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Analogies Between Digital Radio and *Chemical Orthogonality* as a Method for Enhanced Analysis of Molecular Recognition Events

Peter J. Edmonson ¹, William D. Hunt ^{2,*}, Desmond D. Stubbs ³ and Sang-Hun Lee ⁴

1 Zen Sensing, LLC, Atlanta, GA, 30030; E-mail: edmonson@zensensing.com

2 School of Electrical and Computer Engineering, Georgia Institute of Technology, Atlanta, GA 30332, United States of America; E-mail: bill.hunt@ece.gatech.edu

3 Oak Ridge Center for Advanced Studies, P.O. Box 2008, Building 5100, MS-6173 Oak Ridge, TN 37831-6173

4 416 Maetan-3dong, Yeongtong-gu, Suwon-si, Gyeonggi-do, 443-742, Korea

* Author to whom correspondence should be addressed.

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Abstract: Acoustic wave biosensors are a real-time, label-free biosensor technology, which have been exploited for the detection of proteins and cells. One of the conventional biosensor approaches involves the immobilization of a monolayer of antibodies onto the surface of the acoustic wave device for the detection of a specific analyte. The method described within includes at least two immobilizations of two different antibodies onto the surfaces of two separate acoustic wave devices for the detection of several analogous analytes. The chemical specificity of the molecular recognition event is achieved by virtue of the extremely high (nM to pM) binding affinity between the antibody and its antigen. In a standard ELISA (Enzyme-Linked ImmunoSorbent Assay) test, there are multiple steps and the end result is a measure of what is bound so tightly that it does not wash away easily. The fact that this “gold standard” is very much **not** real time, masks *the dance that is the molecular recognition event*. X-Ray Crystallographer, Ian Wilson, demonstrated more than a decade ago that antibodies undergo conformational change during a binding event[1, 2]. Further, it is known in the arena of immunochemistry that some antibodies exhibit significant cross-reactivity and this is widely termed *antibody promiscuity*. A third piece of the puzzle that we will exploit in our system of acoustic wave biosensors is the notion of *chemical orthogonality*. These three biochemical constructs, *the dance*, *antibody promiscuity* and *chemical orthogonality* will be combined in this paper with the notions of

in-phase (I) and quadrature (Q) signals from digital radio to manifest an approach to molecular recognition that allows a level of discrimination and analysis unobtainable without the aggregate. As an example we present experimental data on the detection of TNT, RDX, C4, ammonium nitrate and musk oil from a system of antibody-coated acoustic wave sensors.

Keywords: chemical orthogonality, digital radio, antibody promiscuity, conformational change, biosensors, acoustic wave biosensors

1. Introduction

Molecular recognition is at the core of it all. It may sound adventurous but it is, we believe, safe to say that there are **no** biochemical interactions, which do not depend strongly on molecular interaction. DNA is methylated when it serves the cell to cease transcription of a particular gene. Similarly, the histone cores, around which the DNA is wound, are at times methylated so as to shut off transcription. Such a small molecular moiety, CH₃, that if we were to attempt to glean its presence on DNA using standard analytical chemistry techniques in the presence of complex media we would be hard pressed to find success. But in the molecular world of the cell this small modification changes everything. All this thanks to the mysteries of molecular recognition.

In an effort to achieve a perspective of the many complexities of life, researchers have been striving for governing principles to describe the details in a coherent manner and at least one subfield for these endeavors is systems biology. The area of systems biology has been intense since the early 20th century with the advent of research on quantitative modeling of enzyme kinetics. Systems biology casts a wide net and at its most general definition can be described as “the study of the interactions between the components of a biological system, and how these interactions give rise to the function and behavior of that system[3].” A subset of the breadth occurs at the intersection of telecommunications and biology. Perhaps the first investigator to find his way to this junction was H.P. Yockey[4-9], jumping on board shortly after the publication of Watson and Crick’s landmark papers on the structure of DNA[10, 11]. Of particular interest to Yockey has been the application of Information Theory, specifically the concept of Shannon’s Entropy[12]. It is well beyond the scope and purpose of this paper to review hallmark achievements in systems biology or applications of Information Theory to biology. Rather it is our intent to put into a broader systems biology context our recent findings regarding the very critical *molecular recognition* component of systems biology. Our specific work and data are most directly related to the vapor phase detection of nitrous oxide-based explosives using surface acoustic wave (SAW) biosensors. Though ours is an *ex vivo* system we believe that our findings cast light on the intricacies of molecular recognition.

Almost every biomolecular event in living systems involves the following three principle components:

- Molecular recognition---the lock and key interaction whereby one biomolecule or receptor (e.g. a protein) recognizes with a high degree of specificity another molecule. In the case of electrophysiology, this extends to the recognition of an

ion, say Na^+ , by a channel protein which has been incorporated into the plasma membrane

- Conformational change---the change in the molecular structure of the receptor molecule. At times it helps to think of this as the phase change of the molecule. No additional chemical groups have been added to the molecule but the internal structure of the molecule has changed. Condensed matter physics is replete with examples of crystal structure radically affecting macroscopic physical characteristics
- The hydrolysis of nucleotide triphosphates (ATP, GTP, UTP and CTP) as an energy source.

As we have made known in our previous publications[13-15], acoustic wave biosensors are a technology well suited for the translation of the above first two principles of the canon of living systems into detectable electrical signals. These two principles of conformational change and molecular recognition are *The Dance* introduced in the abstract of this paper.

Combined, these principles manifest themselves as mass attachment to the sensor surface and stiffness changes in the biological receptor layer. These in turn will shift the resonant frequency of the device (e.g. 10MHz for a QCM or quartz crystal microbalance or 250 MHz for a SAW resonator based oscillator). The biological receptor layer can, and has, taken on many forms such as aptamers, peptides among others. It should be understood that though much of what is presented in this paper directly references antibodies, the work can easily be extrapolated in general to other molecular recognition elements (MRE).

The affinity of the antibody immobilized onto the surface of the acoustic wave device will alter the time course of the resonant frequency signature, $\Delta f(t)$, when the antigen binds to it. When the affinity is large, as is the case for monoclonal antibody-antigen interactions, the analyte will bind tightly to the immobilized antibody resulting in a baseline frequency shift for the sensor. Dissociation constants, $K_d = \frac{[A][B]}{[AB]}$, for these antibody-antigen interactions tend to be in the picoMolar (pM) range. When the analyte is a close chemical analog of the original antigen against which the monoclonal antibody was generated, the affinity is not so high. In immunology this concept of cross-reactivity is referred to as *antibody promiscuity*[16]. This will serve the relevant biological underpinning for this paper in which we present both our results on the detection of nitrous oxide-based substances and extrapolate to what hints it may give us about the dynamics of biomolecular interactions. Further, the utilization of one antibody which is promiscuous (anti-TNT in our case) and one which is not promiscuous (anti-RDX) gives us a technique to translate or map *chemical orthogonality* into an electrical signal. The fundamental starting point of this analysis will be the frequency signatures, $\Delta f(t)$, obtained from two different SAW immunosensors oscillator systems. A method of differentiating, identifying and characterizing structurally analogous chemical or biological substances from these frequency signatures is accomplished by implementing simple circuitry and mapping the multiple output signals of the circuitry onto a state-space diagram. Time will no longer be an explicit detail of the response but the mapping will cluster the data into the various binding affinities between the nitrous oxide-based analytes from the two different semi-orthogonal antibody coatings used. Signal state-space diagrams have been used in digital communication systems to map binary information onto magnitude-phase (phasor) plots commonly referred to as constellation diagrams.

Digital communications systems are commonplace and are unknowingly used everyday whenever a cell phone, music or video device or a global positioning system (GPS) functions. Using the cell phone as an example, the caller's voice is the source and the person's ear that is answering the called cell phone will be the destination. A similar example could be drawn in the chemical world where an explosive device is the source and an explosive detector would be the destination. The caller's voice is converted from an analog to a digital format and clusters of these 1's and 0's then modulate a radio frequency (RF) to propagate through free space to a local base station. Here, the clusters of 1's and 0's are removed from the RF and placed on another suitable carrier that could be optical fiber or a microwave link. The digital data travels throughout the phone carrier system to another base station located in the vicinity of the destination cell phone. The digital data is modulated back onto an RF signal to transmit to the cell phone. Once answered, the clusters of digital data are removed from the RF, processed to compensate for loss of signal strength and any deformation due to impairments during the journey and finally converted to an analog signal to be fed via a speaker into the person's ear. The key to digital radio techniques is that the clusters of digital data allows for a more efficient mode of transmission where several digital bits are concatenated into a single communication event. This single communication event encompasses two orthogonal signals, the in-phase (I) channel, $A\sin(\omega t + \phi)$ and the quadrature-phase (Q) channel, $B\cos(\omega t + \phi)$, where A and B are the amplitudes, ω is the frequency (f) component ($\omega = 2\pi f$) and ϕ is a phase offset. The digital data within each cluster controls the settings of the amplitude and phase offset of each of the I and Q channels to complete a combined phasor of amplitude and phase that essentially points to this cluster. The frequency is controlled by Government agencies and is dependent on certain frequency allocations.

Therefore, if a transmitter were to map the digital data into clusters and each cluster were to control independently the orthogonal I and Q signals, information would be embedded within these orthogonal I and Q signals that is representative of the original data. Similarly, if a receiver were to have a two channel (I and Q) detection system and if the amplitude and phase offset were gleaned from each channel, then the original digital data could then be reconstructed by using the received phasor to point to the digital clusters within a mapping system. Again, with reference to the chemical example, if an explosive vapor or particle were to be presented to a two channel (I and Q) detection system and information was gleaned from each channel, then the original chemical composition could then be reconstructed by using the detector's phasor to point to the cluster containing the chemical composition within a mapping system.

1.1 A Precursor to Vapor Phase Molecular Recognition:

For centuries scientific ingenuity and innovation have been influenced by nature's ideal design. One of the elusive designs is that of the sensory olfactory system---an array of highly sensitive receptors responsible for chemical vapor recognition. In the animal kingdom, this ability is magnified among canines where ppT (parts per trillion) sensitivity values have been reported. Today, detection dogs are considered an intricate part of the US drug and explosives detection force. However, growing concerns about their susceptibility to extraneous scents have inspired a rapidly developing ensemble of analytical detection tools. In previous publications, we have demonstrated the ability to detect and differentiate among analogous molecules using an acoustic immunoassay vapor sensor termed "dog-

on-a-chip” [15]. It was our perspective at the time that this analytical tool was limited by the availability of an antibody toward a specific target molecule. This point of view was derived from the conventional gestalt in immunology, that for every new protein one wishes to assay, a new antibody must be raised against it if one is to get the requisite specificity. However, what we discovered is that one can reduce the number of antibodies “required” for a particular application by exploiting the intrinsic promiscuity common to all antibodies[18-20].

Behring, a physicist who won the Nobel Prize in 1901, and Kitasato, his long time colleague, first introduced the theory of antibody-antigen binding specificity[21]. Later that century another Nobel laureate, Karl Landsteiner published the structure and mechanisms involved in antibody-antigen interactions. These works formed the canon of antibody-antigen reactions, which can be succinctly expressed as the precise fit model. This model has been subsequently revised following innovative work by Cameron and Erlanger[22] who introduced the cross reactivity phenomenon between antibodies, antigens and their structural homologues. The mechanism is said to be comprised of both electrostatic and hydrophobic interactions due the large number of hydrophobic residues in the antigen binding site. In addition, some promiscuous antibodies can cross-react through hydrogen bonding with antigens that are not structural homologues of the target antigen [23]. James and Tawfik *et al.* concluded that the promiscuous nature of antibodies suggests that each protein may have its own unique pattern of multispecificity and each activity stands alone and may be highly specific[23]. In this paper we identify this pattern of promiscuous activity as a molecular signature that is unique and quantifiable. Herein, we report evidence of multispecificity in an anti-TNT clone when exposed to vapors of 2,4,6-trinitrotoluene (TNT) and Royal Demolition Explosives (RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine) by introducing a novel method for the treatment of the data using digital radio concepts to unveil the real-time dynamics of antibody/antigen interactions.

2. Experimental Set Up and Configuration

The immunosensors utilized herein are based on two port surface acoustic wave (SAW) resonators fabricated in the laboratories of the Microelectronic Acoustics Group at Georgia Tech. These devices have a center frequency of nominally 250 MHz and are fabricated on ST-cut quartz substrates. ST-cut quartz is a particular cut of quartz for which interdigital transducer (IDT) structures generate a Rayleigh wave in the crystallographic X-direction. For sensor applications, the ST-cut of quartz provides excellent temperature stability near room temperature which minimizes the need for additional temperature control circuitry. Fig.1 schematically illustrates a single channel vapor phase biosensor with an immobilized monolayer of antibodies on the surface of a SAW device. The device is then connected into an oscillator circuit, and frequency changes can then be precisely measured. The sensing mechanism of an acoustic wave sensor is based on the surface perturbation of the sensor, *i.e.*, any changes on the SAW propagation path affect the velocity of the wave which in turn leads to the change of the resonant frequency. The perturbation arises from a mass adsorbed to the sensor surface and/or changes of physical properties of the contacting medium, and the resulting frequency changes generally follow the Sauerbrey equation[24]. For more complex coating, *i.e.*, antibody layer and/or other biomolecules, Hunt *et al.*[14] developed an analytical relationship from the complex reciprocity relation and time-dependent perturbation theory.

$$\Delta f = -\frac{2f_u^2 h_f}{\sqrt{\rho_q \mu_q}} \left[\Delta \rho - \frac{\Delta \mu}{V_s^2} \right]$$

where V_s is the acoustic velocity; ρ is the density of the film; h_f is the thickness of the film; μ_q and ρ_q are the shear stiffness and density of the quartz crystal, respectively; μ is the stiffness of the film; Δ is the difference between perturbed and unperturbed (denoted by subscript u) quantities.

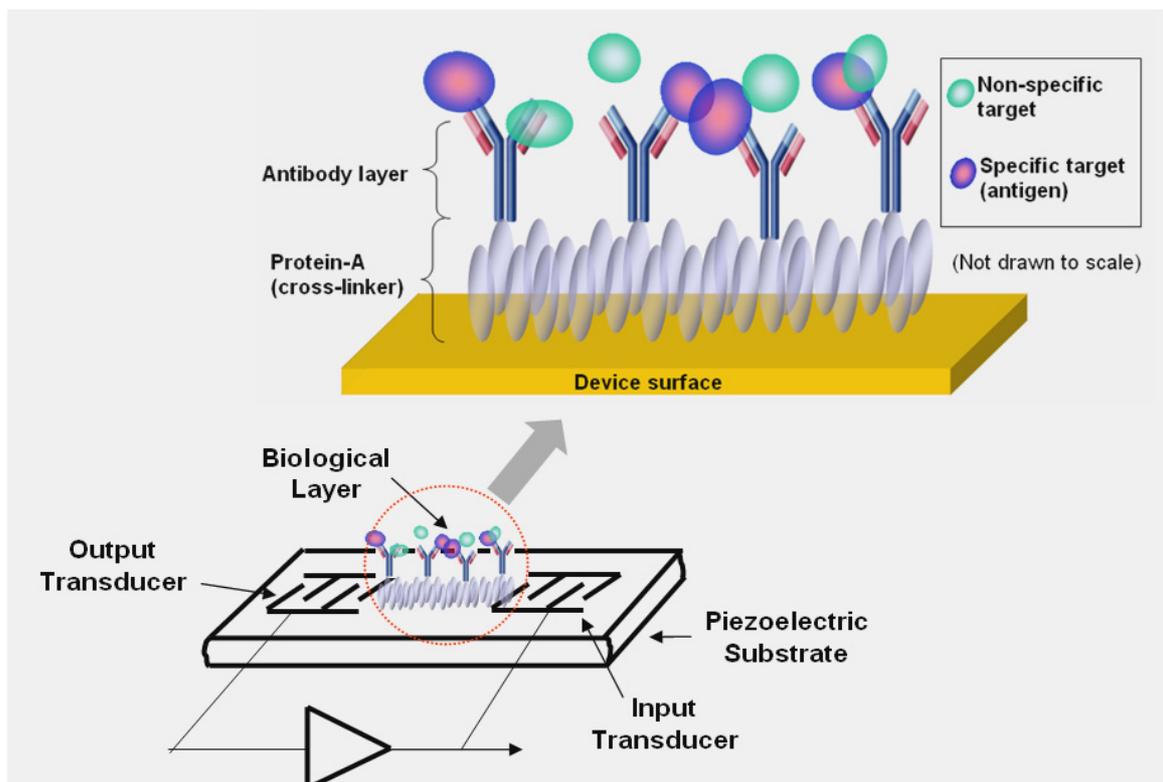


Figure 1. Schematic of a SAW immunosensor

A detailed discussion of our antibody immobilization protocols, measurement methods and verification of molecular recognition can be found in our previous publications[13-15] and will not be repeated here. In the discussion that is to follow, one can assume that we have a sensor array of containing one reference SAW sensor and up to three SAW sensors, each coated with a different antibody. A vapor, which has not been pre-treated or pre-concentrated, containing the analyte as well as other molecules is passed through the sensor array and the frequency shift of each SAW sensor is recorded on a laptop computer. The algorithm we present represents a post-processing of this data whereby the frequency signature data, $\Delta f(t)$ is mapped into an IQ constellation diagram.

3. Experimental Methodology and Results

Initially, we will discuss the vapor phase biosensor detection system configuration in which there are two antibody coated SAW sensors and one reference sensor. To introduce the analogy with digital radio, we will speak of these antibody-coated sensors as “channels.” Each channel consists of an orthogonal or semi-orthogonal SAW immunosensor shown previously in Fig. 1[25]. This orthogonal

biosensor detection system is illustrated in Fig. 2A where the X channel output is from oscillator X implementing the SAW immunosensor coated with biolayer X and the Y channel output is from oscillator Y implementing the SAW immunosensor coated with biolayer Y.

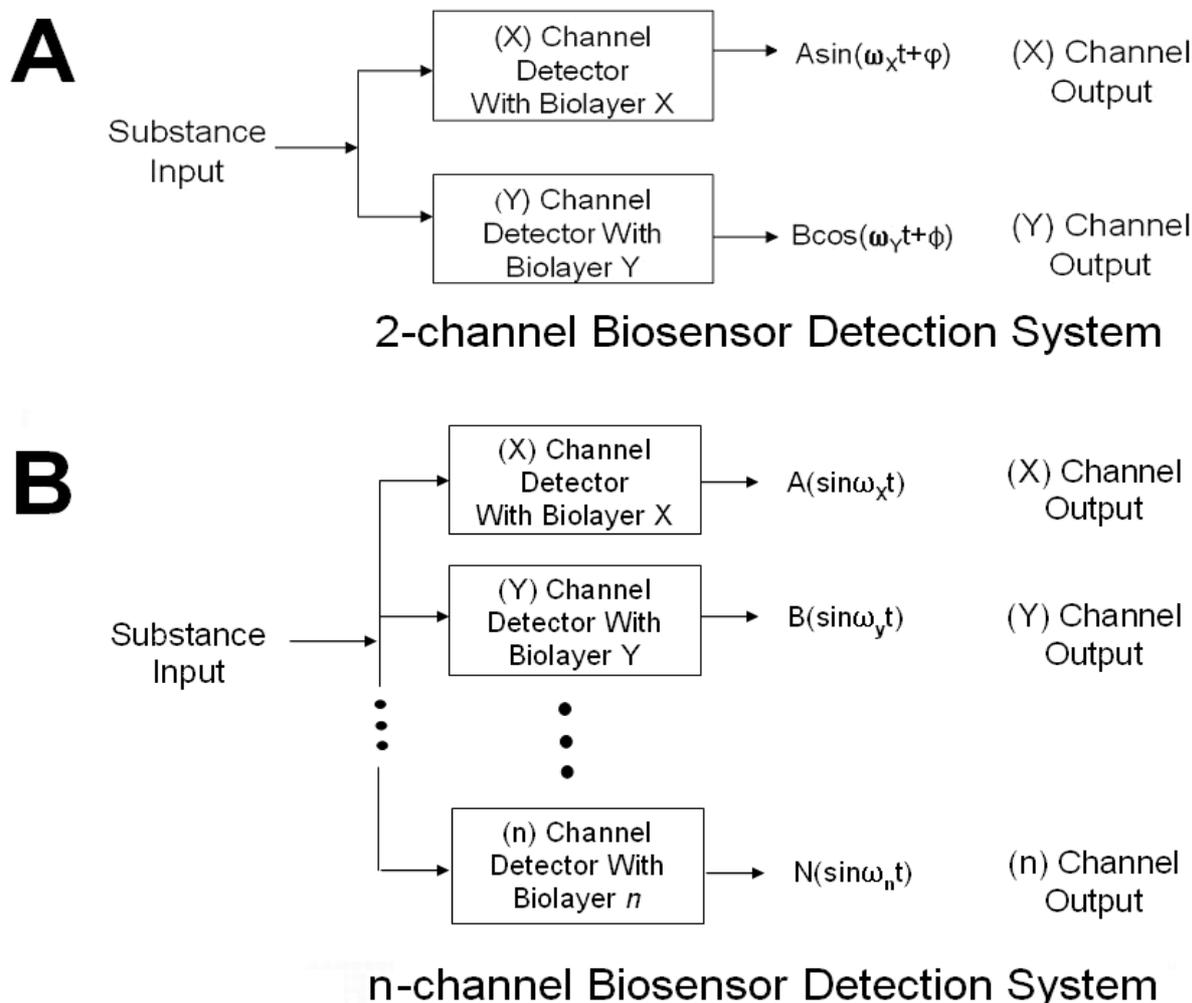


Figure 2. A schematic of 2-channel (A), and n-channel (B) biosensor system

Figure 3A illustrates the domain conversion technique where each separate time-frequency characteristic of the X and Y oscillators are mapped onto a single quadrature frequency domain, with both axes having the units of Hz. The sampling of the time-frequency characteristics can be strategically controlled depending on the system parameters such as the time duration of the analyte injection.

There is a strong analogy between the detection techniques of a quadrature digital communication system and that of our orthogonal biosensor system. Fig. 3B shows the constellation plot of 8-ary

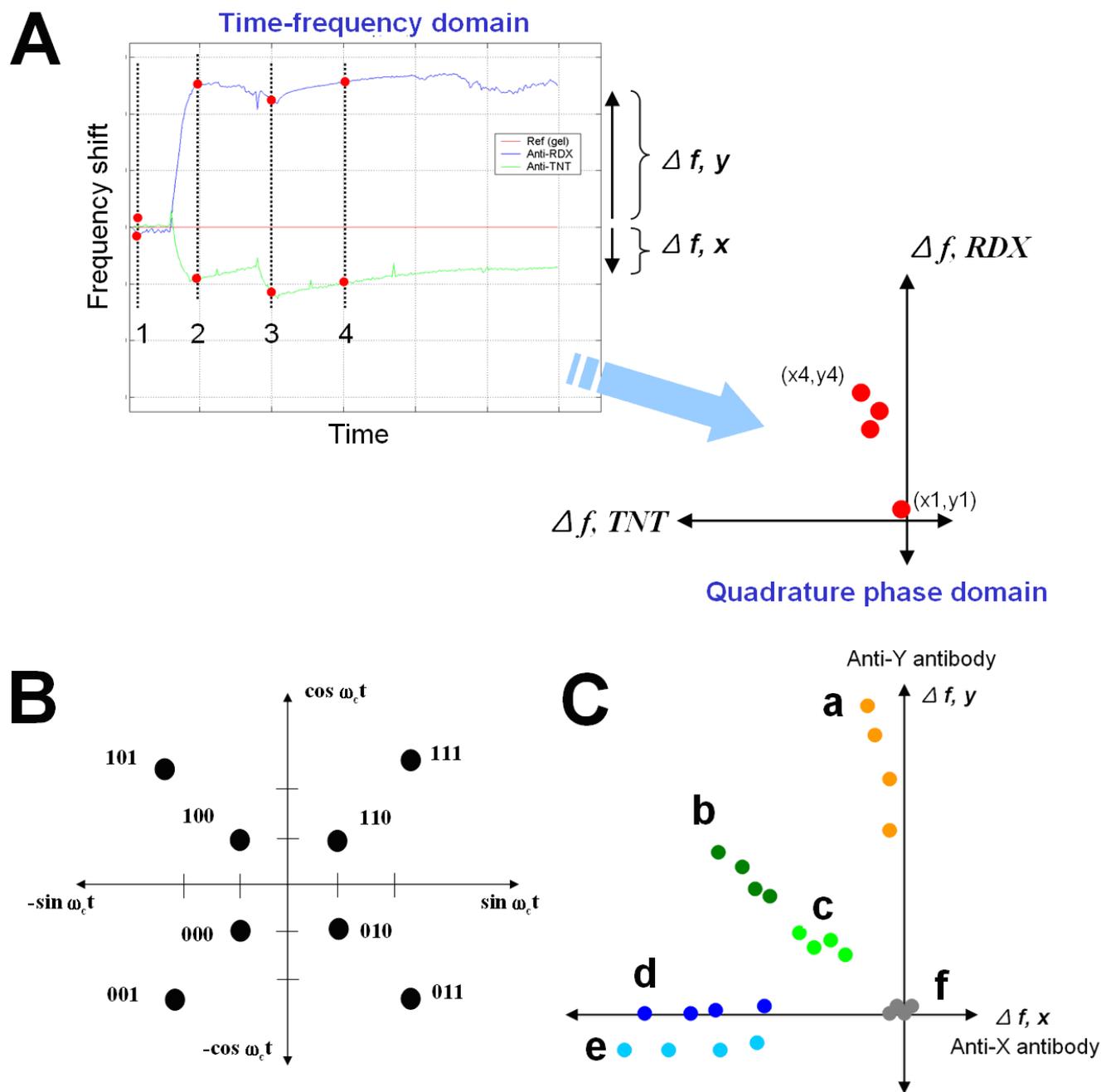


Figure 3. (A) Conversion from time-frequency domain to the quadrature phase domain. (B) 8-ary QAM constellation diagram. (C) two channel immunosensor signal state-space map

QAM system, where the first two bits of the tribit binary data defines the phase position and the least significant bit defines the amplitude position. For a QAM signal, the binary data is combined into two separate carriers, each having the same frequency but mathematically orthogonal in phase with respect to one another. At the radio receiver, the received signal is separated based on its orthogonal phase information into two separate orthogonal channels. The binary data is then characterized from each channel and mapped according to the phase and modulation information.

Likewise, one can create a state-space map from the immunosensor system to characterize and map the target analytes. Just as the binary data defines its position on the constellation plot, the analyte data also defines its position on the state-space map by the degree of affinity between each analyte and the two system antibodies. For example, on the signal state-space map in Fig. 3C, sample **a** is specific to the anti-Y antibody, thus can be considered as the substance Y, but also has a slight affinity to anti-X antibody. Samples **b** and **c** are different concentrations of the same substance with affinity to both anti-Y and anti-X antibodies. Sample **d** can be considered as the substance X, and **e** has a very similar chemical structure to **d** but has different concentration or vapor pressure. Sample **f** is in totally different category and is not an analogue of X or Y. This analysis scheme greatly reduces the mathematical complexity, which is required for most chemometric methodologies. (The analogies between the interaction of the antibody/antigen binding event and the detection techniques of a quadrature digital radio receiver system are summarized in Table 1.)

There also exists a multi-dimensional communication system that implements orthogonal frequency domain modulation (OFDM). Here, the digital data is split amongst a multitude of narrowband channels such that the data in channel (n) is “orthogonal” to the data in channel (n+k), where (k) is a number greater than 1. The greater the (k) value, the more orthogonal the data in each channel will be with respect to each other and hence can be described as interacting less with each other. The two-channel biosensor system previously shown in Fig. 2A can also be extended to a general nth dimensional system (Fig. 2B). Here, the degree of chemical similarity between analytes of individual channels decreases as (k) increases. Just as each narrowband channel of the OFDM system would have a defined bandwidth and the difference between adjacent channels would define adjacent channel interference, the multi-dimensional biosensor system also has similar traits. The affinity of each channel’s biolayer (bandwidth) can be defined along with the degree of multispecificity between the adjacent channel antibodies (interference). The grouping of (n) and (n+1) biosensor channels would produce a 2-dimensional map, and the grouping of (n), (n+1) and (n+2) biosensor channels would produce a 3-dimensional map. The system can then be designed such that there is a minimal amount of cross-reactivity between channels.

Fig. 4A shows the explosive substances and the analogues that were presented to the two-channel biosensor system. An antibody monolayer was immobilized on each surface of the SAW resonators using heterobifunctional linker *e.g.*, protein A[15]. The final preparation involved the deposition of a thin hydrogel layer to support the antibody layer and provide a semi-aqueous environment on the sensor surface. Both the mouse anti-TNT clone (lot #107415) and the mouse anti-RDX (lot #200202-3-4) were obtained from Strategic Biosolutions. This lot of anti-TNT was reported to cross-react to other TNT analogs[16, 17, 26]. Zeck *et al.*[16] reported higher binding affinities among the TNT analogs with nitro-groups on the ring suggesting an electrostatic binding mechanism maybe the key to its molecular specificity. The substances in this case are related via an NO₂ branch. Our experiments have shown that these substances, TNT, RDX, Musk Oil or Musk Xylene, and ammonium nitrate all bind differently with respect to TNT antibodies and RDX antibodies. Our experimental setup included a pneumatic apparatus that drew vapor from the various substances into the two-channel biosensor system. In this experiment, the X channel detector implemented the TNT antibody layer and the Y channel detector implemented the RDX antibody layer. The frequency components of both channel outputs were stored.

After sampling and accumulating several of the frequency offset data from each of the X and Y immunosensor channels and applying the aforementioned domain conversion, a signal-state map was constructed which is shown in Fig. 4B.

The signal state-space map of Fig. 4B illustrates the grouping or clustering of substance data. The TNT vapor was introduced using an INEEL vapor generator, with a flow rate of 150 cc/min at 13.2 °C, and released 50 pg of TNT per pulse with duration of 0.3 seconds. The RDX vapor was introduced using the same type of generator, with a flow rate of 150 cc/min at 53 °C, and released 50.3 pg of RDX per pulse with duration of 3.2 seconds.

The C4 data was obtained in an open lab with an unheated 5 micron filtered sampling head positioned 2.0 inches from the material at the bottom of a sample bottle. The Musk Oil data was similarly recorded with the filtered sampling head positioned 3.5 inches from the sample. The ammonium nitrate data was derived at the same distance. Looking again at Fig. 4B, it is clearly shown that each substance is distinctively mapped onto a region of the signal state-space map.

The dimension of the signal state-space can be expanded by introducing additional semi-orthogonal channels. As an example, we show a 3D cluster map, which was produced from three semi-orthogonal immunosensor channels. The sensor system setup and the domain conversion schematics remain the same, but an additional sensor coated with anti-dinitrophenol (anti-DNP) antibody was employed to the sensor array. For this experiment, soil samples containing TNT and several analogous compounds (nitrophenols) obtained through the EPA (Environmental Protection Agency) were examined as a target. The result is a three-dimensional map (Fig. 5) defining the characteristic signature of each of the chemical analogs.

4. Discussion

In summary, antibody multispecificity, termed *antibody promiscuity*, is common occurrence in biological systems. The ability to cross react with multiple antigens is believed to be due to the conformational diversity (“conformation isomerism”) among antibody clones[23]. The polyspecific nature of antibodies has been linked to the occurrence of high background noise during an immunoassay termed interference and is closely correlated to its binding affinity. We report the observed multispecificity of anti-TNT and anti-RDX towards nitrous oxide (NO₂) groups of molecules and the categorizing of multiple substance data within a state-space map by use of a two-channel detection system. The addition of an anti-DNP immunosensor provides the user with a 3-dimensional state-space detection map. The results suggest that chemical orthogonal methods may be used as an analytical tool for the detection and differentiation among analogous molecules based on quadrature detection techniques for digital radio systems. Using Table 1 as a comparison between digital radio systems and semi-orthogonal state-space immunosensing, well known techniques to optimize a digital radio system would be transformed over to the chemical realm for better molecular recognition strategies. Also, this detection scheme can be accomplished in real time and with minimal computational effort.

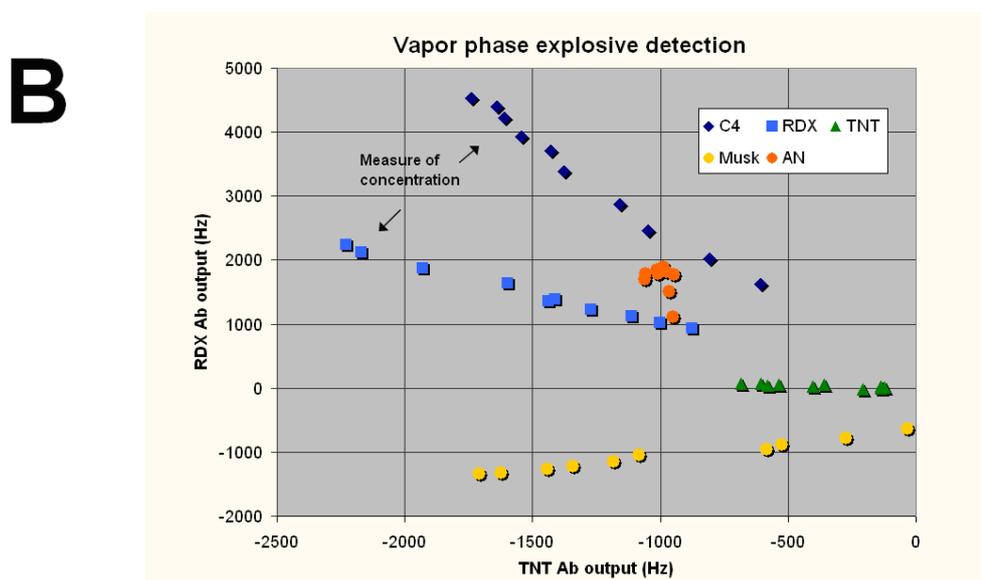
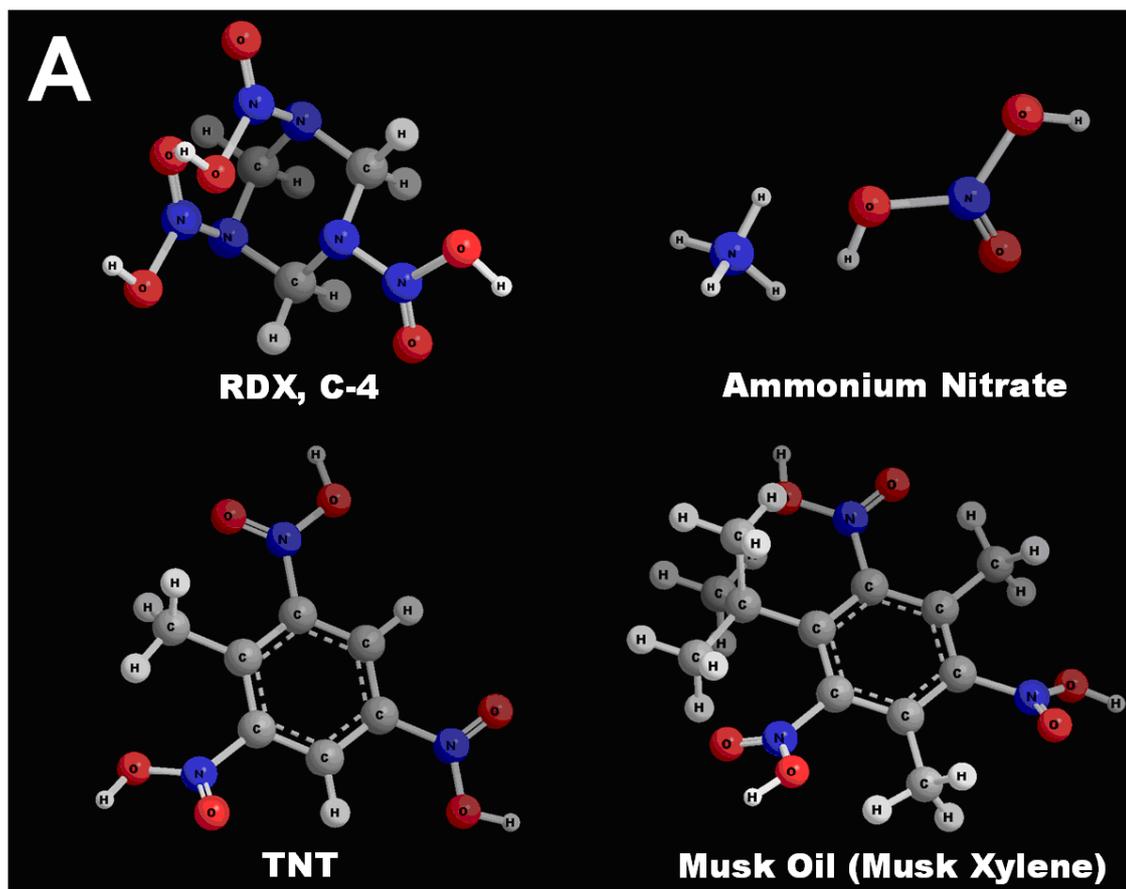


Figure 4. Explosive substances and analogues used for the experiments (A), and the signal state-space map of explosive samples (B).

Table 1. Comparison table between digital radio systems and semi-orthogonal state-space immunosensing.

Parameters	Digital Radio Systems	Semi-orthogonal state-space immunosensing
Map	Group showing the relationship between binary data and the signals magnitude and phase	Group showing the relationship between analyte data and the signals frequency
Axes of the map	Two orthogonal signal channels with embedded phase and magnitude information dependent on binary data	Two orthogonal or semi-orthogonal signal channels with embedded frequency information dependent on analyte data
Separation between data clusters within the map	Degree of similarity between the binary coded signals	Degree of chemical similarity between analytes
Magnitude of data clusters within the map	Dependent upon binary data characteristics such as bit value	Dependent upon analyte characteristics such as concentration and/or vapor pressure
System Selectivity	Dependent of front-end and baseband filtering to reject close-in signals	Dependent on the antibodies inherent ability to distinguish antigenic differences to reject similar analytes
System Sensitivity	Ability of the system to respond to weak RF signals (measured in microvolts)	Ability of the system to respond to low concentrations (measured in parts per billion)
Channel Interference	Semi-orthogonality of pairwise signals and/or cross channel rejection characteristics	Immunoglobulin multispecificity of antibodies to analytes
System Link Budget	Receiver's energy per bit to the noise power spectral density ratio, E_b/N_0 , dependent on data rate, transmit power, and path loss	Sensor's bound antigen concentration (b) to total antibody concentration (a) ratio per concentration of total antigen (c) ratio, b/ac (Scatchard equation), dependent on analyte concentration, vapor pressure and path loss

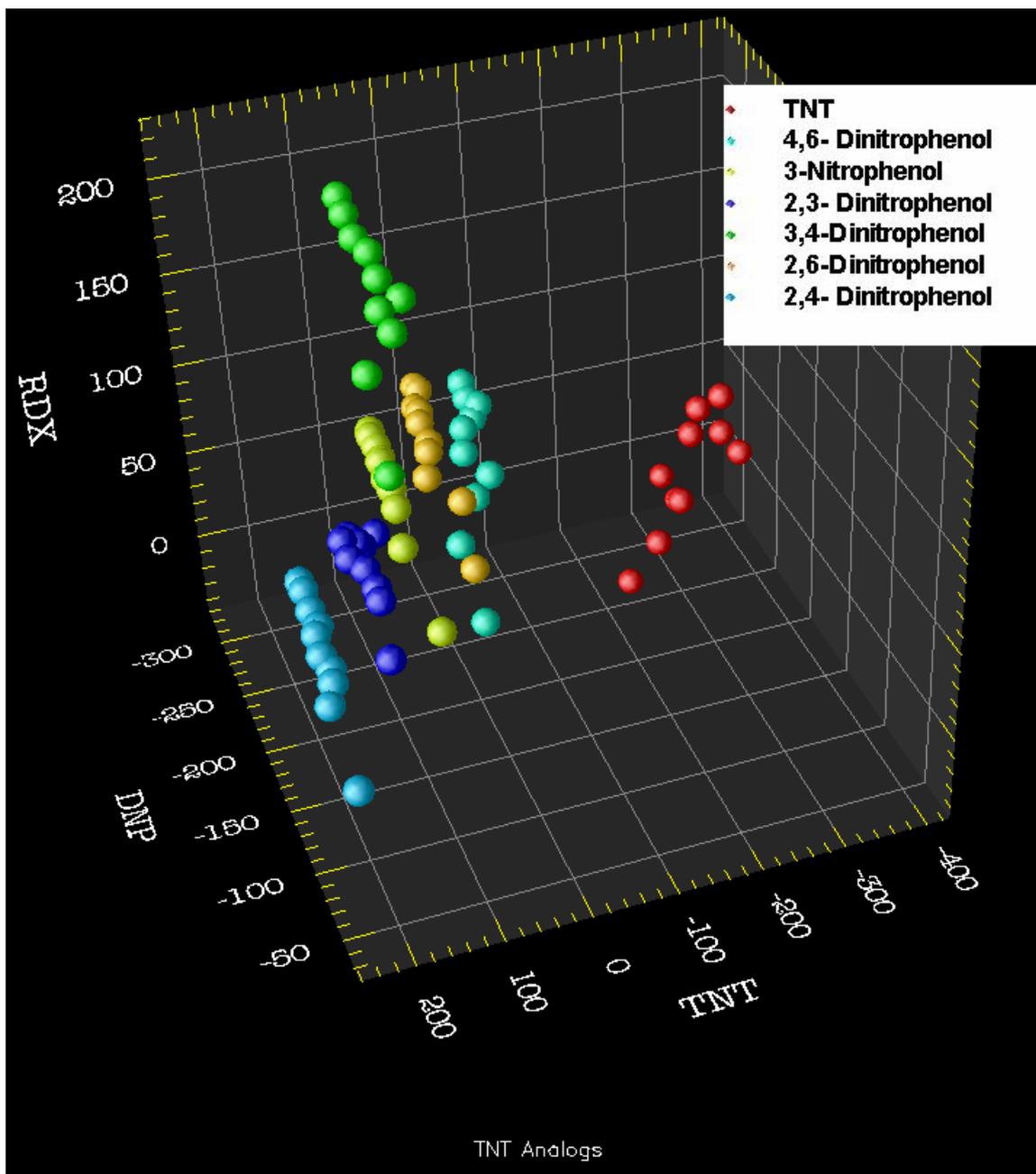


Figure 5. A three dimensional signal state-space map generated from the signature of three channel semi-orthogonal immunosensors system. Each individual sensor is coated with anti-TNT, anti-RDX, and anti-DNP antibodies respectively. The headspace vapor of various soil samples containing TNT and nitrophenol analogues were analyzed.

5. Conclusion

In this paper three biochemical constructs, *The Dance* (i.e. conformational change and molecular recognition), *antibody promiscuity* and *chemical orthogonality* were combined with the concepts of in-phase (I) and quadrature (Q) signals from digital radio to manifest an analytical biochemistry approach

that allows us to convert a molecular recognition event into an electrical signal. This approach establishes a level of discrimination and analysis unobtainable without this aggregate of biochemistry and digital radio. As an example we presented experimental data on the detection of TNT, RDX, C4, ammonium nitrate and musk oil from a system of antibody-coated acoustic wave sensors. This paper introduces the notion of chemical orthogonality and its use to implement multiple orthogonal immunosensors to detect and map several analogous analytes. This chemically orthogonal system takes advantage of antibody promiscuity to discern mapped clusters of data with respect to analytes differing only slightly in chemical composition. From an analytical biochemistry perspective it opens the prospect of being able to discriminate between close chemical analogues that would be unobservable with a tool such as Mass Spectroscopy. It also raises questions as to whether the approach can be used to detect glycosylated proteins or methylated histone cores. This remains a question of interest to us. Further, we may move to speculate that the menagerie of *The Dance*, *antibody promiscuity*, *chemical orthogonality* and *digital radio* may well lead to a deeper understanding of the processes underlying molecular recognition itself.

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