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Bio-Batteries and Bio-Fuel Cells: Leveraging on Electronic Charge Transfer Proteins

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Bio-fuel cells are alternative energy devises based on bio-electrocatalysis of natural substrates by enzymes or microorganisms. Here we review bio-fuel cells and bio-batteries based on the recent literature. In general, the bio-fuel cells are classified based on the type of electron transfer; mediated electron transfer and direct electron transfer or *electronic charge transfer* (ECT). The ECT of the bio-fuel cells is critically reviewed and a variety of possible applications is considered. The technical challenges of the bio-fuel cells, like bioelectrocatalysis, immobilization of bioelectrocatalysts, protein denaturation etc. are highlighted and future research directions are discussed leveraging on the use of electron charge transfer proteins. In addition, the packaging aspects of the bio-fuel cells are also analyzed and the found that relatively little work has been done in the engineering development of bio-fuel cells.

Keywords:

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1. INTRODUCTION

Bio-fuel cells are energy-conversion devises based on bio-electrocatalysis leveraging on enzymes or micro-organisms.[1–4] Chemical reactions can proceed by direct electron transfer (DET), in which case the electron transfer occurs directly between enzymes and electrodes,[5] or through shuttle mediated electron transfer (MET), in which electron transfer mediators shuttle the electron between enzymes and electrodes to reduce the kinetic barrier in the electron transfer between enzymes and electrodes. Direct electron transfer (DET) is desirable for efficient communication between enzymes and electrodes, and eliminating the need for mediators may simplify the construction of bio-fuel cells. In terms of applications, of bio-fuel cells (BFC) will most likely be use in miniature cells to derive power from biological macromolecules to power small devices. It may be possible to implant miniature BFCs within a human patient to power micro sensor/transmitter devices e.g., glucose sensors for diabetics, to monitor blood pressure, temperature, metabolite concentrations, etc. or to power a pacemaker or bladder control valve. It is also conceivable that these miniature BFCs may have defense applications.

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2. BIO-BATTERIES AND BIO-FUEL CELLS

Even though fossil fuel (petroleum) meets the majority of global energy demands, the increasing difficulty of sustained supply and the associated problems of pollution and global warming are acting as a major motivation for research into alternative sustainable energy technologies, like solar, wind and hydrogen fuel cells.[6] Fuel cells offer a possible (and partial) solution to this problem, with the fuel needed for conventional cells usually being either hydrogen or methanol with operating temperatures <100 °C.

In a hydrogen fuel cell, electricity is generated efficiently from the oxidation of hydrogen, coupled to the reduction of oxygen, with water as the only by-product (Fig. 1(a)). The most commonly used electrocatalyst in the fuel cells is platinum. Platinum is very efficient in oxidizing hydrogen and enabling high currents to be produced in a fuel cell. The major disadvantage is that platinum is expensive and its limited availability, making hydrogen fuel cells an expensive method of energy production. Platinum is also poisoned by carbon monoxide (CO) impurities that are often found in industrially produced hydrogen. Removal of CO adds to the cost of the fuel cell system.

After Galvani first noticed the twitching of a frog's leg upon application of an electric current, it has been

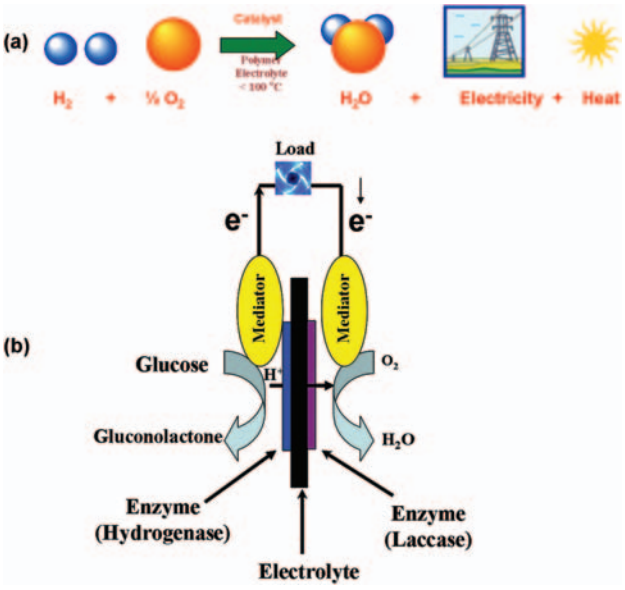

Fig. 1. (a) Principle of a low temperature H_2/O_2 fuel cell, (b) operating principle of a biofuel cell involving hydrogenase and laccase enzymes.

known that several biological pathways have a bio-electrochemical facet.[7] As an electrical signal can induce a biological reaction; the reverse in is also true in most of the cases and in this way biological processes can be used to generate electricity for powering electrical equipment. Even though the BFCs have been known for almost a century since the first microbial BFC was demonstrated in 1912,[8] the first enzyme-based bio-fuel cell was reported only in 1964 using glucose oxidase (GOx) as the anodic catalyst and glucose as the bio-fuel.[9] The ability of a hydrogenase coated electrode to catalyze efficient oxidation of hydrogen suggests exciting possibilities for use of such an electrode in a BFC, in which the conventional platinum fuel cell anode is replaced by a precious metal-free hydrogenase electrode. When the hydrogenase anode is coupled with a cathode incorporating the fungal enzyme laccase, which catalyses reduction of oxygen to water, the hydrogenase biofuel cell produces a small, but measurable power output. The operating principle of a bio-fuel cell is given in Figure 1(b).

A bio-battery generates electricity from carbohydrates (sugar) utilizing enzymes as the catalyst, through the application of power generation principles found in living organisms. The bio-battery incorporates an anode consisting of sugar-digesting enzymes and mediator, and a cathode comprising oxygen-reducing enzymes and mediator, either side of a cellophane separator. The anode extracts electrons and hydrogen ions from the sugar (glucose) through enzymatic oxidation as follows:

$$\text{Glucose} \rightarrow \text{Gluconolactone} + 2H^+ + 2e^-$$

The hydrogen ion migrates to the cathode through the separator. Once at the cathode, the hydrogen ions and electrons absorb oxygen from the air to produce water:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

During this electrochemical reaction, the electrons pass through the outer circuit to generate electricity (Fig. 1(b)). It is interesting to note that the catalytic four-electron reduction of oxygen to water could take place at an enzyme electrode in a neutral solution. Due to the selective reactivity of the enzymes at each electrode, no cross reaction occurs between the anode and cathode.[10] In general the BFC could be classified into many types based on fuel containment, fuel and catalyst sources, origin of the catalytic enzymes and the method of electron transfer between reaction site and electrode.[11]

Very recently, INHABITAT[12] has reported that the Sony Corporation (Japan) has developed a bio-battery with a peak power output of 50 mW, which could power a portable MP3 player. The Bio Battery, based on the work of Professor Kenji Kano (Kyoto University), is a type of battery that uses energy sources such as carbohydrates, amino acids and enzymes from a variety of sources. The anode consists of sugar-digesting enzymes and mediator, and the cathode composes of oxygen-reducing enzymes and mediator. The mediators in this case are Vitamin K3 for the anode and potassium ferricyanide for the cathode. When sugar is added to the mixture, the anode garners

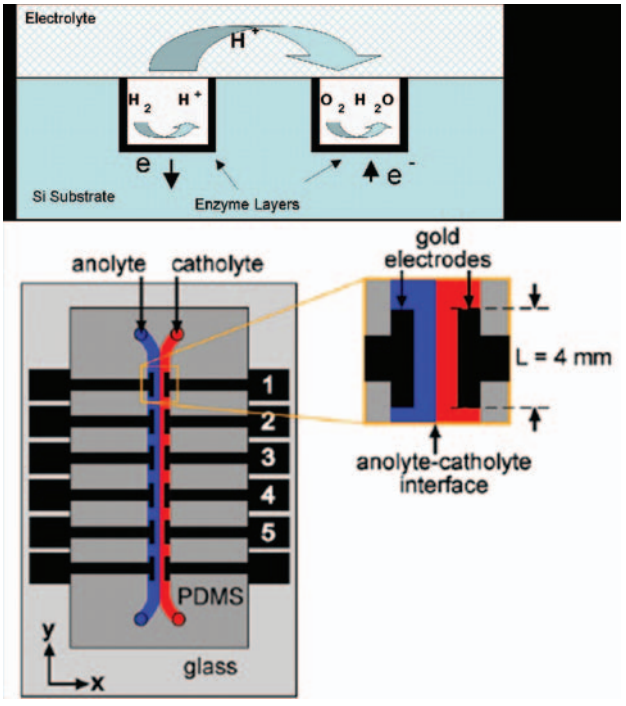

Fig. 2. (a) Design concept of Silicon wafer based micro bio-fuel cell and (b) schematic of plan view including enlargement of anolyte and catholyte flowing past a pair of electrodes (multiple cell design). Reprinted with permission from [13], K. G. Lim and G. T. R. Palmore, *Biosens. Bioelectron.* 22, 941 (**2007**). © 2007, Elsevier Ltd.

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the electrons and hydrogen ions. When the battery generates power, the protons travel to the cathode through the electrolyte to combine with the oxygen to produce water.

Since the biocatalysts (enzymes) are very selective catalytically, the miniaturized bio-fuel cell could in principle be fabricated as a membrane-less fuel cell. Lim and Palmore at the Brown University have reported a microfluidic BFC with many channels connected in parallel (Fig. 2). In this configuration, the design allows streams of fuel and oxidant to flow in parallel within a microchannel without using a membrane as a separator and showing a power density $>25\ \mu W/sq \cdot cm$.[13]

3. PREVIOUS ATTEMPTS OF BIO-FUEL CELLS

Several potential applications of BFCs have been reported or proposed in the literature for implantable devices, remote sensing and communication devices as a sustainable and renewable power source.[14] However, there are no BFC design formats or templates that allow for the production of a working device with a size on the order of 1 cc, which are needed for several "real world" applications. An enzyme based BFC is very attractive, however it has been shown that electron flow is too slow to make a viable fuel cell. This is due to the difficulty for enzymes to attain direct electrical contact with the electrodes of the cell and catalyze reactions effectively.

The two largest obstacles with bio-fuel cells which must be overcome are increasing the power density and increasing the enzyme stability. In addition, understanding of the determinants governing the direct electron transfer reaction and mutation of enzymes to tune the redox potential, to improve DET kinetics, or to reduce the enzyme size are also very important challenges facing the commercialization of bio-fuel cells.[15] To address these key issues, various enzyme immobilization methods have been attempted for constructing BFCs, such as adsorption, entrapment, and covalent attachment. Recent advances in bionanotechnology are promising to improve the performance and stability of immobilized enzymes beyond the scope of these traditional approaches.[16] The large surface area provided by nanomaterials for the attachment of enzymes will increase enzyme loading and possibly improve the power density of BFCs. Additionally, various nanostructured materials have shown great potential for stabilizing enzyme activity, which can be further employed in improving the lifetime of BFCs.[16]

4. TECHNICAL CHALLENGES OF BIO-FUEL CELLS

Bio-fuel cells are attracting increased attention mainly due to promising advances from the research laboratories around the world. However, there are several issues to be resolved before bio-fuel cells become commercially viable for practical applications. The main challenges are:

(1) Nanostructured bioelectrocatalysis.
(2) Immobilization of bioelectrocatalysts.
(3) Protein denaturation induced by CNT.

The following sections briefly describe these issues:

4.1. Nanostructured Bioelectrocatalysis

Traditional direct hydrogen fuel cells require noble metal catalysts both for hydrogen oxidation and oxygen reduction.[17] Similarly, the bio-fuel cells also need catalysts (bio-catalysts) for the conversion of chemical to electrical energy. One approach is to use microorganisms and/or enzymes as biological reactors for the fermentation of raw materials to fuel products (similar hydrogen fuel reformers); the second approach is to use the microorganisms and/or enzymes as catalysts directly in the bio-fuel cells. The second approach, using purified redox enzymes for the targeted oxidation and reduction of specific fuel and oxidizer substrates, is more efficient for bio-fuel cells. Also, bio-catalysts are an attractive renewable and less expensive alternative to transition metal catalysts for mediated electron transfer (MET).[18]

MET-type bioelectrocatalyt based BFCs offer the current density advantage over the direct electron transfer (DET) type, but require that mediators and enzymes be immobilized on electrode surfaces. The construction of DET-type bio-fuel cell is relatively simple as the system is free from several restrictions concerning mediators. The cell would not require separators because the crossover of fuels (substrates) would not occur in principle due to enzymatic substrate specificity as long as the enzymes are immobilized on electrodes and dehydrogenases (that is, redox enzymes reacting with electron acceptors except dioxygen) are utilized as anode catalysts. Kamitaka's group have reported a construction of single compartment bio-fuel cell, with no separators, using D-fructose dehydrogenase (FDH) from *Gluconobacter* sp. and laccase from *Trametes* sp. (TsLAC) as DET-type bioelectrocatalysts in the two-electron oxidation of D-fructose and four-electron reduction of oxygen in the anode and cathode, respectively.[19]

There is also a recent study of utilizing tungsten carbide as an electrocatalyst towards the oxidation of several common microbial fermentation products (hydrogen, formate, lactate and ethanol) for microbial fuel cell conditions (e.g., pH 5 at ambient temperature and pressure). Current densities of up to 8.8 mA cm^{-2} are achieved for hydrogen (hydrogen saturated electrolyte solution), and up to 2 mA cm^{-2} for formate and lactate, respectively, with cell voltage values between 0.15 and 0.3 V.[20]

It is also worth mentioning virus-based lithium-ion biobatteries. There is an increasing need for smaller and more flexible Li ion batteries and for methods to assemble battery materials in various applications. However, realizing

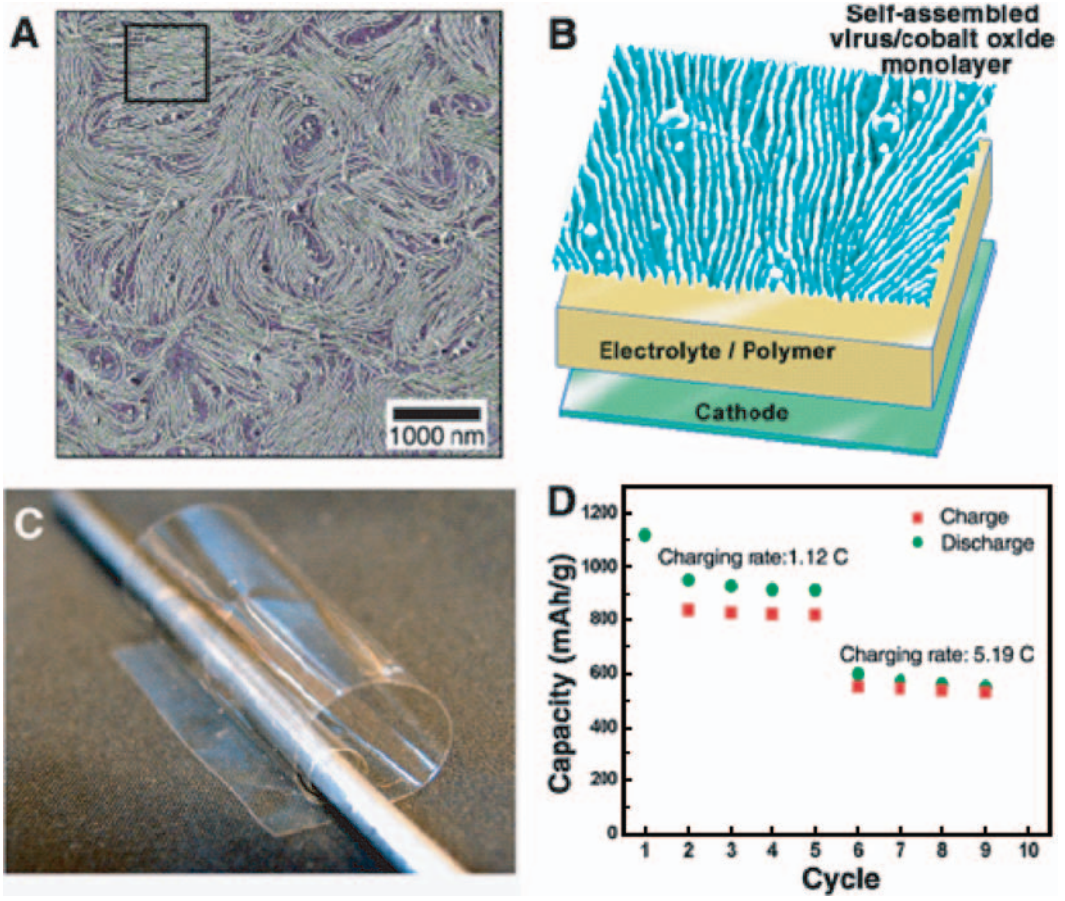

Fig. 3. Two-dimensional assembly of Co_3O_4 nanowires driven by liquid crystalline ordering of the engineered M13 bacteriophage viruses. (A and B) Phase-mode atomic force microscope image of macroscopically ordered monolayer of Co_3O_4-coated viruses. The Z range is 30- (C) Digital camera image of a flexible and transparent free-standing film of (LPEI/PAA)100.5 on which Co_3O_4 viral nanowires are assembled into nanostructured monolayer with dimensions of 10 cm by 4 cm. (D) Capacity for the assembled monolayer of Co_3O_4 nanowires/Li cell at two different charging rates. Reprinted with permission from [21], K. T. Nam et al., *Science* 312, 885 **(2006)**. © 2006, AAAS.

smaller and flexible battery systems need monodisperse, homogeneous nanomaterials and hierarchical organization control. Biosystems have the inherent capabilities for molecular recognition and self-assembly and thus are an attractive template for constructing and organizing the nanostructure. Combining virus-templated synthesis at the peptide level and methods for controlling two-dimensional assembly of viruses on polyelectrolyte multilayers provides a systematic platform for integrating these nanomaterials to form thin, flexible lithium ion batteries.

The engineered viruses are ordered by competitive electrostatic interactions, the interdiffusion of the polyelectrolyte, and the anisotropic shape of the M13 virus.[21] As seen in Figures 3(a) and (b), by using this technique to spontaneously order M13 viruses and subsequently grow Co_3O_4 on the virus coat proteins, 2D organized ensembles of nanowires on a 10-cm length scale can be produced. The spatial distance and ordering behavior between viral nanowires can be manipulated by controlling both surface charge and fluidic forces. This assembly process produces lightweight, flexible, and transparent active material/substrate multilayers, constructed as free-standing films by a simple dipping method (Fig. 3(c)). Thus, the assembled layers compose a negative-electrode material grown upon a solid electrolyte or separator membrane. For electrochemical evaluation, 100 nm of Cu, which functions as a current collector, was deposited by E-beam evaporation on the assembled Co_3O_4 nanowires/polymer layer. This assembly was then tested in Swagelok cells with a Li foil negative electrode separated from the multilayer by a separator dipped in liquid electrolyte. The capacity for the assembled monolayer of Co_3O_4 nanowires/Li cell at two different charging rates is shown in Figure 3(d). The cell was found to sustain and deliver 94% of its theoretical capacity at a rate of 1.12 C and 65% at a rate of 5.19 C, demonstrating the capability for a high cycling rate.

These results demonstrate that basic biological principles can be applied to the rational design and assembly of nanoscale battery components, exhibiting improved performance in properties such as specific capacity and rate capability. The simplicity of genetic modification allows for the growth and assembly of other functional nanomaterials for applications such as photovoltaic devices, high–surface area catalysts, and supercapacitors.

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4.2. Immobilized Bioelectrocatalysts on CNTs

In order to promote DET of the protein and to improve the stability, it is necessary that the protein is immobilized in the form of clusters.[4] In this context, glucose oxidase (GOx) can be attached as crosslinked enzyme clusters (CECs) onto the surface of carbon nanotubes (CNTs). In has been demonstrated in a recent study that CEC-GOx did not manifest any decrease in activity for 250 days.[4] The CEC-GOx based BFC was characterized by potentiostatic polarization in an unbuffered solution. The open circuit voltage (OCV) was 0.33 V while a maximum power output of 120 μWcm^{-2} occurred at a cell potential of 0.1 V (Fig. 4(a)). The long-term performance of the miniature BFC with CEC-GOx, constant voltage measurements at 0.1 and 0.25 V (Fig. 4(b)) showed some transient behavior initially and the cell performance stabilized after about 2 hours. More importantly, at 0.1 V where a heavy load was applied to the BFC, the performance of the BFC was very stable without any significant performance decay for more than 16 hours (Fig. 4(b)).

Using CNTS inside the carbon matrix (nano-particles were inserted to a carbon cloth) results in better enzymes immobilized and more reproducible output currents were obtained. For example, mediator-free bilirubin oxidase (BOD) modified CNT-carbon electrodes, the glucose-air battery showed the open-circuit potential of ca. 0.5 V, the short-circuit current of ca. 0.6 to 4 mA$\cdot$cm^{-2} (with respect to an apparent geometrical surface area) and an impressive peak power of 0.075 to 1 mW$\cdot$cm^{-2} between 0.25–0.3 V.[10]

Carbon nanotubes have the potential to allow novel 3-dimensional CNT-GOx anode structures that can be useful in BFC devices. CNT allows for building surface architectures with distinct levels of hierarchical

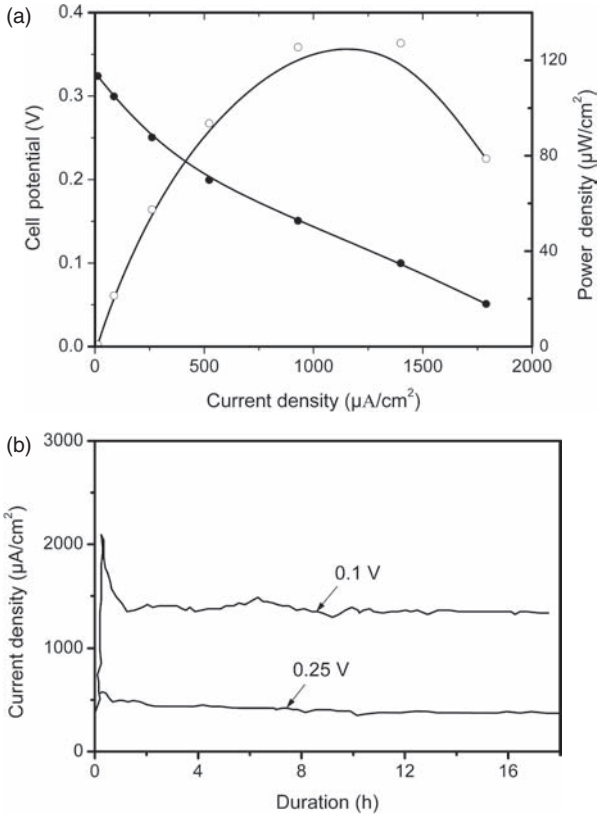

Fig. 4. Electrochemical characteristics of BFC; (a) potentiostatic polarization and (b) Stabilized performance at 0.1 and 0.25 V. Reprinted with permission from [4], M. B. Fischback et al., *Electroanalysis* 18, 2016 **(2006)**. © 2006, Wiley-VCH Publishers, Inc.

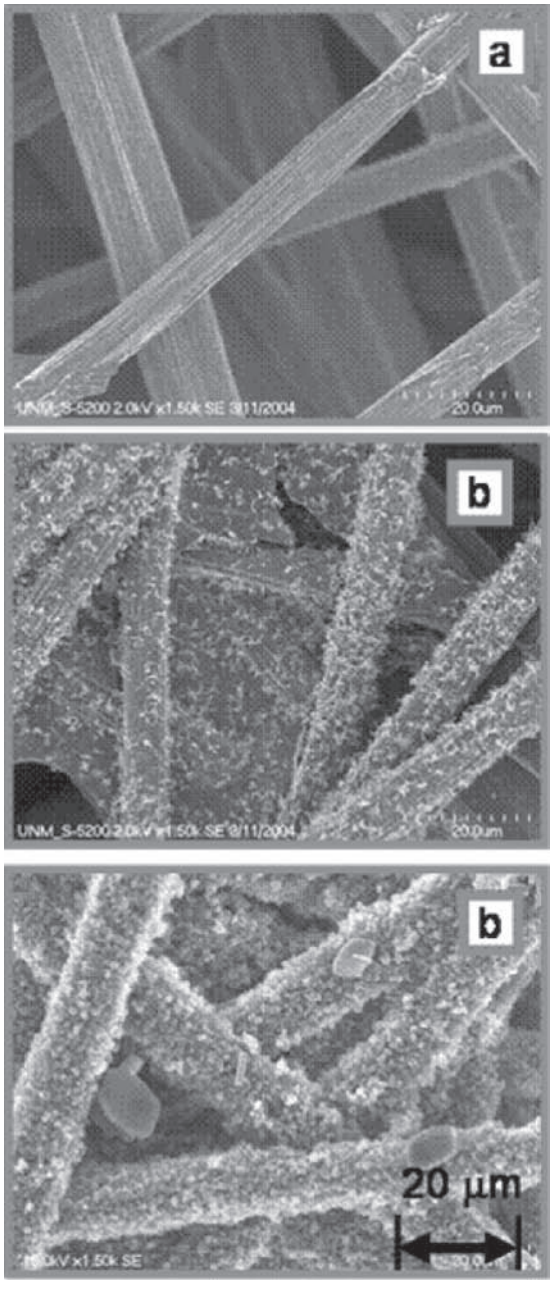

Fig. 5. Toray carbon paper (a) with CVD deposited multi-walled CNT (b) and the final glucose electrode structure after PEI coating and (c) Glucose Oxidase immobilization in modified Nafion matrix. Reprinted with permission from [22], B. Branch et al., *209th ECS Meeting*, Abstract #571, May, Denver, CO **(2006)**. © 2006, ECS–The Electrochemical Society.

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organization of pore structures. MWCNTs grown on the carbon paper (Figs. 5(a) and (b)) matrix by chemical vapor deposition (CVD) with the length of the individual CNT filaments and the spacing between them is at the nano-meter scale. The layer-by-layer immobilization of the negatively-charged charged GOx on the CNT-carbon paper composite (Fig. 5(c)) was carried out by using positively-charged surface modifier such as, polyethylenimine (PEI). Nano-structured supports derived from such nanotubes demonstrate pore structures that provide unique capabilities due to large surface-to volume ratio combined with substantial porosity. This is of particular importance in designing flow-through anodes for bio-fuel cells. The BFCs evaluated using glucose (fuel) solution various concentrations of 5 to 50 mM corresponding to that of potentially environmentally available sources (plant sap or blood/tissue supplies from the animals), showed limiting current density values up to 2 $mA \cdot cm^{-2}$.[22]

In order to maximize the operating cell voltage as well as minimize the polarization losses of the BFCs, direct electron transfer (DET) in the cathode (oxygen reduction) is critical. It has been demonstrated that the electrode surface chemistry is of particular importance for creating favorable conditions for DET in bio-electroreduction of oxygen catalyzed by laccase and BOD from *Myrothecium verrucaria*.[23] With various seeding times of Co for the growth of MWCNTs on the carbon paper, the BOD was immobilized on the hydrophilic site of an anisotropic hydrophilic/hydrophobic composite membrane. As seen from the galvanostatic polarization curves (Fig. 6), the limiting current density values depend strongly on the density of MWCNT used as a base for this electrode design and a limiting current of >2 $mA \cdot cm^{-2}$ was observed for the BOD based cathode with 14 m^2/g active area.

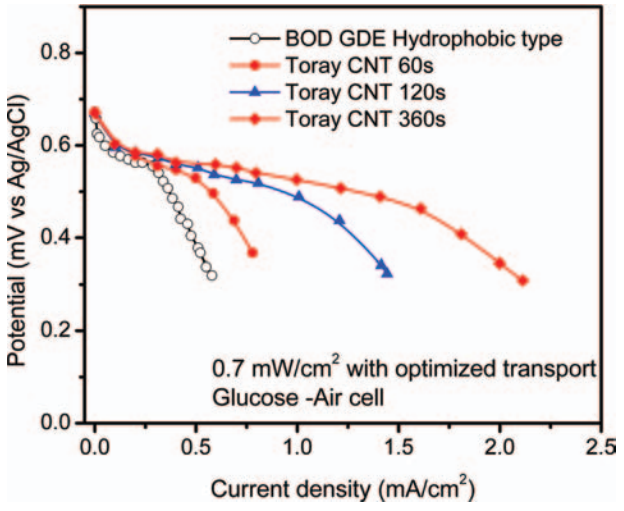

Fig. 6. Galvanostatic polarization curves of the bio-fuel cell gas-diffusion air cathode with BOD as oxygen reduction electrocatalyst designed on the carbon paper/MWCNT. Reprinted with permission from [23], P. Atanassov et al., *209th ECS Meeting*, Abstract #562, May, Denver, CO **(2006)**. © 2006, ECS–The Electrochemical Society.

4.3. Protein Denaturation Induced by CNTs

Recent observations from our laboratories[24] have highlighted the possibility of CNT inducing denaturation of attached proteins. The most likely mechanism is CNT induced dehydration of proteins resulting in denaturation of the protein. This observation presents a challenge in covalent attachment of proteins to CNT.

5. ELECTRONIC CHARGE TRANSFER: AN ALL PERVASIVE UBIQUITOUS MECHANISM IN NATURE

Electron transfer, spatial dislocation of an electron, is the simplest of chemical processes underlying all chemical reactions. The process of efficiently and controllably moving electrons around is one of the primary regulatory mechanisms in biology. Sustenance of life depends on constant shuttling of electrons. Almost all chemical reactions involve transfer of electrons and so in principle any one of them can be leveraged for technological applications. The cardinal goal of bionanotechnology, a fusion of life sciences and nanosciences, is to exploit awesome power of biological macromolecules by converting them into functional devices.[25, 26] Whilst all chemical reactions can be utilized to exploit electron transfer, proteins due to their unique 3D structure may have certain distinct advantages which are not fully understood.

Photosynthesis, nitrogen fixation and the mitochondrial transport chain are driven by electron transfer processes. Therefore it is not surprising that much effort has been placed on understanding the fundamental principles that control and define the simple act of adding and/or removing electrons from chemical species. We propose to christen this electron transfer process as *electronic charge transfer* (ECT). Most of the bio-molecular electronic or protein based devices[25] hinges on ECT in one way or another. Since the early efforts of Gamow, 1928, electron tunneling has been postulated to be a plausible mechanism underlying ECT.[27, 28] Electron tunneling has been postulated to be a preferred mechanism for electron charge transfer in a multitude of chemical and biological phenomena. Structured water molecules near redox cofactors have been noted to accelerate ECT kinetics. Electron transfer is effective at long range[28] and seems to be an underlying basis for a number of biochemical phenomena e.g., allosteric effect.[29]

5.1. Cornucopia of Proteins Manifest ECT

The oxidation of intermediary metabolites by molecular oxygen in the mitochondria of animal and plant cells require the successive transfer of hydrogen atoms or electrons, first to NAD^+, then from NADH to an ubiquinone (Q), next from QH_2 to ferricytochrome *c* and finally from ferrocytochrome *c* to oxygen (Fig. 7) provides

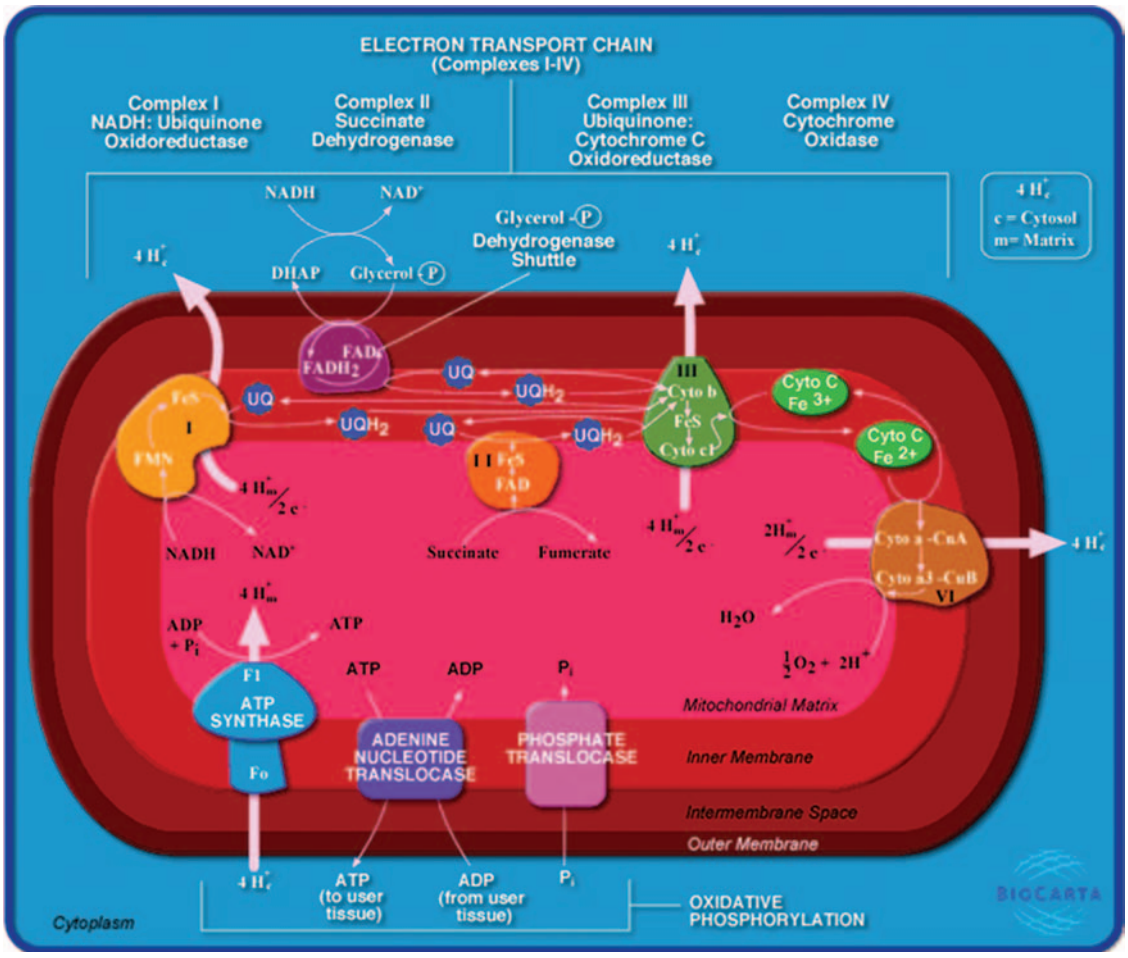

Fig. 7. A plethora of proteins manifest ECT by external stimulation. (Figure adapted from http://www.biocarta.com/pathfiles/h_etcPathway.asp).

an excellent example of ECT. These reactions are catalyzed, e.g., by an oxidoreductase using NAD^+ or $NADP^+$ as acceptor, NADH:Q oxidoreductase, QH_2:cytochrome-*c* oxidoreductase, and ferrocytochrome-*c*:O_2 oxidoreductase, respectively. In some instances, $NADP^+$ is used as the first hydrogen acceptor and an additional enzyme, $NAD(P)^+$ transhydrogenase is required for the initial reduction of NAD^+. In other cases, particularly with substrates of higher redox potential, neither NAD^+ nor $NADP^+$ is required and appropriate enzymes catalyze either the direct reduction of Q, e.g., succinate dehydrogenase, or introduce electrons into the sequence *via* the so-called electron-transfer flavoprotein. Similarly, in the light-driven oxidation of water that occurs in the chloroplasts of green plants (Fig. 8), hydrogen atoms or electrons are transferred successively to plastoquinone (PQ), then to plastocyanin and finally to $NADP^+$ in reactions catalyzed by photosystem II, PQH_2:plastocyanin oxidoreductase, photosystem I, and ferredoxin:$NADP^+$ reductase, respectively.

Proteins bearing transition metals such as Cu^{2+} and Fe^{2+} play leading roles in electron transport as one-electron redox-active centers within proteins that are used to effectively move electrons around. Well-known examples are the blue copper proteins ($Cu^I \leftrightarrow Cu^{II}$),[30] (Fig. 9(A)), cytochrome superfamily, ($Fe^{II} \leftrightarrow Fe^{III}$ porphyrins; Fig. 9(B)), and iron-sulfur proteins ($Fe^{II} \leftrightarrow Fe^{III}$ with sulfur ligands; Fig. 9(C)). Significant efforts have been placed on developing our understanding of how biological systems control which electron transfer processes is feasible and how fast they will occur.

Cytochrome *c* is a small globular protein which transfers redox equivalents in the respiratory chain. It can be productively immobilized on negatively charged surfaces,[31, 32] which serve as a platform for an effective communication of the redox center with electrode. Cytochrome *C′* is a dimeric metalloprotein found in a large number of photosynthetic and denitrifying bacteria. Each monomer contains, as the active site, a five coordinate protoporphyrin IX (Haem) prosthetic group. The Haem iron, postulated to exist in a 'quantum mechanically admixed' ($S = 5/2, 3/2$) spin state,[33] possess a nearby histidine residues as an axial ligand, yet the sixth coordination site remains empty. The haem group is situated in a hydrophobic pocket in the protein structure such that only nitric oxide and carbon monoxide may bind to the reduced Fe 2+) haem iron, and only nitric oxide may bind to the oxidized

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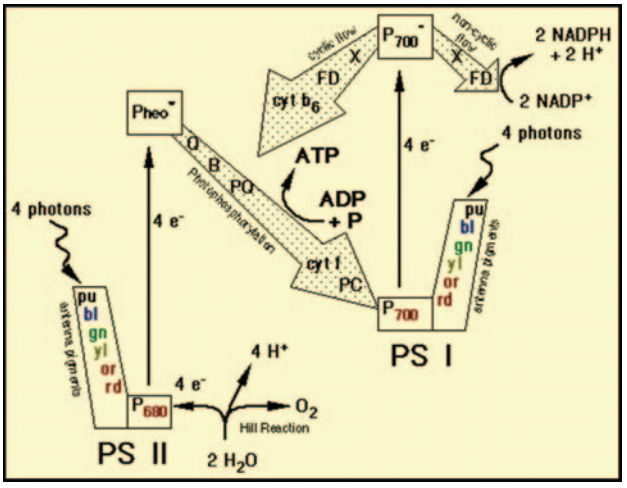

Fig. 8. The photosynthetic process by which biochemical energy and substrates are generated from inorganic precursors and photon energy (light) in the cholorplasts of green plants (Figure reproduced from http://plantphys.info/Plant_Biology/photopart.html).

(Fe 3+)[34, 35] reported the binding of CN-to reduced and oxidized cytochrome *C′* from some bacteria, although with low affinity). Consequently, Cytochrome *C′* may represent ideal biosensing selectivity with potentially no interference for NO when in the native oxidized state.[36]

The fundamental design of electron transfer proteins, with only a few exceptions, is two catalytic sites connected by redox chains. Catalytic sites are defined as multi-electron redox centers or clusters of single-electron redox centers that interact with substrates and act as sources or sinks of pairs of electrons. Chains are defined as groups of single electron redox centers that are more widely separated, with fewer near neighbors. The two catalytic sites and the connecting chain may be entirely within a single protein, as in the quaternary structure of *Saccharomyces cerevisiae* succinate dehydrogenase (Fig. 9(D)).[37] In some circumstances, the protein folding may allow two catalytic sites to be placed sufficiently close to each other that no connecting chain is needed to assure physiologically competent tunneling rates, such as in ascorbate oxidase. It is most common for a protein to contain one catalytic site plus a trail of cofactors to a binding patch on the surface, as in Ni–Fe hydrogenase or aldehyde oxidoreductase. The protein may contain just a portion of a chain between surface docking sites with no catalytic sites. These chains indirectly connect to catalytic sites in other proteins, such as cytochrome *c*554.

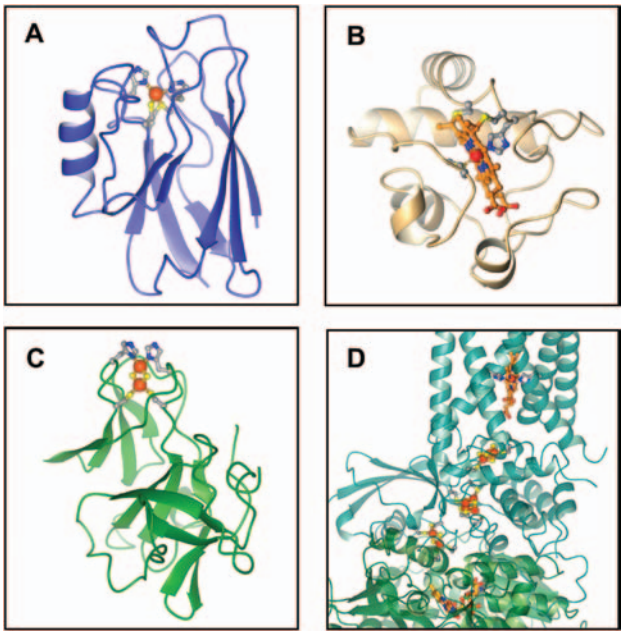

Fig. 9. Several transition metal containing proteins. (A) Azurin (PDB ID 1AZU), is a mononuclear copper containing redox protein. (B). Cytochrome *c* (PDB ID 1HRC) contains a heme cofactor that coordinates an Fe ion. (C) The Rieske Fe-S protein from *Thermus thermophilus* (PDB ID 1NYK) contains a 2Fe-2S cluster coordinated by His and Cys residues of the protein. (D) Succinate dehyrogenase (PDB ID 1YQ4) contains a series of protoporphyrin-coordinated and inorganic Fe ions.

5.2. Flavoproteins

Flavoproteins commonly contain one of two prosthetic groups, FMN (e.g., NADH dehydrogenase, EC 1.6.99.l) and FAD. The FMN is non-covalently bound in all known cases. FAD may be non-covalently bound (e.g., in dihydrolipoamide dehydrogenase (NADH), EC 1.8.1.4) or covalently bound by a methylene bridge between the benzene ring of the benzo[*g*]pteridine-2,4-dione and an amino acid residue, such as cysteine, histidine or tyrosine, in the protein (e.g., succinate dehydrogenase, EC 1.3.99.1), or directly at ring position 6. 8-Hydroxy-pyrimidino [4,5-*b*]quinoline-2,4-dione functions as prosthetic group in methanogens and in deoxyribodipyrimidine photolyase (EC 4.1.99.3). Apart from a few exceptions where the role of the flavin is not clear, flavoproteins carry out oxidation-reduction reactions, in which one substrate is oxidized and a second is reduced. For all these enzymes each catalytic cycle consists of two distinct processes, the acceptance of redox equivalents from a reducing substrate and the transfer of these equivalents to an oxidized acceptor. Accordingly, the catalyzed reactions consist of two separate half-reactions: a reductive half-reaction in which the flavin is reduced and an oxidative half-reaction, in which the reduced flavin is reoxidized.

It should be noted that flavoproteins can act in sequence. The most extreme case is found in the pathway for the β-oxidation of fatty acids. A flavoprotein dehydrogenase first oxidizes the saturated fatty acyl CoA. The dehydrogenase then transfers its electron via a second flavoprotein, the electron-transferring flavoprotein, to the membrane-bound iron-sulfur flavoprotein, electron-transferring protein ubiquinone oxidoreductase. This last protein reduces coenzyme Q, thus delivering electrons to the respiratory

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chain. Similar sequences of reactions are found in the catabolic pathways of several amino acids and in the operation of the mitochondrial one-carbon cycle.

5.3. Oxidoreductases

The 3D structure of Glucose Oxidase (GOx) from *Penicillium amagasakiense*,[38] is shown in Figure 10(a). GOx has been widely used in the construction of BFCs. GOx (β-D-glucose:oxygen 1-oxidoreductase, EC1.1.3.4), a dimeric protein with a molecular weight of ~160 kDa with a tightly bound flavin adenine dinucleotide (FAD) as a cofactor, catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor (Fig. 10(b)). Of the two dimers, first monomer consists of β-sheets and the second dimeric unit consists of 4 α-helices supporting antiparallel β–sheets. The conversion of β-D-glucose to gluconic acid involves the transfer of two protons and two electrons from the substrate to FAD.

5.4. Redox Potential of Electron Transfer Proteins

The redox potential is a measure (in volts) of the affinity of a substance for electrons—its electronegativity—compared with hydrogen (which is set at 0). Electron transfer proteins manifest a wide range of redox potentials although they may contain similar or identical redox center.[39] Such a wide range of redox potential for proteins can be attributed to the net charge on the protein and the intricate 3D structure of the ET proteins. Rees proposed a linear correlation between the net charge of proteins and their redox potential. A review of the factors that influence protein redox potentials makes it clear that this linear correlation is fortuitous. The key factors influencing redox potentials are the contributions to the Gibbs energy difference between the two redox states, resulting from bonding interactions at the redox center, electrostatic interactions between the redox-center charge and polar groups within the protein and solvent, and redox-state conformational changes. The relative importance of these terms is likely to vary from protein to protein. Therefore while we can leverage the electron transfer from even the simplest chemical reactions, proteins offer certain unique advantages in using genetic engineering to modify the redox center and its surrounding polypeptide backbone by mutating critical residues or "hot spots."

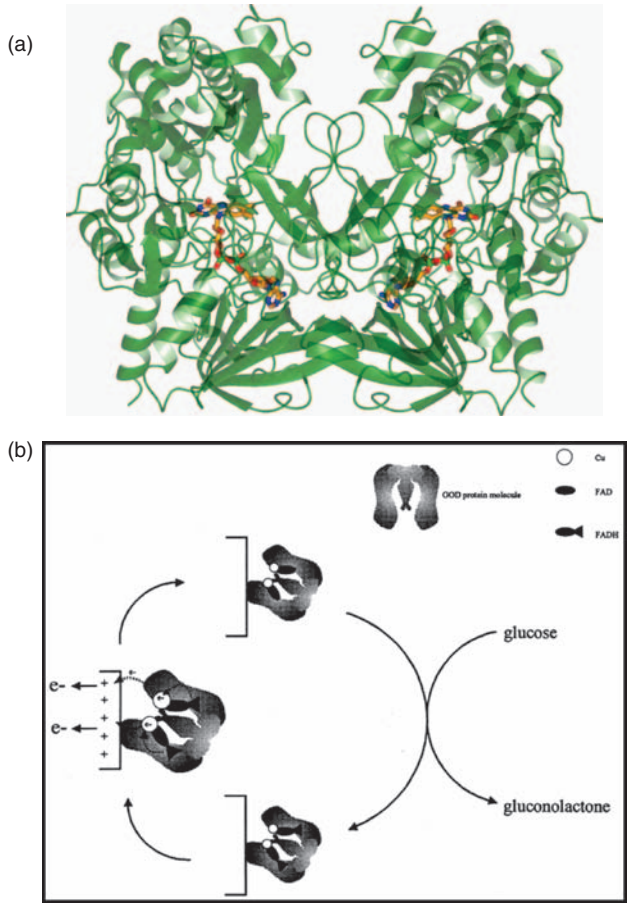

Fig. 10. (a) Glucose Oxidase (GOx) dimeric structure, as generated from PDB code, 1GPE and (b) the electron transfer pathway.

5.5. Immobilization of Electron Transfer Proteins

We will focus on GOx as a test case for covalent attachment to substrates in view of its wide spread use in fuel cells. Covalent attachment of GOx to SWCNT further enhances efficient transfer of electrons. CNTs have unique electronic properties, high mechanical strength and chemical stability, making them attractive for fabricating of GOx coupled CNT based device elements for use in bio fuel cells. For chemically coupling the CNTs with biomolecules, it is critical to functionalize CNT surfaces, which are very inert. Several strategies have been developed for functionalizing CNTs, derivatizing them with biomolecules, and demonstrating the proof of concept of using bacteriorhodopsin mutants -CNT hybrids[24] for sensing via electrical, electrochemical, and electro-optical means. In all the cases, however, the CNTs are functionalized wet-chemically in a spatially random fashion where molecular bonding is mediated by defect creation, or hydrophobic adsorption (Fig. 11(a)). While such conventional treatments may be adequate for demonstrating the proof-of-concept of sensors from CNTs contacted on substrates, or random dispersions of CNTs in solutions, they have serious limitations for realizing *multifunctional* sensor arrays on *a chip* that integrates system level logic operations for data processing.

Forming sensor arrays from *pre-derivatized individual* CNTs may not viable because wafer-scale placement of CNTs at predetermined locations by self-assembly *after* synthesis is difficult. Fabricating sensor arrays from CNT *bundles* grown in aligned configurations followed by subsequent protein derivatization is now possible, but only to create sensors responsive to one type of stimuli

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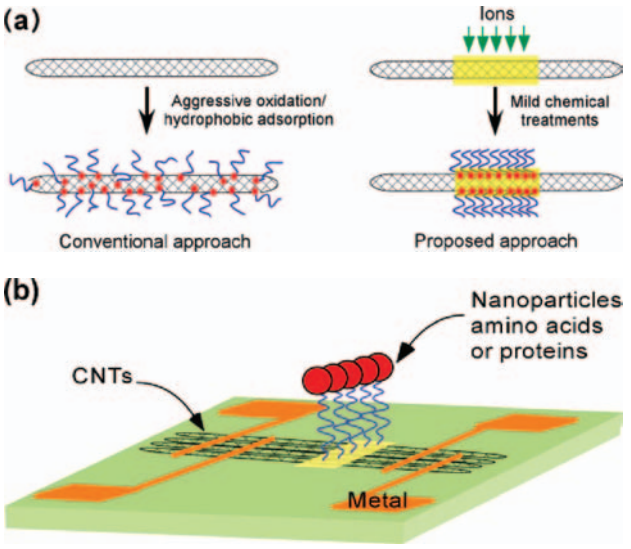

Fig. 11. Comparison of random and site-selective functionalization of CNTs, illustrating the utility of latter for nanobiodevices. (a) Conventional routes using aggressive oxidation with acids and ultrasonication (left) versus FIB irradiation followed by mild chemical treatments (right). The red dots denote defect sites generated, and the blue wavy lines represent functional groups anchored to the defects. Conventional methods result in random defect creation, while ion irradiation allows control over site selectivity, defect types, and concentration. (b) Schematic of an example hybrid nanodevice comprised of site-selectively anchored nanostructures on preselected segments of a CNT assembly (black) on a planar substrate (green, e.g., Si). Arrays of such devices, with each array element potentially containing different nanostructures, open up possibilities for addressing specific CNT segments, for fingerprinting, detection, analysis, and separation of biomolecular structures in a single chip. Reprinted with permission from [24], M. S. Raghuveer et al., *Adv. Mater.* 18, 547 **(2006)**. © 2006, Wiley-VCH Verlag GmbH & Co. KGaA.

(e.g., CNT derivatized by protein A). This is because *aggressive* treatments with reactive acids combined with ultrasonic agitation used in random functionalization are not easily extendable to obtain multiple device elements on a chip from CNTs derivatized with different types (e.g., mutants) of electro-active proteins (e.g., A, B, C etc. at different locations) for orthogonal sensing capabilities. Non-site-specific aggressive CNT derivatization when combined with chip microfabrication can, for-example, destroy biomolecules anchored in a prior step due to chemical attack and ultrasonic agitation.

It is therefore necessary to go beyond the random attachment paradigm to one that allows control over the location of biomolecular attachment in order to fully exploit bio-derivatized CNTs to build device architectures on a chip for *multifunctional* sensing (Fig. 11(b)). Localized creation of high-reactivity zones in CNTs for subsequent derivatization would ensure that the overall properties of the CNTs are retained or controllably altered, and the integrity of other chip components are not compromised or destroyed during device fabrication.

5.6. Attaching GOx with CNTs as Crosslinked Enzyme Clusters (ECE)

In order to promote DET of the protein and to improve the stability, it is necessary that the protein is immobilized in the form of clusters.[4] In this context, GOx can be attached as crosslinked enzyme clusters (CECs) onto the surface of carbon nanotubes (CNTs). In a recent study that CEC-GOx showed no activity decrease for 250 days. The concept is depicted in Figure 12.

5.7. Anchoring Proteins on Suitable Matrices—The Core Issue of Immobilization

The method of oriented binding of photosynthetic proteins to wide band semiconductor electrodes can be broken up into two main strategies: in the first one, a part of the protein is bound to a pre-treated electrode surface and the native protein is floated on top of the functionalized surface while another employs a His-tagged protein than attaches to a pre-treated surface. This new molecular engineering method requires merely an in-house production of a small subunit of the photosynthetic protein to express a short (between 10 and 20 residues) amino acid sequence that preferentially binds directly to the electrode of interest (ZnO, TiO_2, ITO, Au or any other) upon self-assembled recombination, orienting the electron donor part to the electron acceptor of the solar cell. This approach significantly simplifies the process: there is no need for thin film depositions or any other surface pretreatment; there is no preference for the semiconductor or metal used; and this is easily adaptable to nanostructured surfaces. At the

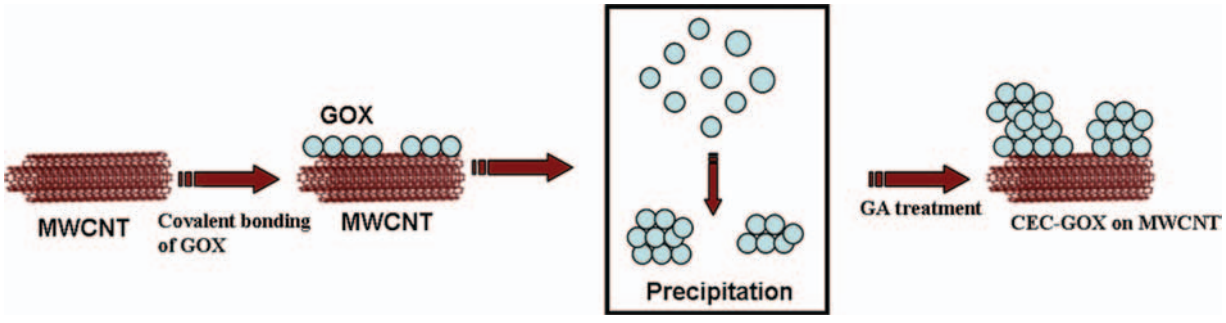

Fig. 12. A schematic representation of processes involved in covalently bonding GOx with MWCNTs.

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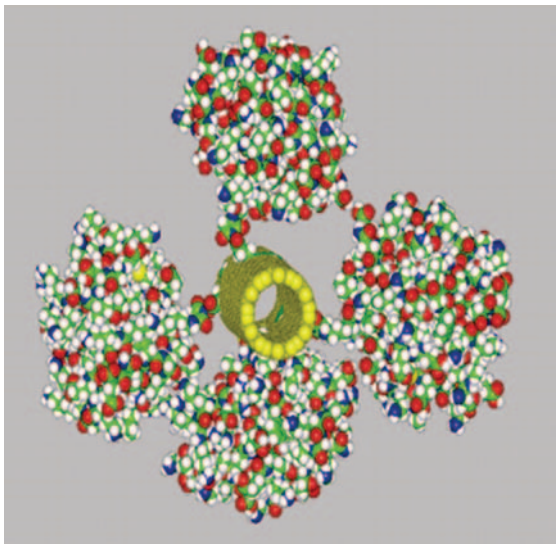

Fig. 13. Molecular dynamics simulation of GOx with SWCNT. Reprinted with permission from [40], S. S. Tatke et al., *Nanotechnology* 15, S684 **(2004)**. © 2004, IOP Publishing Ltd.

same time, it lowers the resistance and increases the available photocurrent, power and efficiency of the device by substantially decreasing the distance of electron diffusion into the photo anode. A molecular dynamics simulation derived covalent attachment of GOx to SWCNT is shown in Figure 13.[40]

6. PACKAGING OF BIO-FUEL CELLS

One of the major challenges in bionanotechnology is merging new nanoscale fabrication tools with classical synthetic methods and delicate biomolecular building blocks to create materials with unique biomedical properties.[41] In order to address the packaging requirement of the biofuel cells, it will be necessary to bridge the disciplines of biology, chemistry, materials science, semiconductor technology and engineering to find optimum packaging solutions for the challenges posed by these devices. *Biological packaging* can be defined as the sum total of the physical device, temperature regulating and monitoring systems, type of preservation solution, and storage protocol(s) necessary to maintain cells or tissues in a "state of suspended animation" during transport or storage.[42]

The packaging issues connected with biological applications pose different set of challenges. The materials used in the medical device industry are extremely robust, and research shows that the failure rate is less than one in one million packages.[43] To achieve the performance levels of this order, the reliability testing procedures for the devices has to be very stout, which is an added cost of the product. The big difference, however, between medical device packaging and other branches of the packaging industry is the role the regulators play. Primarily the medical device industry for the last 30 years has been shaped by the FDA, which oversees all aspects of medical device packaging from material selection, design and manufacturing to labeling and sterilization.[43] Frequently, the packaging solutions for biological applications are tailored to their specific application, which contributes to lack of standardization.

BFC research and development appears to be meager at present to grasp its real packaging challenges. However, understanding of Bio-MEMS's packaging issues that exist today could serve as a foundation to focus on the biofuel cell packaging demands. Small size and low power are crucial for implantable devices. Low power electronics contributes towards minimization of volume and permit insertion of devices into locations such as the eye and ear for a variety of therapeutic and diagnostic applications.[44] Biocompatible materials and processes such as advanced sealing and packaging techniques are also vital for small implantable and diagnostic devices. For example, a major break-through in stents was to treat them with drug-bearing polymers.[45] Usage of drug-bearing and biocompatible plastics for implantable devices is commonly practiced today. The human body is mostly polymers, so selecting polymers based packaging materials makes sense for biopackages. Incorporating common plastic engineering material, like LCP, and automatic injection molding to form micro-channels and ports are an ideal technology for Bio-MEMS and other advanced devices.[45]

The converging paths of microtechnology and biotechnology offer an enticing opportunity that may not require the latest semiconductor processes in order to make cutting-edge biodevices. The development of power sources such as BFCs are essential to meet the power requirements of the devices targeted for biological applications. There exists a possibility in the near future that BFCs powered by sugar solutions using enzyme coated electrodes to oxidize glucose and another to reduce oxygen could have an amazing effect on small, integrated power sources.[46] BFCs are ideal for implant applications because they do not require recharging.[47] Typically the expected longevity of power sources for implantable devices is >5 years.[46] Additional desirable features include smaller size and capacity to provide information on their status in response to an outside query.

The best method to power implantable devices is using harvested energy from natural sources; examples include the sodium and potassium gradients across cell membranes; oxygen gradients within the body; or light-harvesting implants based on mitochondrial chylomicrons and the very efficient photosynthetic organelles in green plants.[46] Biological systems are unique and utilize highly efficient nanostructures to do things differently. The future holds for development of systems that can convert chemical energy into mechanical motion using nanomotor proteins with appropriate synthesis for energy conversion. Enzymes are the dynamic catalytic nanomachines in living organisms to run all synthetic, energy conversion

(metabolic) and animation processes (growth, reproduction, locomotion). The challenge posed here is how to integrate all the pieces together to design a workable nanomachine that is capable of performing a specific task, have an integrated BFC power source for longer operation time, and the capacity to respond to the external queries. A long road is ahead for developing acceptable and workable solutions for BFC powered devices.

The primary function of the device packaging is to provide mechanical support, electrical interconnection between the package and the device, protect the device from environment, and serve as a joining structure to the next level system integration. In addition, the package as a whole must provide qualities such as ease of handling and assembly, standardization, testability, thermal management, electrical performance, and cost. The device packaging has always been a challenge from the start for the microelectronics industry and it continues to be the hurdle even today. This challenge is becoming a major roadblock as the technology is progressing towards nanoscale devices. The device packaging has been plagued by higher cost, device miniaturization, and shrinking board real estate, identifying a process that is reliable and compatible with various materials used in the electronic packaging. Due to these shortcomings, commercialization of the product to the market place is being controlled by the given set of packaging requirements. For example, the advancement of the Micro Electro Mechanical Systems (MEMS) is primarily derailed by the issues related to their packaging. The packaging issues associated with BFCs will also have to address some of these bottlenecks to emerge into a viable solution as a power source for the implantable biomedical devices.

ABBREVIATIONS

Bio-fuel cell: BFC, Direct electron transfer: DET, Mediated electron transfer: MET and Glucose oxidase: GOx

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