

Protein engineering for molecular electronics

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Recombinant DNA technology allows the manipulation of the physical properties of proteins that perform electron transport and photochemical processes. Recent work is reviewed that has a potential impact on the development of molecular electronic devices, within a general framework outlining strategies for device fabrication. This review is also published in *Current Opinion in Structural Biology* 1992, 2:587–592.

Current Opinion in Biotechnology 1992, 3:388–393

Introduction

This review is prompted by the recent enthusiasm for the use of biological macromolecules as building blocks for the construction of molecular electronic devices (MEDs). Molecular electronics is a term that has a dual meaning. One meaning refers to electronic devices that use materials whose unique properties result from their molecular structure. In this regard, proteins can be used in MEDs to sense, respond to, or record chemical, electrical, or physical stimuli. Examples include the use of the bacterial photopigment bacteriorhodopsin as a three-dimensional medium for the storage and readout of optically encoded information [1,2,3]. The second definition of molecular electronics embodies the concepts that individual molecules are functional units responding to stimuli, and that they have the potential to be interconnected in order to replicate electronic circuits functionally. Good examples of such molecular electronic devices do not yet exist, but the principles necessary for their design and assembly are beginning to emerge from a variety of areas.

From the perspective of practical device design, there are several levels at which the ability to modify protein molecules using recombinant DNA technology could have an impact on the fabrication of MEDs. These include alteration of intrinsic optical and electronic properties, increasing protein stability for operation in non-biological environments, and modification of surface properties to facilitate *de novo* design of mesoscale molecular assemblies or molecular circuits. Current activities in each of these categories are described below.

Modification of protein electronic and physical properties

Naturally occurring proteins incorporate a wide range of physical properties that are of potential use in electronic devices. In most cases, the useful properties derive from a prosthetic group such as a metal center, organic redox cofactor, or chromophore. Although naturally occurring redox and photoactive proteins exhibit great functional diversity, this is achieved through a limited number of prosthetic groups whose properties are modulated through interaction with amino acid side chains of the surrounding protein moiety. Attempts to engineer proteins in order to modify their properties in useful ways have followed precedents suggested by the study of structure–function relationships in natural systems. In this respect, heme-containing proteins, which comprise a diverse set of molecules with electron transfer, ligand binding, or catalytic function, are of particular interest. Alterations in the electronics or chemistry of the heme iron center frequently produce large changes in optical spectroscopic or magnetic properties which are useful as indicators for state assignment or device readout. Following pioneering work on cytochrome b5 [4], myoglobin [5–8], and cytochrome p450 [9], recent work reporting site-directed modifications of residues that are heme iron ligands, or can otherwise influence heme spectral properties, include additional studies on cytochrome p450 [10] and myoglobin [11], together with studies on cytochrome c peroxidase [12,13] and iso-1-cytochrome c [14]. Although the study of the structure–property relationships that affect heme proteins remains an area of active interest [15], few systematic efforts have been

Abbreviation

MED—molecular electronic device.

directed at producing unusually stable molecules that could increase the reliability of state assignment. However, a serendipitous enhancement of protein stability was reported for a site-directed mutant of iso-1-cytochrome c [14], in which an internal asparagine had been changed to a hydrophobic isoleucine residue. This alteration resulted in the loss of an internal water molecule whose location in the interior of the native protein was postulated to be energetically unfavorable. Similar hydrophobic enhancements could direct further efforts towards the important goal of stabilizing redox proteins in non-aqueous environments.

A property central to the function of many MEDs is the regulation of electron-transfer rate between proteins or between proteins and an external oxidoreductant. Physical aspects of the electron-transfer processes in proteins are now relatively well understood, and suggest that electron-transfer rates depend primarily upon prosthetic group separation, difference in oxidoreduction potential and molecular reorganization energy [16,17]. This is consistent with recent site-directed modifications of the invariant Phe82 residue in yeast iso-1-cytochrome c, suggesting that intermolecular electron transfer does not require the participation of specific aromatic amino acids as electron wires between protein prosthetic groups [18,19]. Nevertheless, translation of the physical requirements for efficient electron transfer into a specific modification strategy remains complicated, as shown by the results obtained by Barker *et al.* for yeast iso-1-cytochrome c [20]. In addition to heme-containing redox proteins the introduction or regeneration of copper-binding sites in proteins has been investigated. In one study, both type I and type II copper site properties could be obtained by addition of an appropriate external ligand to an azurin mutant in which one of the native histidine copper ligands had been deleted by site-directed mutagenesis [21]. In a second study, site-directed modifications of cytochrome c were performed in order to introduce a copper ligand site [22]. In both cases, the modified proteins appeared to lack the intrinsic stability of the native molecule, but may point the way to successive generations of molecules with useful functions.

Interaction specificity

Because rates of intermolecular transfer depend very strongly on prosthetic group separation distance [16, 17], reactions between reversibly binding electron-transfer proteins depend on interactions that ensure the proper relative intermolecular orientation. Early work that modeled the interactions of cytochromes c and b5 established the importance of solvent exclusion and complementary electrostatic interactions in redox protein interactions [23]. This precedent has since been generalized to many other biological electron-transfer interactions. With the advent of site-directed mutagenesis methods, investigation of intermolecular recognition interactions has intensified [24–29]. Most notably,

methods have recently been developed that discriminate between the relative contributions of the principal components of intermolecular recognition: polar interactions that result from hydrogen bonds and salt bridges forming in the interaction domain, and non-polar van der Waals interactions that occur when protein surfaces dehydrate upon formation of the complementary complex [30]. This methodology also facilitates mapping the interaction domain between two associating macromolecules, a key step in the development of generalized strategies to both study natural systems and engineer alternative interactions among molecules suitable for use in MEDs.

Bacteriorhodopsin in MED applications

Bacteriorhodopsin (molecular weight 26 000 D) functions as a light-driven proton pump in the purple membrane of the salt-marsh micro-organism *Halobacterium halobium* [1,2]. Bacteriorhodopsin incorporates a retinal chromophore, which is covalently bound as a protonated Schiff base in an all-*trans* conformation in the resting state. Upon absorbing a light photon, the retinal photoisomerizes and subsequently undergoes a multistep photocycle that spans much of the visible spectrum. The photocycle can be stopped at specific intermediates by low temperature trapping and recycled by thermalization or application of a second light pulse of the appropriate wavelength [1,2]. Photoactivated retinal switching occurs with high quantum yields at disparate wavelengths, which facilitate state assignment, and produces both changes in protein refractive index and a photoelectric potential in oriented assemblies. These properties, together with the excellent stability of the protein when immobilized in polymer films or gels, form the basis for a variety of prototypes for data storage media, holographic memory, and electro-optic applications [1,2,3,31,32]. Three-dimensional photochromic memories have incorporated bacteriorhodopsin that has been oriented by an external electric field and then immobilized in a solid polyacrylamide matrix [2]. This application exploits the two-photon absorption cross section of bacteriorhodopsin and requires that a small volume of the matrix be simultaneously illuminated by intersecting laser beams for data storage.

Readout is achieved by reillumination, which produces an electrical signal on the cuvette surface that depends on the state of the irradiated volume in the data storage matrix. In this context, it is interesting to note recent experiments demonstrating the maintenance of redox protein properties when immobilized in silicate glasses [33], as well as preservation and control of proteolytic enzyme activity in a photochromic azobenzene copolymer [34]. Although most readily applied in biosensor applications, both schemes could potentially be useful in three-dimensional optical memory applications that utilize photochromic properties of incorporated

proteins or that couple protein catalytic properties to the photochromic matrix.

Current work on bacteriorhodopsin with relevance to molecular electronics involves finding additives, alternative pigments, or site-directed mutants of the protein that will alter the relative stabilities of the photo-intermediates [35], enhance stabilization of the final M intermediate at room temperature [36], or alter other aspects of photocycle-coupled proton translocation [37,38] that might facilitate readout from information storage applications. Consequently, investigations of the Asp96Asn modification [39], which alters coupling of retinal photoisomerization to proton translocation, continue to have substantial practical interest [40]. The physical changes in the modified protein include an increase in the lifetime of the intermediate from 10 ms to 750 ms, together with improved diffraction efficiency and photochromic sensitivity relative to the native protein. Considerable latitude for property improvement exists using a combination of site-directed modifications at the chromophore binding site, introduction of alternative chromophores, or the engineering of modified ion-binding sites, all of which can affect intermediate lifetimes and spectral properties [1,2]. In this regard, the recent structure determination of bacteriorhodopsin by electron diffraction methods [41], coupled with rapidly advancing methods of computational simulation [42], may provide new insights into the engineering of bacteriorhodopsin for MED applications.

Oriented protein immobilization on surfaces

Bacteriorhodopsin is not unique in its ability to order spontaneously in two-dimensional films that resemble its native membrane state and, indeed, other membrane protein systems have been suggested for use or have potential in a variety of MED or related biosensor roles. Recent work includes studies of engineered bacterial transmembrane pores [43] and isolated bacterial reaction centers immobilized on electrode surfaces [44]. In these cases, surface orientation of the protein complexes results from preexisting interactions between the protein complexes or their membrane environments. However, a number of experiments have been carried out that involve tethering proteins to electrode surfaces through polymeric 'wires' [45,46] or through surface electrostatic interactions [47] in order to enhance electron-transfer efficiencies. Related studies of interest examine properties of soluble proteins conjugated to photo- or redox-active organic substituents that enhance enzyme catalytic function in the absence of natural cofactors or regeneration systems [48–50]. It is easy to envision hybrid systems that incorporate proteins with conjugated cofactors immobilized on electrode surfaces. Although the forgoing studies emphasize electron conduction between device components, the efficiency of many devices incorporating

prosthetic groups in sensor or non-linear optical applications depends critically on the ability to control prosthetic group orientation precisely relative to the electrode or optical waveguide surface. Site-specific introduction of anchoring points in a protein of known three-dimensional structure provides a straightforward solution to this problem. As an example, recent site-directed modifications of the heme protein cytochrome b5 carried out at the University of Illinois introduced cysteine residues at specified positions on the molecular surface. This allowed oriented coupling to a silane substrate and produced immobilized molecular arrays demonstrating a high level of heme prosthetic group orientation as determined by linear dichroism spectroscopy.

Methods to introduce two-dimensional periodicity on surface molecular arrays depend either on engineering both intermolecular interactions [30] and anchoring sites for surface immobilization, or on schemes that erect molecular assemblies on a scaffold already possessing periodic order on the few tens of angstroms scale. Scaffold possibilities include natural two-dimensional lattices [43], DNA lattices [51], or the very highly ordered synthetic lattices formed from streptavidin anchored to surfaces through biotinylated phospholipids [52]. As streptavidin is a tetrameric protein with four biotin-binding sites arranged with approximate tetrahedral geometry, two biotin-binding sites per tetramer remain free in the two dimensional arrays and provide a regular lattice for attaching additional molecules (Fig. 1).

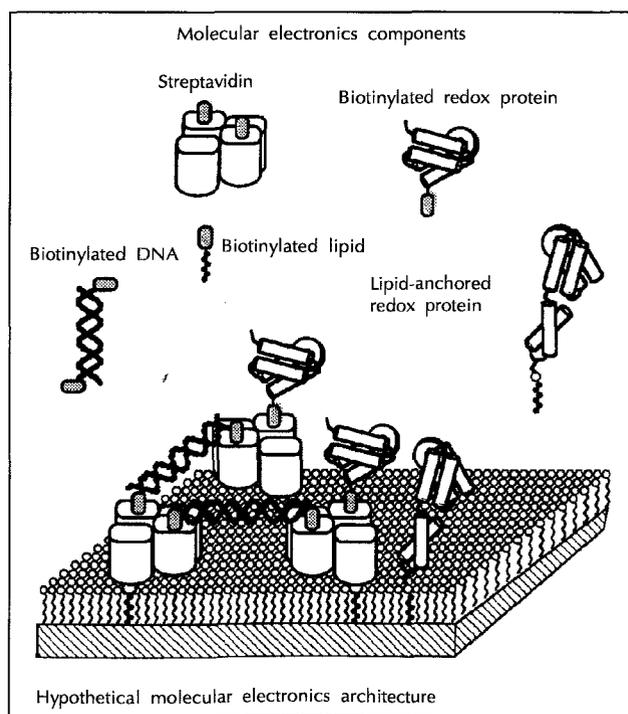


Fig. 1. Illustration of molecular electronics components and potential architectural features of self-assembling systems restricted to diffusion in two-dimensional lipid films.

Strategies for protein patterning and circuit fabrication

Although it appears possible that quite complicated molecular assemblies could be erected on two-dimensional protein lattices using a combination of chemical conjugation, site-directed introduction of anchor points, and molecular fusions at the DNA level (reviewed elsewhere in this issue), the materials described thus far all come under our first definition of molecular electronics as bulk materials. Although these substrates could undoubtedly be patterned using lithography methods similar to those used in fabricating integrated circuits, optical diffraction would limit detail to scales substantially larger than individual molecular assemblies. Such devices would not realize the ultimate in miniaturization of individually assignable devices inherent in molecular electronics of the second definition, whereas individual molecular assemblies would lack the unique addressability and specific connectivity between components commonly associated with electronic circuits. Nevertheless, it is interesting to consider how it might be possible to construct true molecular circuits, as well as the dual problem at the molecular level of how such devices could be addressed and read. Atomic probe microscopy could potentially be used to manipulate and assemble molecules as a means of constructing devices, as well as a means of setting molecular states or reading out information [53]. The limitations of manipulating only one or a few components at a time, however, would seem ultimately to defeat the objectives of creating a molecular device. Alternatively, it may be possible to 'condition' a preexisting molecular lattice of uniformly interconnected molecules so that it is able to store or process information as a neural network analog. Information processing using a distributed approach requires each molecular assembly to sense the state of its nearest neighbors and alter its configuration accordingly. With phasing to an external clock, this scheme has the formal structure of a cellular automaton operating with individual macromolecules as building blocks. In such a highly cooperative device, it may not be necessary to manipulate individual molecules directly, but utilize intermolecular communications common to many cooperative multimeric proteins to perform some analog computing function. Fabrication possibilities also exist using defect tessellation automata [54], where very complicated structures can be generated by defect introduction into a periodic lattice subject to a regular site replacement protocol. Although presently only the subject of computer simulation, it seems possible to embody the required properties in ligand-capture and displacement schemes using symmetric protein molecules with multiple binding sites. Access and read-out could potentially be achieved by any of the methods outlined above. While these devices are presently wholly conceptual, and may ultimately be limited by molecular noise that will necessitate distributed or redundant processing to ensure reliability, it seems clear

that genuine approaches exist to investigate the limits of this technology.

Conclusion

Practical application of engineered proteins in molecular electronics and sensor materials applications are clearly on the near horizon. Protein molecules represent the ultimate miniaturization possible in individual devices whose state can potentially be controlled independent of its neighbors. This property, together with the emergence of a knowledge base allowing protein properties and interaction specificity to be engineered with relative ease, will undoubtedly lead to progressively more sophisticated assemblies, whose functional limitations can only now be guessed at. Multidisciplinary programs such as those in the Soviet Union that pioneered development of bacteriorhodopsin based devices [1,3], or the Frontier Research Program of the Riken Institute in Japan [55], represent focused efforts to realize the potential of this technology.

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