Elastic molecular machines in metabolism and soft-tissue restoration

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Elastic protein-based machines (bioelastic materials) can be designed to perform diverse biological energy conversions. Coupled with the remarkable energy-conversion capacity of cells, this makes possible a tissue-restoration approach to tissue engineering. When properly attached to the extracellular matrix, cells sense the forces to which they are subjected and respond by producing an extracellular matrix that will withstand those forces. Elastic protein-based polymers can be designed as temporary functional scaffoldings that cells can enter, attach to, spread, sense forces and remodel, with the potential to restore natural tissue.

Voet and Voet define metabolism as ‘the overall process through which living systems acquire and utilize the free energy they need to carry out their various functions’. Every utilization of free energy constitutes an energy conversion; that is, for every free-energy input, there is a free-energy output. For animals, the free-energy input initially comes in the form of food, and the free-energy output is the particular function. Accordingly, metabolism involves a remarkably integrated family of free-energy conversions and constitutes the major part of biological energy conversion. Another element of energy conversion in biology would be the mechanical stretching of an elastic fiber, which stores the deformation energy, followed by recoil and return to the non-deformed structure.

Biomolecular machines

Metabolism is performed by the cell but, in general, free-energy conversions are performed by proteins. Consequently, proteins can properly be called biomolecular machines, because they convert energy from one form or location to another, and so the cell can be seen as an integrated set of biomolecular machines. To encompass the whole of biological free-energy conversion, however, the proteins of the extracellular matrix must also be included. In particular, the relationship of a cell to the proteins of its extracellular matrix becomes fundamental to the energy conversions involved in tissue development and function. This article addresses the principles and applications of protein engineering in two ways. First, I will outline the mechanism by which energy conversion, more properly called free-energy transduction, can be performed by proteins and protein-based polymers (polymers composed of repeating peptide sequences that are often inspired by the repeating sequences of natural proteins). Second, I will address the use of elastic protein-based polymers in tissue restoration from a unique vantage point, viewing the cell as a mechanochemical transducer. By attachment to an elastic extracellular matrix, the cell senses the forces (as a mechanical-energy input) to which its tissue is subjected and then produces an extracellular matrix sufficient to withstand those forces (as the chemical-energy output).

Tissue restoration

The basis of biomedical engineering to restore tissue function arises from the energy transductions of cells. The aim is to design temporary functional scaffoldings that mimic essential elements of the structural and functional state of a tissue that is to be restored. These should then induce cells to enter, attach, spread, sense forces and remodel the temporary scaffolding into a natural functional tissue competent to sustain the forces. This is tissue restoration, and the biomedical-engineering canon on which it is based is, I believe, a consult principle of biology and medicine; that is, it makes use of the fundamental principles on which biology and medicine are based.
REVIEWS

Tissue injury arising from too little or too much exercise

When a tissue is under exercised, the cells are not sufficiently mechanically stimulated to produce a tissue of adequate strength. In other words, there has not been a sufficient mechanical-energy input to stimulate conversion to the chemical-energy output of a strong extracellular matrix. Because of this, even when the range of use is reasonable, the tissue fails.

When a tissue is over exercised, there can be either chronic or acute failure. In moderate chronic overuse, the cells may be unable to keep up with the required restoration, as may occur for the articular cartilage of a runner’s knees. In acute failure, the stresses (or mechanical forces) applied to the tissue exceed the load capacity of even a strong tissue. The consequence is catastrophic failure, followed by the inability of even a strong tissue to repair itself. Thus, free-energy transduction is central to soft-tissue restoration.

Each year in the USA, low-back pain results in more productivity loss than any other medical condition, and in health-care costs of more than US$33 billion. When disability costs and lost productivity are added into the calculation, the economic losses exceed US$100 billion per year. The common cause of low-back pain is pathology of a soft tissue, the intervertebral disc.

The restoration or repair of an injured intervertebral disc can occur at two levels: first, to improve the outcome of a discectomy or laminectomy procedure by using materials to prevent adhesions and fibrosis, which result in failed-back-surgery syndrome; second, to use a material to restore the correct disc dimensions and viscoelastic properties, and, at the same time, to use a material to provide for cellular attachment so that cells can sense the forces that an approximately configured disc would sustain. Bioelastic materials (elastic protein-based polymers) are being developed for use at both levels.

A perspective on biological energy conversion

Thermodynamics

The science of thermodynamics is the study of temperature changes. It grew out of the need to design machines capable of performing work. The steam engine is a historical example. For a steam engine, the primary factor is the water-to-vapor phase transition; a heat source introduces the substantial energy required to convert water to steam, and the pressure resulting from steam formation drives a piston that produces motion. In a somewhat analogous manner, El Niño is an engine: a large body of warm water causes the formation of large amounts of water vapor in warm air; on meeting a cool air mass, the water vapor condenses to a liquid, releasing the considerable energy that had been stored as a consequence of the water-to-vapor phase transition and this released energy causes temperature and pressure gradients that result in strong winds and associated rains.

In contrast to this, metabolism occurs without significant changes in temperature or pressure. Instead of raising the temperature from below a phase transition to above it in order to perform work, living organisms can be thought of as functioning by lowering the temperature at which a special phase transition occurs from above to below physiological temperature. It is as though the organism had found a way to perform mechanical work by lowering the boiling point of water from 100°C to 0°C while remaining at atmospheric pressure and 37°C.

The energy to lower the transition temperature generally comes from chemical energy (i.e. changes in the concentration of chemicals). The most dramatic lowering of the transition temperature is due to the dephosphorylation of proteins side chains that were previously phosphorylated using the universal biological energy source adenosine triphosphate (ATP).

The nature of the special phase transition that can achieve energy conversion in living organisms is, however, quite different from the water-to-vapor phase transition; it is a phase transition in which the hydrophobic groups of a protein go from being hydrated in water to being hydrophobically folded and assembled. The hydrophobic groups of the protein become associated intramolecularly, as in the hydrophobic folding of a globular protein, and/or associated intermolecularly, as when a uniform solution separates into two distinct phases. Crucially, in both cases, the mixture of ordered and disordered components is called an inverse temperature transition (ITT).

Control of the temperature at which the intervertebral disc can achieve energy conversion is, however, quite different from the water-to-vapor phase transition; it is a phase transition in which the hydrophobic groups of a protein go from being hydrated in water to being hydrophobically folded and assembled. The hydrophobic groups of the protein become associated intramolecularly, as in the hydrophobic folding of a globular protein, and/or associated intermolecularly, as when a uniform solution separates into two distinct phases. Crucially, in both cases, the mixture of ordered and disordered components is called an inverse temperature transition (ITT).

Because the focus is on the catalytic protein, and because the protein becomes more ordered as a result of this phase transition, the temperature of the assembly transition achieved on raising the temperature from below a phase transition can be thought of as functioning by lowering the temperature at which a special phase transition occurs from above to below the physiological temperature. Among other factors, the function of muscle contraction and transport of oxygen by hemoglobin, and the energy conversion of the inner mitochondrial membrane and the thylakoid membrane of chloroplasts have been discussed elsewhere in this light.

Accordingly, the principles of protein engineering required for performing energy conversions of the types that occur in biology are derived from a full understanding of those factors that change or control the temperature of the hydrophobic folding and/or assembly transition. This is called the ΔTf mechanism, where ΔTf is the temperature of onset of the inverse temperature transition of hydrophobic folding and/or...
assembly. Interestingly, the key factors that affect $T_t$ are principal players in metabolism. These factors may be changes in the state of functional groups of the protein (e.g. a change in the charge on a side chain), the state of an attached redox group that receives and releases electrons, or in the phosphorylation of a side chain) or they may be extrinsic to the protein (e.g. changes in the concentrations of chemicals in the surrounding media). Importantly, the inverse temperature transition is capable of performing several kinds of work in addition to mechanical work, such as chemical and electrochemical work. Experimental studies on the inverse temperature transitions exhibited by elastic protein-based polymers, as they relate to the performance of various forms of work or energy conversions of relevance to biology, have resulted in a set of five phenomenological axioms (Box 1).

Out of an understanding of protein engineering for energy conversion (which is really an understanding of the means by which proteins fold hydrophobically and function upon interaction with other molecular and ionic species) comes a new biotechnology, in which protein-based polymers are designed for numerous medical and non-medical applications.

Elastic protein-based polymers in energy conversion

The initial elastic protein-based polymer for our studies is (Gly-Val-Gly-Val-Pro)$_n$, or (GVGVP)$_n$. This sequence is the most striking repeating sequence of the bovine elastic-fiber protein, repeating eleven times within a single subunit$^{11}$. When this polymer (with $n$ of the order 200) is dissolved in cold water and the temperature is raised above $T_t$ of 25°C, a phase separation occurs$^2$. The denser, viscoelastic, polymer-rich phase can be cross-linked by $\gamma$-irradiation to form an elastic matrix that has an elastic modulus approximately $10^2$ N m$^{-2}$; the elastic modulus can be varied from $10^7$ to $10^8$ N m$^{-2}$ by varying the compositions and conditions. This makes it possible to match elasticity over the wide range of biological soft tissues.

The nature of the elasticity

Elastic protein-based polymers fold and assemble by relatively unrestricted contacts involving bulky hydrophobic side chains. This leaves most of their backbone peptide moieties without structurally constraining inter- and intramolecular hydrogen bonding, and so these moieties are free to undergo large-amplitude, high-entropy rocking motions that become damped on extension.

These protein-based elastomers are nearly ideal elastomers, with limited straining and breaking of bonds on extension resulting in the potential for extraordinary functional lifetimes. For example, the half-life of the mammalian elastic fiber is of the same order as the lifetime of the individual, more than 60 years$^{19-20}$, in spite of the relentless heavy elastic demands placed on such tissues as the aortic arch and descending thoracic aorta. This decrease in entropy, caused by the damping of internal chain dynamics on extension (i.e. a decrease in the amplitude of the peptide-moety rocking motions), has been called the peptide-librational-entropy mechanism of elasticity$^{21-23}$.

Pumping iron, protons and electrons

When a weight is hung on a strip of cross-linked elastic protein-based polymer and the temperature is raised above $T_t$, the elastic matrix contracts and lifts the weight; a thermal-energy input can be used to ‘pump iron’. Alternatively, chemical-energy inputs (e.g. protonation of a carboxylate) and electrochemical-energy inputs (e.g. reduction of an attached redox function such as a nicotinamide) can also be used to ‘pump iron’, because they lower $T_t$ from above to below the operating temperature (e.g. physiological temperature$^{24}$).

The mechanical-energy input of stretching a hydrophobically folded elastic protein-based polymer matrix containing a carboxylate can raise the $pK_a$ of the functional group and cause the uptake of a proton$^{25}$; the affinity of an unaltered state of an attached redox couple for an electron can be similarly increased by increasing the hydrophobicity (L. C. Hayes, PhD thesis, University of...

Box 1. Five axioms for the protein engineering of protein-based polymers capable of inverse temperature transitions of hydrophobic folding and assembly

| Axiom 1 | The manner in which a guest amino acid residue, or chemical modification thereof, alters the temperature, $T_t$, of a hydrophobic folding and/or assembly transition is a functional measure of its hydrophobicity. A decrease in $T_t$ represents an increase in hydrophobicity and an increase in $T_t$ represents a decrease in hydrophobicity. |
| Axiom 2 | Raising the temperature above $T_t$ results in hydrophobic folding and assembly, and can be used to perform useful mechanical work (e.g. organization into a structure or lifting weights); this is thermomechanical transduction. |
| Axiom 3 | At constant temperature, lowering the value of $T_t$ from above to below an operating temperature (i.e. increasing the hydrophobicity) also results in hydrophobic folding and assembly, and can be used to perform useful mechanical work. |
| Axiom 4 | Variables including temperature, pressure, chemical concentrations, the charged state of a functional side chain, the redox state of a biological prosthetic group and light-excited changes in chemical structure can be used to alter the value of $T_t$ to perform mechanical work by producing folding and assembly. Any two distinct functional groups responsive to these variables can be coupled one to the other by being part of the same hydrophobic folding and assembly domain. |
| Axiom 5 | The above energy conversions can be demonstrated to be more efficient when carried out using more hydrophobic protein-based polymers. |
The underlying physical mechanism
The underlying physical mechanism that gives rise to the diverse energy conversions is a completion for hydration between apolar (hydrophobic) and polar (e.g. charged) species. This competition is directly responsible for the large hydrophobicity-induced pKₐ shifts, as much as six or more units, and for the effectiveness with which a charged species can lower the temperature of the inverse temperature transition (i.e. lower the value of Tₛ). For example, the addition of one charged moiety per 100 residues can lower the value of Tₛ by as much as 100°C. This process results from an apolar–polar repulsive free energy of hydration.

Bioproduction, purification and biocompatibility
In order for elastic protein-based polymers to become viable materials for medical applications, three elements must be demonstrated: sufficiently low production costs; purification to the extraordinary levels required for use as a resorbable biomaterial; and biocompatibility with tissues, tissue fluids and blood.

Bioproduction by recombinant DNA technology
Low production costs can be achieved by using recombinant DNA technology to produce the designed sequence via the machinery of the living cell. When considering a plant source and agricultural products, this places the lower limit of cost near that of other agricultural commodites, such as starch and soy-bean oil, <$US1 kg⁻¹. A striking demonstration of the capacity of E. coli to produce elastic protein-based polymers is shown in Fig. 2, which shows cells with approximately 80% of the cell volume containing (GVGVP)₁₂₁ in inclusion bodies. Although the costs are presently orders of magnitude higher than US$1 kg⁻¹, unofficial information from the producers of industrial proteins indicates that a few dollars per kg should be an attainable cost. Of course, for certain medical applications, costs as high as US$1000 g⁻¹ are acceptable.

Purification based on phase transitions
An advantage of the protein-based polymers considered here is their ability to undergo phase separations. Using this approach in a direct manner, purifications to less than 1 part per million (ppm) impurity, as judged by western-immunoblot techniques, have been attained. This is a high purity, as 10 ppm is considered adequate for the pharmaceutical use of insulin produced in E. coli.

Using the product in larger quantities as a structural biomaterial, in the role of a device rather than as a potent drug, requires a higher standard of purity than this, however. For use as a biomaterial, one million times more material may be required. The crucial issue becomes the time period over which the impurities may be released to the host. It is clear from animal studies of subcutaneous implants that purification to 5 ppm is grossly insufficient when the material can be dispersed within a month. In fact, sufficient purification has been achieved that a 30 mg implant that disperses within days can be used without evidence of the protein-based polymer having ever been present (Fig. 3).

Thus, purification has been demonstrated for the most

The capable of inverse temperature transitions. (Adapted with permission from Ref. 3.)

The pairwise interconversions of energies that are possible using molecular machines. (Adapted with permission from Ref. 3.)

The electromagnetic paradigm for protein folding and function. This diagram indicates the diverse energy conversions with the possible exception of magnetic-field effects.

Figure 1

The δₐp hydrophobic paradigm for protein folding and function. This diagram indicates the pairwise interconversions of energies that are possible using molecular machines capable of inverse temperature transitions. (Adapted with permission from Ref. 3.)

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stringent case, in which all the impurities from *E. coli* can be released within a few days without any sign of a tissue response. These results demonstrate the remarkable biocompatibility of these protein-based polymers. A full battery of eleven tests, recommended by the American Society for the Testing of Materials had previously been carried out on (GVGVP) \(_n\) to demonstrate its extraordinary biocompatibility. Furthermore, monoclonal antibodies to (GVGVP) \(_n\) have not yet been produced, despite intensive efforts to do so. Thus, it has been clearly demonstrated that the parent elastic protein-based polymer has the requisite biocompatibility for medical use as a biomaterial in contact with tissue, tissue fluids and blood.

**Cellular function in tissue maintenance**

A tissue is composed of two major components: the cells and the extracellular matrix that the cells construct. Each tissue has a particular set of physical and chemical functions in fulfilling its role of sustaining the organ and/or organism of which it is a part. The central question in tissue maintenance or restoration is, ‘what causes a cell to remodel the particular extracellular matrix of a differentiated tissue?’ The answer is that cells of the correct phenotype attach to the extracellular matrix at multiple sites, thereby sensing the forces to which the matrix is subjected and consequently producing an extracellular matrix that can sustain those forces. Appreciation of this began more than two decades ago with studies by Glagov and co-workers and, more recently, van der Leij, Ingber and Neren and their co-workers, using the terms ‘cellular tensegrity’ and ‘mechanotransduction’ to identify the phenomena involved.

In the cases of a simple bioelastic matrix and the elastic fiber, stretching increases the Gibbs free energy by causing a decrease in chain configurational entropy and in solvent entropy. The result is an elastic recoil as the extending force subsides. Figure 4 illustrates a more complex case in which there is cell attachment to a bioelastic matrix. By attaching to the bioelastic matrix at each end of a cytoskeletal fiber, the mechanical-energy input of stretching the elastic matrix is borne in part by the cell’s cytoskeletal components. The stretched cytoskeletal fibers produce chemical-energy outputs, such as an increase in the free energy of a carboxylate or a bound phosphate, or the uptake of protons (i.e. pumping protons, as demonstrated experimentally by a stretch-induced increase in \(pK_a\) of glutamate-containing elastic matrices).

The increase in \(pK_a\) arises from the increase in Gibbs free energy of the negatively charged carboxylate as stretching increases the exposure of hydrophobic groups to water and results in competition between the charged and hydrophobic groups for hydration. Similarly, the chemical-energy output of a stretched cell could be an increase in the Gibbs free energy of an attached negatively charged phosphate. This stretch-energized phosphate, or some other manifestation of a stretch-induced chemical-energy output, would be the immediate chemical-energy response; the eventual chemical-energy response would be the activation of genes to produce an extracellular matrix sufficient to sustain the dynamic range and temporal aspects of the applied forces. Cellular tensegrity is one term used for these aspects of molecular biophysics.
Tissue restoration

Cells of a tissue have the remarkable ability to receive an energy input from their environment and to respond by improving the capacity of the tissue to accommodate or process that energy input. Stretching a vascular wall causes its cells to produce extracellular matrix to sustain those same tensional forces better; exercising musculoskeletal tissues causes increased muscle and bone mass to meet the next physical challenge better. Sights, smells, sounds and touch represent energy inputs that stimulate cells of the brain tissue to develop neural networks that can better sense those same energy inputs. The common related aphorism is 'use it or lose it', but equally relevant might be 'use it to improve it'.

I will focus on the kind of biomaterial that would be required to make use of this exceptional capacity of cells for tissue restoration. The biomaterial should be able to be made into an appropriate temporary functional scaffolding – for example, it should be biocompatible, elastic when the tissue to be restored is elastic and able to handle energy inputs in a manner similar to natural tissue. Bioelastic materials composed of elastic protein-based polymers have these requisite properties for soft-tissue restoration.

Using protein-based machines

In this approach, the prosthesis should match the mechanical properties of the tissue to be restored, and, as in Fig. 4, it should contain sites to which the normal cells of the tissue can attach. By multiple attachments of the cell to the temporary functional scaffolding, the mechanical stresses sustained by the functional artificial matrix would be appropriately transmitted to that cell, which can discern the magnitude of the force, its dynamic range and the time course of any changes. This mechanical sensing by the cell would result in the chemical remodeling of the matrix into a natural tissue sufficient to sustain those forces, that is, in the production of the required extracellular matrix. Although the foregoing general discussion may be expected to be
applicable to both hard and soft tissues, elastic protein-based polymers are most relevant to soft-tissue restoration.

Soft-tissue restoration

In soft-tissue restoration, the prosthesis should have the correct compliance, elastic modulus and viscoelasticity for the tensional or compressional force changes it will feel, as well as cell-attachment sequences as discussed above.

Urinary incontinence

A conservative estimate of the medical costs of urinary incontinence in the USA is over US$10 billion per year. The problem of stress urinary incontinence can be overcome by adding a bulking agent at the base of the bladder to provide sphincter support by enhancing urethral compression. Many inert substances have been injected at the base of the bladder as space or volume fillers [e.g. polytetrafluoroethylene (PTFE)40, paraffin41, autologous fat42 and bovine dermal collagen (a connective-tissue component) treated to decrease immunogenicity43,44] with unsatisfactory results29.

Soft-tissue restoration provides a most effective way to fill volume by inducing the generation of natural tissue. Replacing a Glu residue of the elastic protein-based polymer used in Fig. 3 with a GRGDSP cell-attachment sequence causes the generation of a natural tissue (Fig. 5) with the normal distribution of elastic and collagen fibers29, rather than simply disappearing (Fig. 3). Generating a natural, long-lasting tissue provides an attractive means of sphincter support to restore urinary continence.

Urological prosthesis

The same elastic protein-based polymer with the GRGDSP cell-attachment sequence can be used in the development of a urological prosthesis. When cross-linked, this polymer forms a matrix with an elastic modulus that matches that of the natural urinary bladder. When a human ureter explant is placed on the matrix, uroepithelial cells grow out onto the matrix. This provides the opportunity to test the consilient approach to tissue engineering: can the filling and emptying of a simulated urinary bladder stimulate outgrowth of the uroepithelial cells and their elaboration of an extracellular matrix?

The simulated urinary bladder is a chamber that can be filled and emptied at defined rates (e.g. filling in 3 h and emptying in 25 sec), this treatment continuing for several days. Filling stretches the elastic matrix, which contains cell-attachment sequences and the attached human ureter explant with its cellular outgrowth. The outgrowth of human uroepithelial cells was compared with and without filling and emptying, and it was found that simulating the tensional-force changes of bladder filling and emptying stimulated the outgrowth, producing a greater density of cells and extracellular matrix45.

Prevention of post-surgical or post-trauma adhesions

Restoring an injured tissue to its normal state in many cases demands a means to prevent adhesions; that is, preventing abnormal bands of connective tissue binding tissues together inappropriately. Using a blood-
The bioelastic materials (which are pressure-responsive, viscoelastic, protein-based polymers) are used to restore the normal dimensions and swelling pressure by injecting into a disc previously deformed by gentle stretching and relaxation of the vascular wall. The bioelastic materials are biocompatible and, in this important respect, can function like natural tissues. Normal cells sense the forces to which their tissues are subjected and respond by remodeling the extracellular matrix into a tissue better suited to the demands placed on reasonably structured tissue.

Conclusion
Bioelastic materials (elastic protein-based polymers) make possible a compliant approach to soft-tissue engineering, in which temporary functional scaffolding can be designed that favor remodeling into a natural tissue. Normal cells sense the forces to which their tissues are subjected and respond by remodeling the extracellular matrix into a tissue better suited to meet the sensed demands. This is known as cellular mechanochemic transduction. The process of sensing an energy input (the applied force) and responding by restructuring the environment (the energy output) represents a specific case of energy conversion. In the best-described case (arterial cells), the energy input derives from cyclic stretching and relaxation of the vascular wall caused by pulsatile blood flow.

Bioelastic materials can be designed to convert the same set of energies as are interconverted in metabolism and, in this important respect, can function like natural tissues. The bioelastic materials are biocompatible and can be designed to match the elastic modulus of the tissue to be restored. As the bioelastic materials are protein based, cell-attachment sequences can be included as a continuous part of the sequence, as occurs in the protein that naturally contains the cell-attachment sequences. These sequences, being part of an elastic matrix with the correct elastic modulus, allow cells to attach, sense the applied forces and function as though they were in their natural extracellular matrix. Because they are protein based, bioelastic matrices can be degraded in the same way as protein is degraded, releasing amino acids as their breakdown products. In this way, the temporary functional scaffolding can become remodeled to restore the natural tissue.

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References
Facts and more facts

Molecular Biology Lab Fax: Recombinant DNA (Vol. I); Gene Analysis

Chapter 3 provides a useful but incomplete discussion of PCR, including a trouble-shooting guide and a very handy table showing the sensitivity of restriction enzymes to nucleoside length. A variety of PCR-based methods are omitted, leaving the reader to consult the more detailed texts cited in the references. Methods for isotopically labelling nucleic acids are discussed in some length in Chapter 4 and an introduction to methods of isotopic detection is included, as is a useful section on safety.

Chapter 5 is an elaborate treatment of agaro-gel electrophoresis, providing comparisons of agarose variants, and a 26-page list of plasmid digests that can be used as markers. Curiously, there is no mention of native polyacrylamide gel electrophoresis of nucleic acids.

Chapter 7 is a list of the molecular weights and structures of a wide variety of reagents used in molecular biology and, in this chapter, the author adheres more closely to the stated purpose of the book. The final chapter concerns laboratory safety and some of the hazards associated with various chemicals.

Lab Fax does fulfill its stated purpose of providing numerous pieces of information. The question is whether reading these volumes will enable a person in the molecular biology lab either to choose better techniques or to be able to apply more effectively the techniques described in the numerous lab manuals and the primary literature. Regrettably, these volumes fall short in both regards.

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