# Lessons from the Immune System: From Catalysis to Materials Science

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## Introduction

Nature has produced a remarkable array of molecules that perform the complex processes of living organisms, from the immune response and catalysis to signal transduction and gene regulation. However, in contrast to chemists who typically synthesize and characterize a single molecule at a time, Nature draws upon a vast combinatorial library of precursor molecules and screens them for desired properties. Perhaps the most notable example of this strategy is the immune system, which is capable of generating tremendous molecular diversity via gene rearrangements and somatic mutation and screening this diversity for high-affinity, selective receptors to foreign antigens. This natural example of the power of combinatorial processes has inspired chemists and biochemists alike to apply this strategy to other problems, ranging from catalysis and drug discovery to materials science. In this Account, we will describe a number of ongoing efforts in our laboratory which may help to illustrate the potential of combinatorial libraries in chemistry.

## Catalytic Antibodies

One of the first examples in which the chemical potential of large combinatorial libraries was exploited was the generation of catalytic antibodies.[1] The large repertoire of antibody molecules in the humoral immune system provides the chemist with the ability to rapidly evolve selective receptors for a wide array of chemical structures. Consequently, an appropriately designed immunogen can direct the formation of an antibody that stabilizes the rate-limiting transition state for virtually any reaction of interest. Indeed, antibodies have been generated that catalyze a large number of transformations, ranging from pericyclic and redox reactions to cationic rearrangements.[1] In a number of examples, most notably metalation reactions,[2] transacylation reactions,[3] and Claisen rearrangements,[4] the catalytic efficiencies of antibodies rival those of the corresponding enzymes. Antibodies have also been shown to accelerate reactions that lack enzymatic precedent, such as the Diels−Alder reaction[5] and Cope rearrangement,[6] as well as reactions that utilize abiological cofactors, including periodate-dependent oxidation,[7] borohydride-dependent reduction,[8] and peracid-dependent epoxidation reactions.[9] In addition, antibodies can catalyze reactions which are difficult to perform using conventional chemical methods, including kinetically disfavored cyclization,[10] Diels−Alder,[11] elimination,[12] and aldol reactions.[13] The broad scope of reactions amenable to antibody catalysis underscores the enormous diversity and chemical potential of this immunological library.

Antibody catalysis not only provides a wide range of highly selective catalysts but also offers insights into the nature and evolution of enzymatic function. The combinatorial optimization process whereby the immune system evolves binding and catalytic function shares many features with the natural evolution of protein function. An examination of the phylogenetic history of proteins supports a scenario of exon shuffling to generate novel protein frameworks, followed by natural selection acting on point mutations to improve function. By comparison, the recombination and selection of antibody gene segments followed by somatic hypermutation recapitulate these processes on a dramatically reduced time scale during the immune

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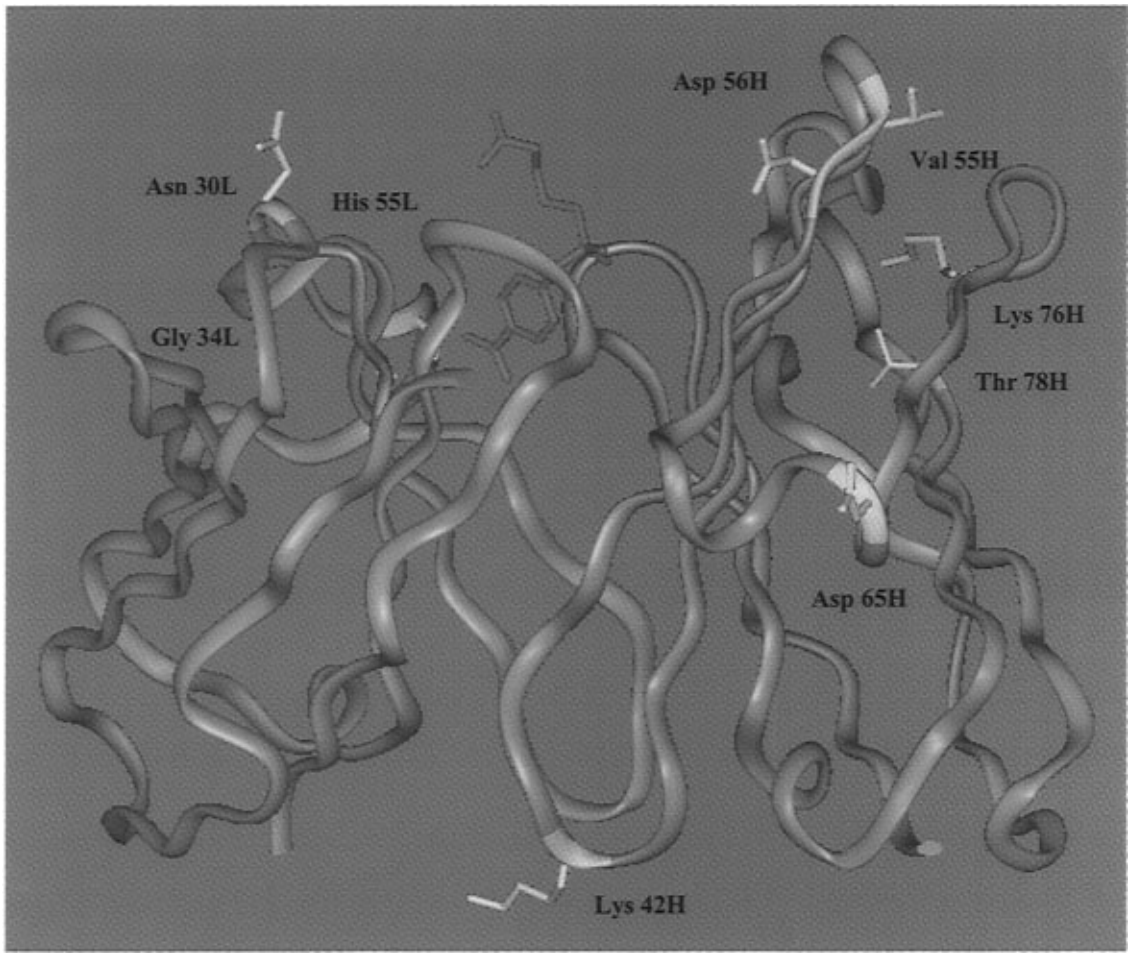


**Figure 1.** Somatic mutations fixed during the affinity maturation of antibody 48G7. Mutated residues are labeled and shown in yellow; the hapten is depicted in red.

response. An example of the immunological evolution of a simple esterolytic antibody[14] will be overviewed to illustrate what can be learned by studying a natural combinatorial optimization process.

Antibody 48G7 was generated against a *p*-nitrophenyl phosphonate transition state ($TS^{\ddagger}$) analogue and found to catalyze the hydrolysis of the corresponding *p*-nitrophenyl ester and carbonate with $10^4$-fold rate accelerations over the uncatalyzed reactions.[15] In order to reconstruct the immunological evolution of this esterolytic antibody, the germline genes of antibody 48G7 were cloned and the corresponding antibody was characterized.[14] Nine replacement mutations were fixed during affinity maturation, the process whereby antibody−antigen recognition is optimized through the somatic hypermutation of antibody genes (Figure 1).[16] These mutations, six in the heavy chain and three in the light chain, resulted in a $10^4$-fold increase in affinity for the transition state analogue, due largely to decreases in the dissociation rate of the hapten−antibody complex. The effects of these mutations on binding free energy appear to be additive, which may help to explain how binding is optimized through a process that sparsely samples sequence space (there are $>10^{22}$ ways to distribute nine single-step point mutations in the variable region of an antibody).

Paralleling the increase in affinity for the $TS^{\ddagger}$ analogue, the $k_{cat}/K_M$ increased approximately 100-fold, consistent with the classic notions of enzymatic catalysis put forth by Pauling and Haldane in which binding energy lowers the activation energy for reaction of enzyme with substrate.[17]

In order to understand the functional consequences of affinity maturation in structural terms, the X-ray crystal structure of the $F_{ab}$ fragment of 48G7, complexed with the phosphonate $TS^{\ddagger}$ analogue, was solved to 2.0 Å resolution.[14] The crystal structure along with a mutagenesis study of active site residues strongly supports a mechanism in which the antibody catalyzes the reaction primarily through selective transition state stabilization. The residues Tyr $33^H$, Arg $96^L$, and His $35^H$ form an oxyanion hole which can stabilize the tetrahedral negatively charged transition state by both electrostatic and hydrogen-bonding interactions. A number of these structural features are shared by other catalytic antibodies generated from distinct phosphonate $TS^{\ddagger}$ analogues, underscoring the importance of transition state stabilization in the immunological evolution of these esterolytic antibodies.[18]

Strikingly, an examination of the crystal structure of the 48G7 $F_{ab}$−hapten complex reveals that eight of

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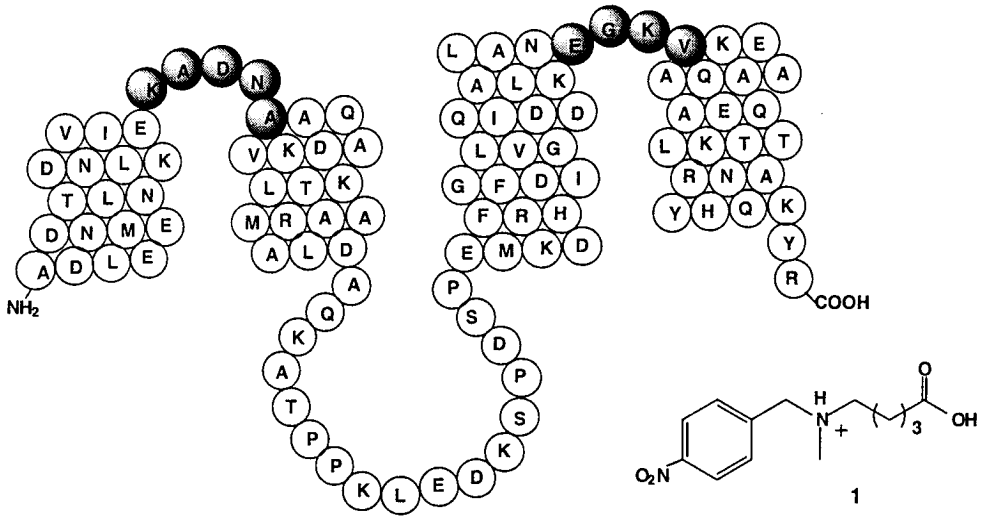


**Figure 2.** Structures of cytochrome $b_{562}$ (shaded amino acids are those randomized in the loop libraries) and ligand **1**.

the nine somatically mutated residues are not in direct contact with the bound TS‡ analogue. Although some of these mutations may be neutral or involved in binding to the carrier protein, it is likely that a significant number are involved in hapten binding, in light of the $10^4$-fold increase in the affinity for hapten. Thus, large improvements in hapten-binding affinity can result from somatic mutations distant from the antibody combining site. One possible explanation for this observation is that these mutations play an important conformational role in reorganizing active site side chains to the optimal geometries for binding hapten, and that the structure of the variable region is highly sensitive to point mutations in the hypervariable loops in comparison to most protein frameworks. Another possibility is that germline antibodies can adopt a large number of nearly isoenergetic conformations resulting from the flexibility of six complementarity-determining regions (CDRs) and the side chains, and that mutations fixed during affinity maturation act to "freeze" out the optimal hapten-binding conformation. In either case, this study suggests that conformational diversity plays a key role in the affinity maturation of this antibody. Further structural and functional studies of 48G7 and other catalytic antibodies should provide increased insight into the mechanism of this natural combinatorial optimization process.

## Antibody Mimics

Despite the power of using the immune system to evolve selective antibody receptors, smaller and more compact protein scaffolds would be desirable for a number of chemical and therapeutic applications. Such frameworks might have enhanced properties, including increased stability, improved expression, and altered pharmacokinetics. Moreover, the characterization of antibody-like receptors could provide additional insight into the remarkable binding properties of the antibody molecule. Previously, combinatorial approaches have been successfully applied to screen libraries of peptides and small proteins for their ability to bind a range of molecules.[19] In our own laboratory, we have used this approach to study carbohydrate−protein interactions[20] and to modify the specificity of DNA-binding proteins containing helix−turn−helix[21] and leucine zipper[22] motifs. Most recently, we have used this method to address the question of whether alternative protein frameworks might function as antibody mimics.[23]

The basic fold of an antibody molecule is that of an eight-stranded $\beta$-sheet onto which hypervariable loops, or CDRs, are grafted.[24] Binding affinity and specificity arise almost exclusively from the interaction of the CDR loops with the antigen. Furthermore, semisynthetic libraries in which the CDR loops of antibodies are randomized either independently or in combination have been screened for high-affinity binding to protein and small-molecule ligands.[25] As a first step toward using an alternative protein scaffold to mimic the antibody combining site, we generated a library in which the two loops of the four-helix bundle protein cytochrome $b_{562}$ were randomized.[23] This structurally well characterized, α-domain fold was chosen for several reasons. The four helices of cytochrome $b_{562}$ diverge to form a potential ligand binding site composed of the two loops connecting the helix termini (Figure 2). In addition, the well-packed, four-helix core should provide a stable framework structure capable of accommodating variations in the loop regions without significant overall structural perturbation.[26] Finally, cytochrome $b_{562}$ is relatively small (approximately 100 amino acids) and thermally stable and can be expressed at high levels, making it preferable to antibodies for a number of applications.

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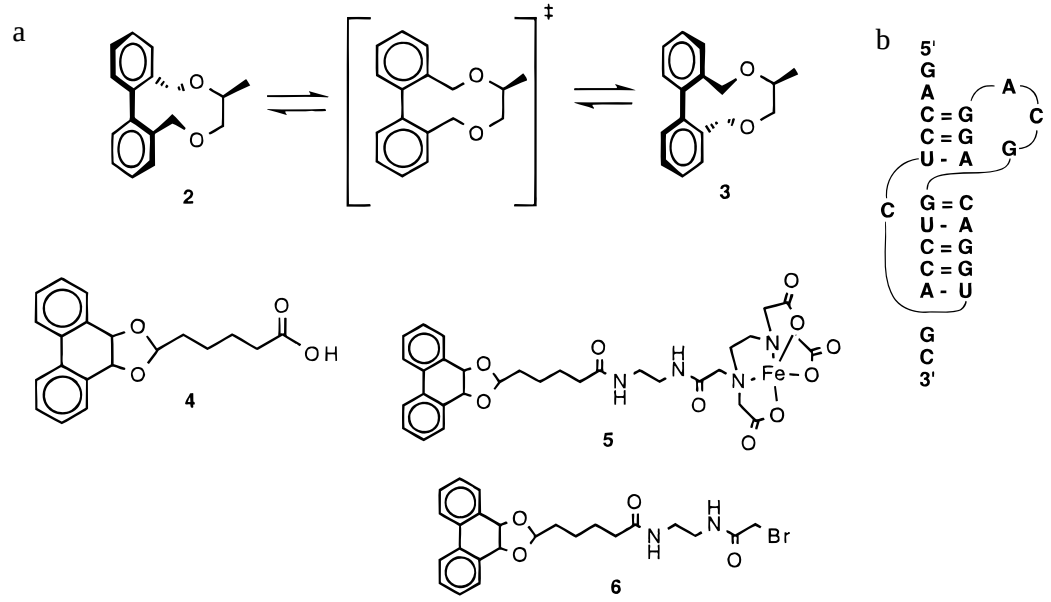


**Figure 3.** (a) Isomerization reaction of diastereomeric biphenyl **2** to product **3**, transition state analogue **4**, and affinity cleavage reagents **5** and **6**. (b) Proposed secondary structure of the 24 nt consensus sequence, bases 37−60 of AA6.

A library of cytochrome $b_{562}$ mutants, in which five amino acids in one loop and four in the second were randomized, was screened against a BSA conjugate of the nitrobenzylamine derivative **1** using phage display methods. Mutant cytochromes were isolated with conserved residues, including Trp-20, Arg-21, and Ser-22 in loop A and Arg-83 and Trp-84 in loop B. The tryptophan residues may be involved in $\pi$-stacking interactions with the nitroaryl ring of ligand **1**. The individual mutants, which fold into a native-like structure, bind selectively to the BSA−**1** conjugate with micromolar dissociation constants ($K_d$). By comparison, a monoclonal antibody was generated which binds selectively to **1** with a $K_d$ of 290 nM. These results suggest that the four-helix bundle of cytochrome $b_{562}$ can approximate the antibody in some respects. However, the binding affinity and specificity may be more restricted, limiting the usefulness of this framework for selectively binding a broad range of ligands. Future work will include the examination of ligand specificity, optimization of the loop and library sizes, and extension of this methodology to other frameworks.

## Nucleic Acid Libraries

One is not limited to polypeptide frameworks in the application of combinatorial methodology to the study of molecular recognition and catalysis. Large libraries of nucleic acids can be readily manipulated to assess the structural and functional roles of RNA and DNA. For example, these methods have been used to identify nucleic acids that bind a variety of ligands, including synthetic dyes,[27] nucleic acids,[28] and proteins.[27] Furthermore, combinatorial approaches have demonstrated that RNA molecules are capable of catalyzing reactions beyond those involved in RNA splicing, an example of which is illustrated below.[29,30]

RNA has been shown to efficiently catalyze reactions involving phosphoryl group transfers,[31] but if a prebiotic world with RNA as the primitive macromolecular catalyst once existed, other basic chemical reactions should be amenable to RNA catalysis. Although mechanism-based screens have been used to identify RNA molecules with hydrolytic, ligase, kinase, and aminoacyl transfer activity,[29] these screens typically require nucleic acid substrates as well as modification of the RNA itself over the course of the reaction (often resulting in single turnover events) and therefore are limited with respect to the transformations which can be examined.

We have pursued an alternative strategy for generating RNA catalysts based on the principles used to generate catalytic antibodies. A large library of RNA molecules was screened for its ability to bind the near-planar transition state analogue **4** and catalyze the isomerization of biphenyl **2** to its diastereomer **3** (Figure 3a).[30]

A 165 nucleotide (nt) RNA (AA6) was isolated from a library of approximately $10^{14}$ sequences that catalyzed the isomerization reaction with a $k_{cat}$ of $2.8 \times 10^{-5}$ min$^{-1}$ and $K_m$ of 542 $\mu$M ($k_{uncat} = 3.2 \times 10^{-7}$ min$^{-1}$). Catalysis was competitively inhibited by the TS$^{\ddagger}$ analogue, with a $K_i$ value of 7 $\mu$M.[30] The similarity of $k_{cat}/k_{uncat}$ to $K_m/K_i$ suggests that the RNA is largely functioning by preferentially stabilizing the transition state.

In an effort to gain greater insight into the nature of this RNA-catalyzed reaction, a series of chemical

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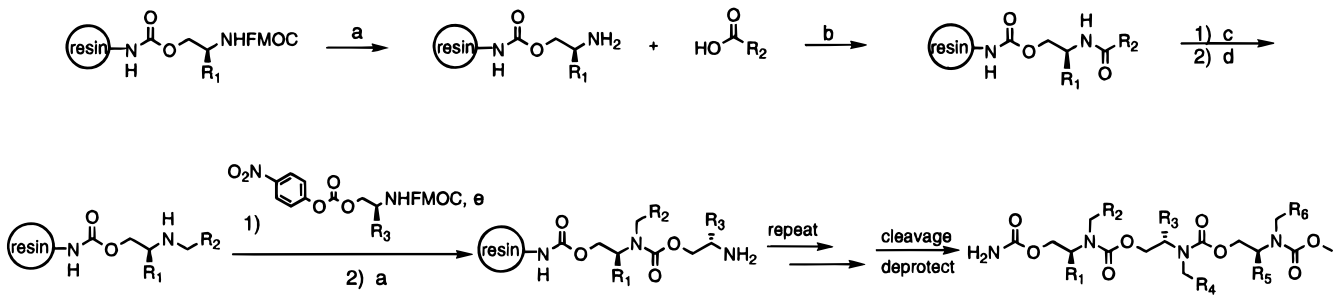


**Figure 4.** Solid phase synthesis of *N*-alkylcarbamates: (a) 20% piperidine, NMP; (b) HOBt, HBTU, DIEA, NMP; (c) $BH_3$·THF; (d) DBU, NMP, methanol; (e) HOBt, DIEA, THF.

modification and mutagenesis experiments was performed.[30] Boundary experiments located a 69 nt section of the initial 165 nt RNA (AA6) that was sufficient for catalyzing the isomerization reaction. Partial randomization of 46 nts within this sequence followed by an affinity-based selection with the TS‡ analogue defined a 24 nt consensus sequence (Figure 3b). Analysis of the cleavage patterns produced by affinity cleavage reagents **5** and **6** indicates that this sequence is an integral part of the transition state analogue binding core. Nuclease digestion experiments in the presence and absence of the TS‡ analogue suggest that this region does not undergo a significant conformational change upon ligand binding. Although essential for catalysis, this structure alone was incapable of catalyzing the isomerization reaction. Additional experiments are presently underway to explore appropriate folding conditions and/or mutations within this structure in order to facilitate further structural analysis and enhance catalytic activity. Moreover, this approach is being applied to other reactions as well and has recently resulted in the isolation of a small RNA with ferrochelatase activity.[32]

## Libraries of Unnatural Oligomers

In addition to their applications in protein and nucleic acid chemistry, combinatorial approaches have allowed for the construction of small molecule libraries, an advance that promises to revolutionize the process of drug discovery. The pharmacological obstacles associated with peptide-based therapeutics have stimulated considerable effort in the generation and screening of combinatorial libraries of small molecules such as benzodiazepines, $\beta$-turn mimics, and protease inhibitors.[33] Efforts in our laboratory have focused on generating structure-based libraries of purine derivatives to inhibit the cell cycle kinase CDK2.[34] However, generally the ease with which large diversity can be generated in these systems is limited in comparison to that of polymeric structures such as peptides. This raises the intriguing possibility that polymeric backbones alternative to peptides may exist with improved pharmacological properties and similar antagonist, agonist, or inhibitory effects.

In order to investigate this issue, we and others[35] have begun to develop efficient methods for the synthesis and screening of libraries of unnatural biopolymers (oligocarbamates, oligoureas, oligosulfones) for their ability to interact with receptors both *in vitro* and *in vivo*. For example, methodology has been developed for the efficient solid phase synthesis of oligocarbamates and *N*-alkyloligocarbamates.[36] These oligomers are synthesized from the sequential coupling of chiral N-protected *p*-nitrophenyl carbonate monomers, which in turn arise from the corresponding amino alcohols. The solid phase synthesis of oligocarbamates involves either base-catalyzed or light-dependent deprotection of the terminal amino group of the growing polymer chain followed by coupling to the next protected *p*-nitrophenyl carbonate monomer. In the case of *N*-alkylcarbamates, the general synthetic scheme involves four steps per coupling cycle: deprotection of the terminal amino group of the growing oligomer, acylation of the free amine with a carboxylic acid monomer, reduction of the resulting amide bond with borane, and coupling of the secondary amine to the next $N^{\alpha}$-Fmoc protected *p*-nitrophenyl carbonate monomer (Figure 4).

Libraries of oligocarbamates have been generated and screened using both a light-directed parallel synthesis method[36] and a bead-based divide and recombine strategy.[37] These libraries were used to map the epitope of an anti-oligocarbamate specific antibody and to identify oligocarbamates that bind specifically to integrin receptors and seven transmembrane receptors.[36,37] The pharmacological properties of these oligomers are currently under investigation; however, oligocarbamates have been shown already to possess greater hydrophobicity than the corresponding peptides and increased protease resistance.[36] In addition to affording potential frameworks for improved drug design, unnatural biopolymers may provide new polymeric folds for testing current notions of polypeptide structure and folding.

## The Periodic Table

Recently, we have taken the combinatorial approach from the realm of biology and organic chemistry to the remainder of the periodic table in an effort to discover materials with novel electronic, magnetic, optical, chemical, and mechanical properties. In the past, such discoveries have led to the development of new

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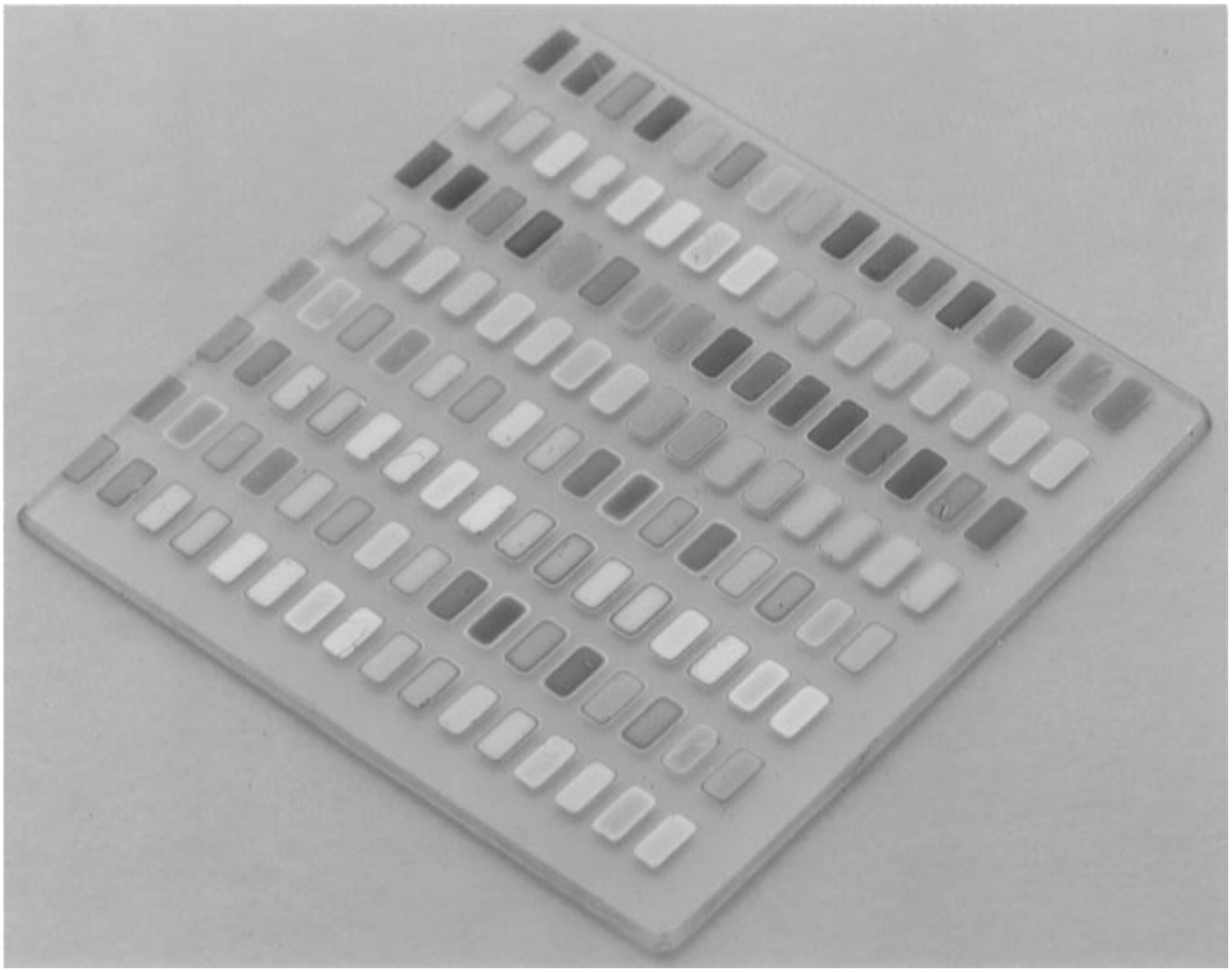

**Figure 5.** A 128-member binary library containing CuO superconductor precursors prior to sintering. Each site is 1 mm × 2 mm; the color of each is the natural color of reflected light from a white light source.

technologies. For example, the preparation of single-crystal semiconductors over 40 years ago transformed the electronics industry. Currently, there is tremendous interest in superconducting, magnetoresistive, luminescent, thermal electric, catalytic, and nonlinear optical materials. However, even though the chemistry of extended solids has been extensively explored, few general principles have emerged that allow one to predict composition, structure, and reaction pathways for the synthesis of such solid state compounds. Consequently, the discovery of new materials depends on a combination of empiricism, intuition, and trial and error synthesis. The fact that many of the exciting new materials being discovered consist of four or more elements makes this process even more daunting. Given approximately 60 elements in the periodic table that can be used to make compositions consisting of three, four, five, or even six elements, the universe of possible new compounds with interesting physical and chemical properties remains largely unchartered. Indeed, J. C. Phillips pointed out that as of 1989 only 7200 true ternary compounds had been characterized.[38] The question then arises whether there is a more efficient, economical, and systematic way to discover materials with novel properties.

In light of these challenges, we have recently applied the combinatorial approach to the discovery of new solid state materials. We have developed methodology for the parallel synthesis and analysis of spatially addressable arrays of a variety of thin films.[39] Thin film deposition methods are synthetically quite versatile; they offer the ability to construct artificial lattices, epitaxial overlayers, and patterned films of a variety of materials. By sequentially depositing the individual precursors of interest through a series of physical masks, it is possible to generate a spatially defined library of solid state thin films. Each sample can be varied with respect to elemental composition, the sequence in which the layers are deposited, and the thickness of each layer (including thickness gradients). Subsequent thermal processing provides a library of materials (or devices) whose physical properties can be screened with contact or rapid scanning probes.

In our initial experiments, arrays containing different combinations, stoichiometries, and deposition sequences of $BaCO_3$, $Bi_2O_3$, CaO, CuO, PbO, $SrCO_3$, and $Y_2O_3$ were generated with a series of binary masks.[39] The arrays were sintered, and BiSrCaCuO and YBaCuO superconducting films were identified using a four point probe array (Figure 5). Samples

(38) *Physics of High-Tc Superconductors*; Phillips, J. C., Ed.; Academic Press: New York, 1989.

(39) Xiang, X.-D.; *et al. Science* **1995**, *268*, 1738.

as small as 200 $\mu$m × 200 $\mu$m in size were generated, corresponding to library densities of 10 000 sites per square inch.

More recently, we have applied the combinatorial approach to the search for new magnetoresistive materials.[40] The discovery of the magnetoresistive effect[41] in Mn-based perovskite oxides $(La,R)_{1-x}A_xMNO_{3-\delta}$, where R = rare earth and A = Ca, Sr, Ba, has attracted considerable attention, due to the potential application of these materials in magnetic storage technology. Colossal magnetoresistance (CMR), where magnetoresistance ratios $\Delta R/R(0) = [R(H=0) - R(H)]/R(H=0)$, as large as 99.0%, 99.9%, and 99.99% has been reported for polycrystalline samples of $La_{0.60}Y_{0.07}Ca_{0.33}MnO_x$ and epitaxial thin films of $La_{0.67}Ca_{0.33}MnO_3$ and $Nd_{0.7}Sr_{0.3}MnO_{3-\delta}$, respectively.[42] A large number of theoretical and experimental efforts have been undertaken to understand this unexpected phenomenon, improve the room temperature sensitivity of these materials, and discover other structures with this intrinsic property.

Our initial library centered around simple perovskite $ABO_3$ and related $A_2BO_4$ or $A_{n+1}B_nO_{3n+1}$ higher order structures, where A = $(La, Y, rare\ earth)^{3+}$ partially substituted with $(Ca, Sr, Ba, Pb, Cd)^{2+}$ and B = Mn, V, Co, Ni, Cr, Fe. A library containing different compositions and stoichiometries of $Ln_xM_yCoO_\delta$, where Ln = Y, La and M = Pb, Ca, Sr, Ba, was synthesized, again using a combination of thin film deposition and physical masking techniques.[40] Large magnetoresistance (MR) has been found in $La_x(Ba,Sr,Ca)_yCoO_\delta$ samples, while Y-based samples exhibit much smaller magnetoresistive effects. The magnetoresistance of the Co-containing compounds was found to increase as the size of the alkaline earth ion increases, in sharp contrast to Mn-containing compounds, in which the MR effect increases as the size of the alkaline earth ion decreases. Thus this approach, even in its current state of development, has shown its effectiveness in material discovery.

There remain tremendous challenges in the development of combinatorial technology for materials science. Improved methods are being explored for the generation of libraries of highly ordered films, including multitarget laser ablation systems. It may even be possible to fabricate libraries of multilattice structures or entire devices using these techniques. Multihead inkjet deposition systems are being tested for generating libraries derived from solution phase precursors. Rapid detection systems are also being developed including a scanning microwave detector with high spatial resolution. Perhaps most importantly, methods are being developed for combinatorial processing in which not only composition and stoichiometry but also processing temperature, pressure, and atmosphere are varied. These methods together with theoretical and empirical input may considerably facilitate both the material discovery process and our ability to test theoretical predictions and map phase diagrams.

## Conclusion

The application of combinatorial strategies to problems of catalysis, molecular recognition, drug discovery, and materials science promises to greatly accelerate our ability to create molecules and structures with novel properties. Moreover, by analyzing the large body of information that results from such experiments, we should significantly increase our theoretical understanding of the relationship between these structures and their properties, enhancing our predictive ability even further. Finally, these efforts underscore the valuable lessons to be learned by chemists and physicists alike from studying the complex processes of living cells.

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