblies to be constructed over various length scales [30]. Efforts to engineer DNA [31] range from the generation of new macromolecular architectures, such as DNA-based dendrimers [32], to the creation of DNA-based machines [33].

The possibility of using protein-based supramolecular assemblies to perform various functions is being examined [27]. For example, filamentous protein assemblies, such as microtubules, have been exploited to serve as masks for the fabrication of metallic nanowires [34], and virion particles have been assembled to create cavities with an 18-nm diameter for materials synthesis and molecular entrapment [35]. In addition, by engineering the coat protein of a bacterial virus to nucleate ZnS or CdS nanocrystals, viral self-assembly has been exploited to create single-crystal inorganic nanowires [7,36] and ordered quantum dots [37].

As illustrated by the above examples, biological materials offer impressive molecular recognition and self-assembly capabilities. These biological components can be coupled to other, non-biological components to generate hybrid systems. For example, peptides and proteins have been coupled to carbon nanotubes to confer molecular recognition [38,39] and self-assembling [40] capabilities.

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**Table 1. Characteristics of biological materials that endow them with useful capabilities for fabrication**

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*aSee text for details and citations.*
Exploiting biocatalysts for fabrication

Useful enzyme reactions in fabrication

In nature, biological materials are synthesized, modified and hydrolyzed by enzymes, and a few studies have used these highly selective ‘biocatalysts’ for fabrication. Most of these examples exploit hydrolytic enzymes to remove a temporary structure selectively under mild conditions [41]. For instance, a protease has been used to remove a gelatin-based ‘resist’ without destroying a polysaccharide sublayer or a previously deposited nucleic acid probe [42]. In another example, a peptide was used to self-assemble into a nanotube, the nanotube was filled with silver, the silver was reduced with sodium citrate, and the peptide scaffold was enzymatically hydrolyzed, leaving behind a silver nanowire (with a diameter of 20 nm) [43].

In contrast to the use of enzymes for hydrolysis, there are fewer examples in which enzymes are used to build polymers or biopolymers (i.e. to increase molecular weight). Physiological pathways to proteins, nucleic acid and polysaccharides are expensive to reproduce in cell-free operations because of the requirement for activated intermediates, such as nucleoside triphosphates or nucleoside diphosphate sugars. But there have been recent efforts to develop alternative, cell-free enzymatic methods for polymer synthesis and modification [44]. Two general approaches are emerging by analogy to traditional polymer manufacturing: hydrolytic enzymes can be used in nonaqueous environments to catalyze condensation, ring-opening or transesterification reactions; and oxidative enzymes can be used to generate reactive intermediates, such as free radicals, that undergo non-enzymatic coupling reactions.

Figure 2 shows two enzymes that catalyze polymer modification reactions in nature: tyrosinase, which oxidizes tyrosine (Tyr) or dihydroxyphenylalanine (DOPA) residues, initiates the crosslinking reactions responsible for ‘setting’ the water-resistant protein adhesive used by mussels to attach to surfaces; and transglutaminase catalyzes a transamination reaction responsible for fibrin crosslinking in blood coagulation. Recent efforts are exploiting tyrosinase and transglutaminase enzymes to construct branched [45] and crosslinked [46] biopolymeric networks, multifunctional biomaterials such as bidomain proteins [47,48] and supramolecular protein networks [49]. In addition, the activity of a Ca\(^{2+}\)-dependent transglutaminase has been controlled by localizing Ca\(^{2+}\) within liposomes and triggering its release by heat [50] or light [51]. Most of these examples have been focused on constructing medical materials, but these enzymes and others, including galactose oxidase, lysyl oxidase, peroxidases and laccases, might offer interesting opportunities for biofabrication.

Examples of biocatalyst-facilitated assembly

The capabilities of biological materials and biocatalysts to facilitate assembly are illustrated by further examples. Figure 3 shows a two-step approach for assembling a model protein, green fluorescent protein (GFP), onto a micropatterned surface. The first step is the enzymatic conjugation of GFP to the aminopolysaccharide chitosan. Specifically, a GFP fusion protein is constructed with a pentatyrosine tail, which provides accessible residues for ‘activation’ by tyrosinase. Once activated, the quinone residues react with the amines of chitosan to yield a GFP–chitosan conjugate that possesses the pH-responsive properties characteristic of chitosan.

In the second step, these properties are exploited to electrodeposit the conjugate onto micropatterned electrodes. In this case, polarization of the electrodes results in proton consumption at the cathode and the generation of a localized region of high pH adjacent to the cathode [52]. When this localized pH is high enough to deprotonate a significant amount of chitosan’s amines (chitosan’s pKa = 6.3) the conjugate becomes insoluble and deposits on the cathode surface. The fluorescence photomicrograph in Figure 3c shows that deposition of the GFP–chitosan conjugate onto the patterned cathodes occurs with high spatial resolution [53].

In a second example, patterning was achieved by using the thermally responsive properties of the protein gelatin. As shown in Figure 4, a reactive polysaccharide sublayer (i.e. chitosan) is first cast on a substrate and the gelatin ‘thermosist’ is then cast over the chitosan. Pattern transfer is achieved by locally heating the gelatin to melt away the thermoresist; for proof-of-concept, heat was applied by a stamp. By using tyrosinase, the GFP fusion protein is then grafted onto the exposed chitosan sublayer. A warm water rinse (1 min at 50 °C) removes the resist, but the GFP patterns on the chitosan sublayer are retained.

The fluorescence photomicrograph and intensity profile in Figure 4 demonstrate the potential of thermo-biolithography for the facile patterning of proteins. Because gelatin is gelled and melted near ambient temperatures, sequential patterning can be performed without destroying the previously patterned GFP [42]. Together, these two examples illustrate the potential for exploiting stimuli-responsive biopolymers and enzymes for assembly.
Enzyme selectivity in nanolithographic patterning

Nanolithographic patterning has been achieved by combining the chemical selectivity of an enzyme with the spatial resolution of the atomic force microscope (AFM). In one study, a supported gel-phase lipid bilayer was patterned by using an AFM tip to induce local deformations in the bilayer that provide access to an interfacially activated phospholipase A2 that is present in solution [54]. In another study, a protease was tethered to an AFM tip and shown to be capable of digesting surface-bound peptides with both spatial and sequence specificity [55].

In the most recent study, an AFM tip was ‘inked’ with a Mg$^{2+}$-requiring endonuclease, which was then written onto a self-assembled monolayer of oligonucleotides. The nuclease was activated by immersion in a Mg$^{2+}$-containing solution, and digestion of the oligonucleotide self-assembled monolayer was localized to hundreds of nanometers in the lateral direction [56]. These examples illustrate the potential for integrating enzymes with other fabrication technologies.

Perspective

The marriage between biology and microfabrication has entered a new phase in which the broader talents of the individual partners are appreciated. Biology contributes materials that offer unique and important structural, recognition and catalytic functions. These materials can be genetically ‘machined’ by biotechnology, assembled through molecular recognition, and acted on by enzymes – all of which can be achieved with biological (i.e. chemo-, regio- and enantio-selective) precision. By contrast, microfabrication contributes devices that can transmit diverse mechanical, electrical, optical and magnetic signals. These devices can be fabricated into a range of shapes and sizes, and their signals can be transmitted with high, in this case, spatial and temporal precision.

Current efforts to couple biology and microfabrication are extending the individual strengths and raising expectations. For example, a self-assembling template for nanowires suggests the potential for two- and three-dimensional nanocircuits, and the deposition of biologically active components from solution presents a simple means of inserting biological activity into pre-assembled MEMS. Potentially there is more.

The marriage could bear even greater fruit if incompatibilities between the individual partners can be overcome. Biomolecular systems such as motor proteins offer impressive abilities to transduce energy and to respond to stimuli. Microfabricated devices are also remarkable

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**Figure 3.** Directed-assembly of protein–polysaccharide conjugates onto micro-patterned electrodes. (a) Tyrosine-tagged green fluorescent protein (GFP) is enzymatically conjugated to the aminopolysaccharide chitosan. (b) The pH-responsive electrostatic properties of chitosan are exploited to electrodeposit the conjugate at the cathode surface. (c) Fluorescence photomicrograph shows deposition of the GFP-chitosan conjugate onto 20-μm gold electrodes separated by 300-μm spaces. Adapted, with permission, from Ref. [53]. © 2003 American Chemical Society.

**Figure 4.** Thermo-biolithographic protein patterning. Chitosan and gelatin are used as the reactive polysaccharide sublayer and the thermally responsive resist, respectively. Pattern transfer is achieved by localized heating to melt selective regions of the gelatin. Tyrosinase initiates conjugation of tyrosine-tagged green fluorescent protein (GFP) to the exposed chitosan sublayer. The gelatin thermosteresist is removed by rinsing with warm water (50 °C for 1 min). Sequential patterning is possible because the gelatin thermosteresist can be applied and removed under sufficiently mild conditions to limit damage to previously patterned components. The fluorescence photomicrograph and image intensity profile illustrate the concept of thermo-biolithography. Reprinted, with permission, from Ref. [42]. © 2003 American Chemical Society.
transducers of energy and can supply a range of stimuli. Unfortunately, the two are not fully compatible because they do not share a common energy currency and often use different signals. If these incompatibilities can be bridged then it might be possible to fabricate implantable devices that are powered by metabolic energy or to engineer biomolecular interfaces that communicate in two directions. Potentially, biofabrication – the marriage between biology and microfabrication – will emerge as the standard for construction at the nanoscale.

Acknowledgements
Financial support for this work was provided by grants from the United States Department of Agriculture (2001–35504–10667) and the National Science Foundation (BES-0114790).

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