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14. ABSTRACT
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15. SUBJECT TERMS
Aptamer, array, deoxyribonucleic acid (DNA), electrophoresis, pattern recognition, universal sensor

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Feasibility of a DNA-Based Combinatorial Array Recognition Surface (CARS) in a Polyacrylamide Gel Matrix

John G. Bruno, John L. Alls, and Johnathan L. Kiel

Abstract—**We report initial attempts at developing a self-assembled combinatorial DNA biosensor array which may be capable of binding and identifying virtually any soluble analyte that binds the array by pattern recognition, in effect making it a universal biosensor surface. Data are presented for differential binding patterns of various analytes to 1-D arrays of combinatorial deoxyribonucleic acid (DNA) concatamer libraries which are spatially separated according to size and charge by electrophoresis in polyacrylamide gels. These DNA concatamer libraries are essentially composed of single-stranded (ss) random DNA 60 mers, which form a "smear" pattern in gels following electrophoresis. When used to bind and detect various analytes or mixtures of analytes in the gel, we refer to the DNA smear as a "combinatorial array recognition surface" (CARS). Differences in intrinsic fluorescence scanning patterns of CARS gel strips were compared before and after addition of various analytes to the arrays to detect binding patterns. Scans revealed a high level of reproducibility for individual CARS arrays in a given gel with or without bound analytes. Scan patterns between different CARS gel strips were initially less reproducible, but purification of the DNA library using spin columns prior to electrophoresis improved gel-to-gel reproducibility.**

Index Terms—**Aptamer, array, deoxyribonucleic acid (DNA), electrophoresis, pattern recognition, universal sensor.**

I. Introduction

FOR WELL OVER a decade, there has been great interest in development of microchip sensor surfaces for detection and identification of a variety of analytes with a major focus on genomic and proteomic applications [1]–[10]. Currently, available DNA arrays on membranes or chips rely on ordered arrays of DNA of known sequence and known location in the array. The main DNA array concept presented herein is referred to as a "combinatorial (DNA) array recognition surface" or "CARS." The CARS approach differs from conventional DNA arrays in several important ways. Current DNA chip arrays can express a maximal diversity of about 10^6 sequences [3]. However, the actual diversity realized is far less. For example, the current Affymetrix gene chips for human single nucleotide polymorphism (SNP) detection cover about 50 000 SNPs per chip. While, this is an impressive achievement, especially in a mass produced commercial device, it is still limited in diversity. In addition, the Affymetrix and related gene chips are based solely upon Watson–Crick base pairing interactions (hybridization) for genomics, so that if one wanted to undertake proteomics studies, one would need an antibody or aptamer array [1].

The CARS concept presented herein can theoretically express far greater sequence diversity than 10^6 permutations, if coupled to computer-assisted pattern recognition, thereby enabling a much broader potential molecular recognition capability. The CARS concept differs from traditional DNA array technology approaches in other significant aspects as well, such as: 1) simplicity and ease of production because it self-assembles and 2) ability to bind molecules other than nucleic acids, including proteins and small molecules, via DNA aptamers. Low levels of aptamers of various affinities for a given target analyte must exist in the array because aptamers can be selected and amplified by SELEX from randomized libraries [11]–[15].

The CARS concept evolved from observations of DNA "smears" and unexpectedly high molecular weight products in random oligonucleotide or aptamer libraries [12]. These "smears" suggested partial hybridization of the random oligonucleotides to form an "array" of much larger pieces of DNA ("pseudoconcatamers") at or below room temperature (Fig. 1). To maximize sequence diversity, such libraries can also be subjected to PCR with a mixture of deoxynucleotides and dideoxynucleotides in a manner similar to the Sanger dideoxynucleotide chain termination sequencing method. These highly diverse oligonucleotides can also be ligated together at points of discontinuity along the phosphate backbone, wherever partial hybrids naturally occur, via *Taq* DNA ligase [16].

Ligation may not have been entirely necessary for the current experiments, as the libraries were confined to a gel matrix, but ligation was performed to ensure that large contiguous pieces would remain intact during analyte binding. In addition, chain ligation may be a useful property of CARS arrays in the future, if larger contiguous DNA molecules are immobilized on chip or membrane surfaces. Data presented here illustrate that both types of DNA arrays (overlapping random oligonucleotides and CARS) can be used to detect and possibly identify unknown analytes by way of the analyte's characteristic interactions (spatial binding pattern) with the array and subsequent neural network or other types of pattern recognition analyses [17].

The CARS concept is illustrated in its 1-D and theoretic 2-D forms in Figs. 1 and 2, respectively. Fig. 1 demonstrates that a completely randomized 60 mer DNA library consisting

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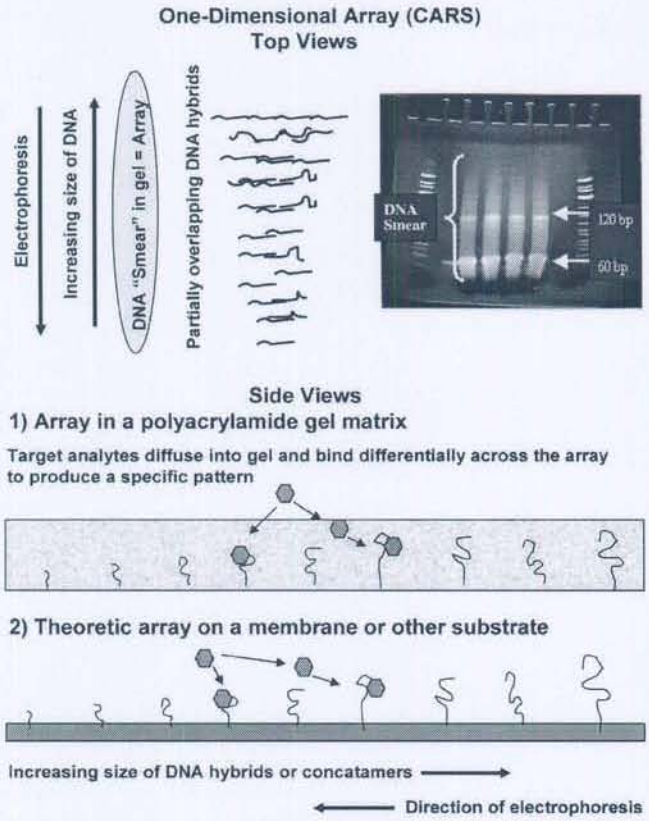

Fig. 1. Conceptual (top left) and actual (top right) appearance of a partially overlapping or partially hybridized collection of random sequence DNA 60 mers (1-D CARS). The gel on the right was a 4%–20% gradient polyacrylamide gel stained with ethidium bromide. DNA ladder standards [50 to 2000 base pairs (bp)] flank four identically run overlapping random 60 mer libraries. In CARS libraries, noncontiguous pieces can be ligated together with *Taq* DNA ligase. The top half of the figure illustrates the appearance of a 1-D CARS from the top view and the bottom half illustrates the conceptual appearance of CARS from the side view.

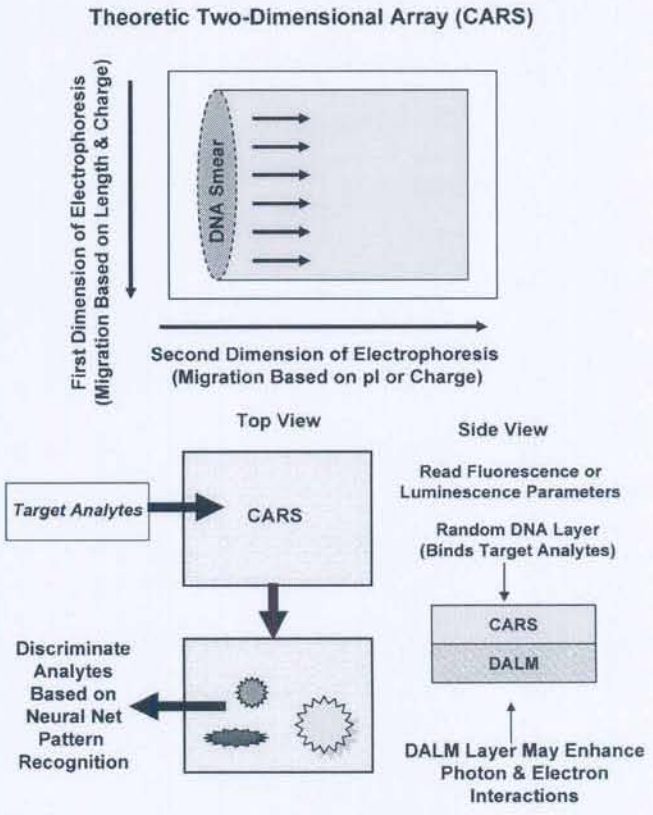

Fig. 2. Conceptual diagram for a theoretic 2-D CARS made by electrophoresing the 1-D array in a second dimension perpendicular to the first to separate DNA based on minor differences in charge or pI [27]. The figure also illustrates how DALM might be conjugated to the array as an underlying layer to enhance photonic and electronic interactions.

of permutations of adenine (A), cytosine (C), guanine (G), and thymine (T) nucleotides at each position in the 60 mer will self-assemble into a smear pattern or "array" of partially and completely overlapping hydrogen bonded strands when electrophoresed through a polyacrylamide gel. This 1-D array of random DNA molecules is size-ordered from shortest at the bottom to longest at the top of the gel. Once assembled and distributed by electrophoresis, the array can be used either in the gel or theoretically transferred to a membrane to examine the complex binding patterns of various analytes including small molecules and macromolecules. Ultimately, specificity of the array can be assured by characterization of known analyte binding patterns, rigid quality control of physical parameters such as array purity, ionic strength, temperature, etc., and "intelligent" pattern recognition algorithms to discriminate various similar binding patterns. For example, one might utilize Bayesian classification schemes, neural networks, or other published spectral pattern recognition techniques [18]. Pattern recognition would be especially important for the theoretic 2-D CARS model (Fig. 2) in which the random DNA library is first electrophoresed in one dimension based on size and charge and then rotated 90° and electrophoresed based on pI as with classic 2-D gel electrophoresis in protein biochemistry laboratories. Recently, Kontos and Megalooikonomou have described rapid and effective algorithms for 2-D and even 3-D spatial patterns that should prove useful for classification of analyte binding patterns on 2-D CARS [17].

It is a little known fact, but DNA does exhibit very low-level intrinsic autofluorescence when excited in the ultraviolet range [19], [20] which yields emissions in the upper ultraviolet and blue regions of the spectrum [Fig. 3(a)]. In this work, we take advantage of the intrinsic fluorescence of DNA in the array, because it is straightforward to scan the fluorescence and despite being of low intensity, the fluorescence demonstrates rather large and fairly reproducible changes upon binding of the DNA to various analytes in the gel.

Changes in the fluorescence scan patterns could be due to quenching of the excitation, quenching of the DNA fluorescence emission by the analyte, or changes in base stacking of the DNA upon analyte binding which have been shown by Komonov and Bukina to effect the fluorescence emission of DNA [19]. The DNA array itself exhibits very low-level autofluorescence when excited in the ultraviolet region of the spectrum [Fig. 3(a)]. However, this fluorescence may be modulated by changes in nucleotide stacking when analytes bind the array (much like a DNA aptamer changing conformation when it binds a target) or by absorption and emission of the analytes themselves. Ideally, the target analyte would absorb the excitation strongly or would

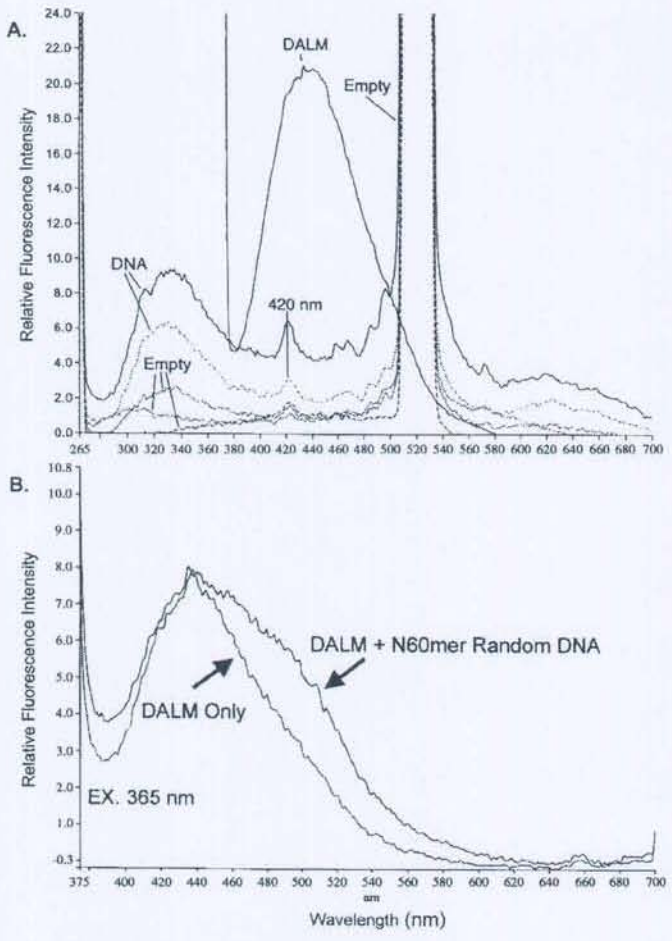

Fig. 3. (a) Comparison of emission spectra using excitation at 260 nm in a Perkin–Elmer LS 50 B luminescence spectrometer for empty 10% polyacrylamide gels (labeled "empty" in the figure) versus random 60 mer DNA (labeled "DNA") following electrophoresis in 10% polyacrylamide gels (at loci in the gels rich in DNA), and bacterial DALM. Strong second-order emission is seen at 520 nm. (b) Comparison of fluorescence emission spectra of DALM (in or adhered onto an epoxy layer) before and after interaction with random 60 mer DNA. Emission enhancement and spectral shifting due to the DALM-DNA interaction are apparent. Excitation was performed at 365 nm (excitation maximum of DALM).

be highly fluorescent itself to enable easy tracking and identification of the analyte's binding pattern to the array. Regardless of the analyte's fluorescence level, one of the major advantages of the current CARS scheme is that is does not require fluorophore labeling, making it simple and straightforward from an experimental standpoint.

Lakowicz *et al.* [20] have shown that metallic surfaces such as silver plates can be used to enhance the intrinsic fluorescence of DNA which may prove to be of value in conjunction with the CARS described herein. Numerous other groups have examined conductive materials for immobilization of DNA arrays to facilitate detection of nucleic acid hybridization events by electrochemical means [21] which could also be of value for rapid readout of the CARS. Similarly, we have investigated the interactions of random DNA with an electrically conductive, fluorescent, chemiluminescent, and electrochemiluminescent polymer known as diazoluminomelanin (DALM). DALM was originally developed for thermal analyses and microwave dosimetry [22], [23]. Data presented herein suggest that DALM may be of value in amplifying the fluorescence intensity and slightly red-shifting the emission spectrum of the CARS DNA-analyte interactions.

II. Experimental Section

Materials: All oligonucleotides were obtained from Ransom Hill Bioscience (Ramona, CA), Sigma Chemical Company (St. Louis, MO), or Genosys Corporation (Woodlands, TX). The BACA1FI and BACA6RI gene probes were synthesized by Genosys Corporation from published sequences [24] for portions of the capsular antigen gene of virulent strains of *Bacillus anthracis*. Precast 4%–20% gradient and 10% homogenous polyacrylamide gels made with Tris-Borate-EDTA (TBE) buffer, as well as DNA ladder (Amplisize; 50–2000 bp) standards were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA), and run on a mini Protean II electrophoresis system (Bio-Rad). All biotoxins were obtained from Sigma Chemical Company (St. Louis, MO). DALM was biosynthesized in *Escherichia coli* strain JM109 bacteria and partially purified as previously described [22]. All PCR reagents, including dideoxynucleotides, were from a "Silver Sequence" kit purchased from Promega Corporation (Madison, WI). *Thermus aquaticus (Taq)* DNA ligase was obtained from New England Biolabs (Ipswich, MA). SELEX binding buffer (BB) was composed of 0.5 M NaCl, 10 mM Tris-HCl, and 1 mM $MgCl_2$ in deionized water (pH 7.5 to 7.6 [11]).

DNA Array Generation: Two types of DNA arrays were generated: 1) a self-assembling overlapping (partially hybridized) random 60 mer (N60) and 2) a ligated (CARS) array with some truncated DNA molecules due to addition of dideoxynucleotides during a chain termination PCR step. The CARS PCR chain termination step involved addition of 6.6 μg of random N60 mer as a self-priming (due to partial hybridization) PCR template with 8 μl of each dideoxynucleotide (i.e., ddA, ddC, ddG, and ddT, premixed by Promega Corporation for sequencing), 20 μl of 5X sequencing buffer (250 mM Tris-HCl (pH 9.0) and 10 mM $MgCl_2$), 5 μl sequencing grade *Taq* polymerase, and autoclaved deionized water to bring the final reaction volume to 100 μl per tube. In addition, 2 μl (80 units) of *Taq* ligase were added per tube and tubes were PCR amplified using the following temperature profile: 96 °C for 5 min, followed by 40 cycles of 96 °C for 1 min, 25 °C for 1 min, and 72 °C for 1 min. PCR extension was completed at 72 °C for 7 min and tubes were stored at 4 °C–6 °C until electrophoresis was undertaken.

For both types of DNA arrays, 3.3 μg (typically, 5–10 μl) of combinatorial library DNA was diluted with 2X nucleic acid loading buffer (Bio-Rad) and loaded into each well of uniform 10% or 4%–20% gradient precast mini TBE polyacrylamide gels and electrophoresed in cold 1X TBE for 1 h at 100 V per gel. If DNA was to be visualized in the gel, gels were stained with 0.5 μg/ml ethidium bromide in TBE for 10 min, followed by rinsing in deionized water for 30 min, and photography on a 300 nm ultraviolet transilluminator using Polaroid type 667 film.

Analyte Binding and Oligonucleotide Hybridization: Gels were carefully cut into strips containing the 1-D DNA arrays of either type and were added to 10 ml of BB. Gel strips were allowed to equilibrate in their respective buffers for 10 min at room

temperature (RT) with gentle shaking and were then scanned as described below prior to addition of analytes. All DNA oligonucleotides were added at a final concentration of $5\mu g/ml$ and all protein analytes were added at a final concentration of $10\ \mu g/ml$ in BB for 1 h at RT with gentle shaking. Gels were gently rinsed twice in 10 ml of BB, carefully repositioned and rescanned on a luminescence spectrometer as described below.

DALM Immobilization in Epoxy and Interaction With Random DNA: Fifty μl drops of slow hardening epoxy resin (Duro Brand, Locktite Corporation, Rocky Hill, CT) were placed in black microtiter plate wells and overlaid with 50 μl of undiluted DALM [23]. The separated layers were allowed to interact in a covered plate for three to four days at ambient temperature. Excess DALM was removed by five washes with 200 μl of deionized water. All fluid was decanted and emission spectra, such as in Fig. 3(b), were acquired, as described below, before and after the addition of 50 μl (30 μg) of random 60 mer DNA.

Fluorescence Scanning: A Perkin–Elmer (Beaconsfield, Buckinghamshire, U.K.) model LS 50 B luminescence spectrometer equipped with a plate reader was used in the thin-layer chromatography (TLC) plate mode to scan DNA arrays in gel slices before and after addition of various analytes. After minor swelling or shrinkage in each of the reaction buffers, gel strips were generally 95–96 mm in length with the DNA array being contained in the lowermost 65 mm of each gel strip. Hence, the postbinding scans were initiated 30 mm from the top of each gel (to account for the stacking gel and well region) and allowed to proceed toward the bottom of each gel strip. Gel strips were scanned with an excitation of 260 nm (10 nm slits), emission of 420 nm (10 nm slits), and 1 mm resolution (i.e., scanned in 1 mm increments). In some experiments, DALM and random 60 mer DNA were scanned separately and in combination using an excitation wavelength of 360 nm (excitation maximum for DALM).

III. Results and Discussion

Generation of DNA Arrays: Gel electrophoresis of random oligonucleotide libraries revealed that a high degree of partial hybridization occurred between individual members of the library, leading to an aggregated collection of hybrids ("pseudo-concatamers") that appear as a smeared lane on electrophoresis gels. This point is illustrated in Fig. 1, which also illustrates that no two smears (essentially 1-D DNA arrays) were absolutely identical, since electrophoretic migration varies slightly from lane to lane in the gel. This fact probably contributes somewhat to the lack of complete reproducibility seen in subsequent experiments (Fig. 5).

Selection of Fluorescence Scanning Parameters: Fig. 3(a) is a comparison of fluorescence emission scans using a 260 nm excitation peak to compare baseline fluorescence of an empty 10% polyacrylamide gel strip, random N60 mer DNA in a gradient polyacrylamide gel (scanned at a locus with high DNA concentration), and bacterial DALM in a black microtiter well. It is apparent from Fig. 3(a) that random DNA in a polyacrylamide gel excited at 260 nm returns most of its energy in the ultraviolet region of the spectrum. We chose, however, to focus on a less prominent blue emission peak (420 nm), because it is in the visible region of the spectrum, where it might be of use in visualizing analyte-DNA array interactions in future work. In addition, if DALM were used as an underlying layer material, its blue fluorescence emission bands might augment the DNA fluorescence emission at 420 nm. Fig. 3(a) also indicates that very high background fluorescence occurs in the range of approximately 500–540 nm (centered at 520 nm) which can be attributed to a second-order emission (i.e., two-times the 260 nm excitation). However, by selecting to monitor the 420 nm fluorescence emission peak with a 10 nm slit, the background fluorescence can be discounted.

Fig. 3(b) compares the fluorescence of epoxy-immobilized DALM before and after the addition of random 60 mer oligonucleotides to the epoxy-DALM surface. The figure indicates enhanced fluorescence intensity and an emission spectrum shift toward the red when random DNA is allowed to interact with DALM excited at DALM's absorption peak of 365 nm. Three scans of DALM-DNA fluorescence interactions similar to those shown in Fig. 3(b) were obtained (not shown) that suggest a fluorescence energy transfer (red shift) from DALM to adherent DNA. The peak wavelength for DALM's fluorescence emission with or without bound DNA remained approximately 440 nm, but there is additional energy output when the DNA is present, because the area under the DALM plus N60 mer DNA curve is clearly greater than the area under the emission curve for DALM only. In addition, the DALM plus DNA curve has spread further toward the red in each of the scans we obtained. These data suggest that DALM may be of use in development of the CARS or other DNA array sensor surfaces to enhance total energy output and shift emissions toward the red (away from much of the biological autofluorescence background encountered in nature).

Comparison of Various Analytes on Both Types of Arrays: Fig. 4 compares the spatial fluorescence scans of six different analyte interactions with two differently prepared DNA arrays. The figure suggests that different analytes can influence the shape of the resultant fluorescence intensity scan as a function of distance along the 1-D array. It is interesting to note that some common features (e.g., peaks and valleys) appear to exist between related scans of each DNA array taken before (solid lines) and after (dashed lines) analyte binding. It is also interesting to note that most of these shared features appear to be dampened upon interaction with the analyte, suggesting energy absorption by the bound analyte. Some instances of very slight fluorescence enhancement (i.e., where the dashed line rises above the solid line) appear to occur, which could be artifacts, but are reproducible within a given experiment (Figs. 4–6).

Array Reproducibility: One issue that could plague the proposed DNA arrays is the question of reproducibility. At present, it appears that the reproducibility of individual scans of the same array with or without bound analytes in polyacrylamide is surprisingly good, as illustrated in Fig. 5. However, the reproducibility between different arrays is not nearly as precise (Fig. 5 comparison of panels A and B). Yet, some shared spatial-spectral features appear to persist between individual trials in Fig. 5. The potential issue of reproducibility could be partially offset by intelligent pattern recognition algorithms capable of correctly discriminating various similar binding patterns [17], [18].

Precast gradient polyacrylamide gels from an industrial source were used in this study to ensure a high degree of

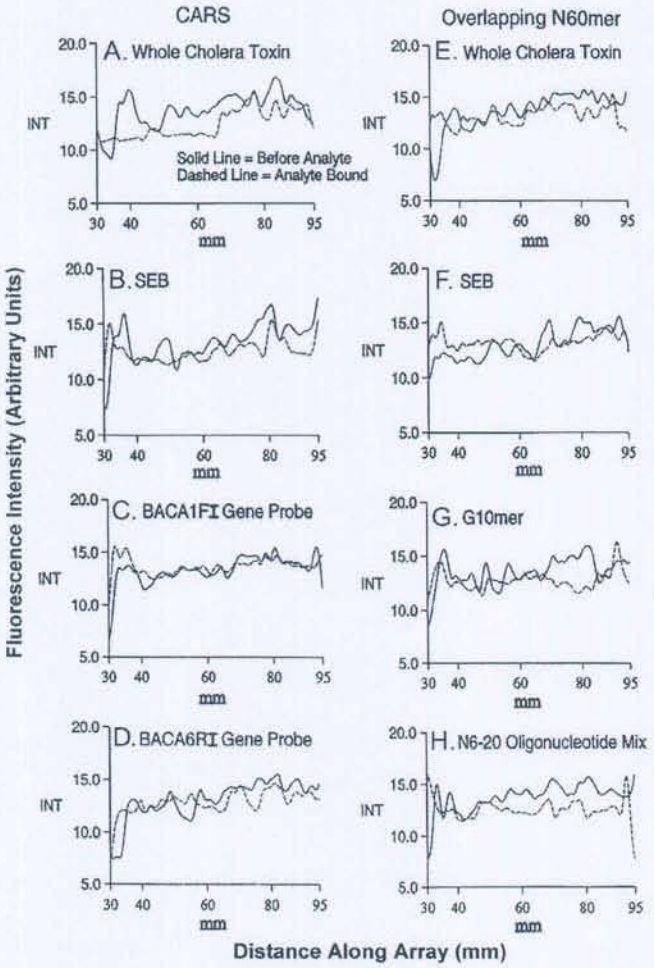

Fig. 4. Comparison of spatial fluorescence scans for two different types of combinatorial DNA arrays electrophoresed in 4%–20% gradient polyacrylamide gels before and after addition of various analytes using an excitation of 260 nm and emission wavelength of 420 nm with 5 nm slits. Analytes were: staphylococcal enterotoxin B (SEB), BACA gene probes (for *bacillus anthracis* capsular antigen), polyG decamer (G10 mer), and N6-20 mers (a DNA ladder standard composed of small DNA fragments from 6 to 20 bp, Sigma Chemical Company). Distance along the array from the bottom of the gel or position of the lowest molecular weight DNA is given in millimeters.

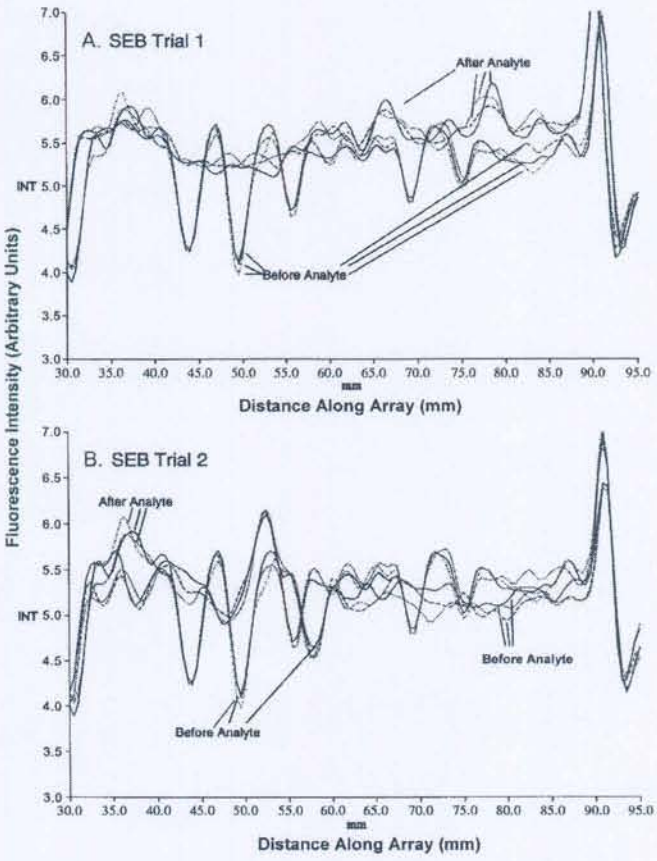

Fig. 5. Analysis of fluorescence scanning reproducibility. CARS DNA was electrophoresed in 4%–20% polyacrylamide gels and scanned before and after addition of SEB, as described in the methods section. Three consecutive scans were made for each trial. Reproducibility between individual scans of a given sample was fairly high. Reproducibility was not as precise between separate trials, although some consistent features may exist between trials. The "before analyte" scans were of two different CARS DNA arrays before addition of SEB and are remarkably similar.

quality control for the separation matrix. In addition, adjacent lanes were used for scan comparisons. Still, as seen in Fig. 1, minor variations in DNA migration exist and may contribute to differences between trials. In addition, factors such as minor differences in osmotic pressure, swelling of gels, potential elution of DNA from the gel during analyte binding and mixing, temperature, and vibrations during the scanning process, may combine to produce noticeable differences between trials. Tighter regulation of these parameters seems attainable and should lead to greater reproducibility of the fluorescence scans.

One parameter which we have recently found to be of great value in reducing baseline variations in the CARS array (Fig. 6) is purification of the self-assembled DNA array through a 10 kD spin column (Nanosep 10 K Omega, Pall Corporation, Ann Arbor, MI). The array is allowed to form as described previously without ligase and is then spun through the Nanosep column at 14 000 rpm on a microcentrifuge to remove excess nucleotides and other "impurities" that may have been effecting the prior baseline fluorescence stability. Fig. 6 exhibits more level baseline fluorescence between three independent experiments using two different genetic probes. These results indicate that a much higher degree of reproducibility can probably be obtained with the CARS approach to sensing, if reagent purity is more tightly regulated. We had originally thought that such purification would not be necessary, because the array was being washed in the gel after analyte binding, but it appears that the centrifugal force of the spin column wash aids in purifying the DNA array material prior to electrophoresis.

IV. Conclusion

The goal of this preliminary study was simply to investigate the feasibility of producing immense combinatorial DNA libraries of varied size from shorter combinatorial oligonucleotides and separating the members of the libraries by electrophoresis to use as a sensor surface. This goal appears feasible based on data obtained in Figs. 4–6. The present data appear to suggest that differences in the spatial fluorescence emission patterns do exist between the before and after analyte interaction scans along 1-D CARS arrays and simple random 60 mer DNA arrays (Figs. 4–6). Analyte interactions with the

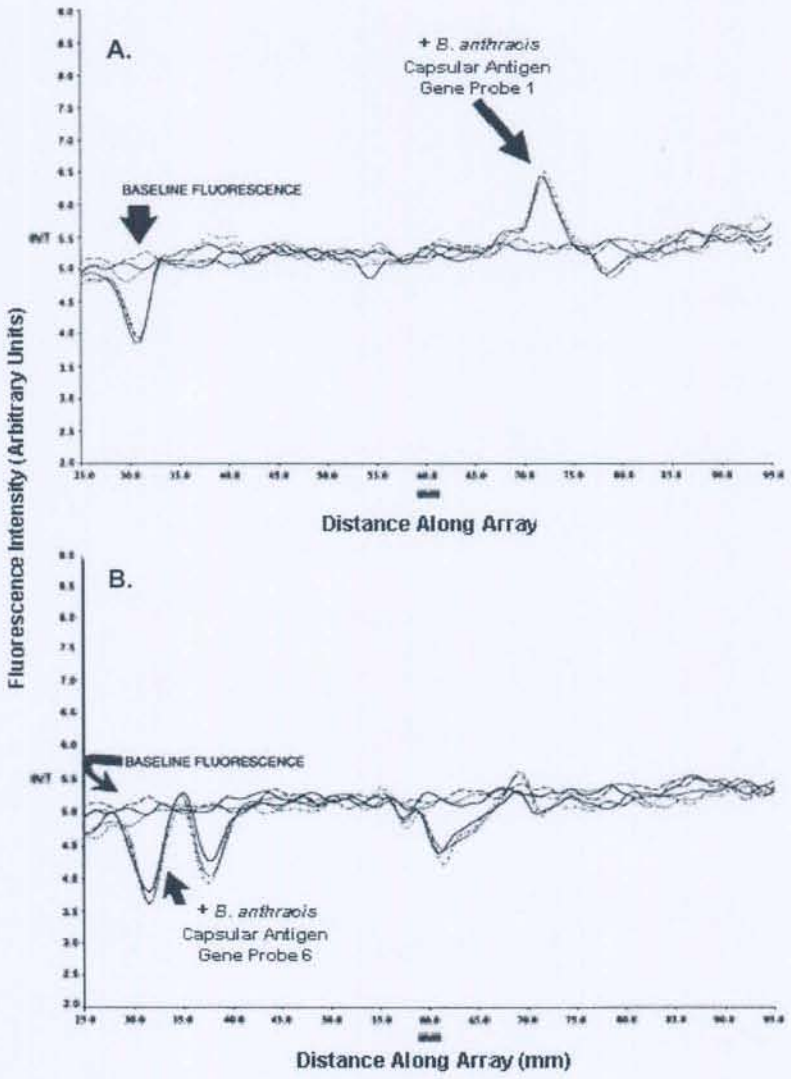

Fig. 6. Latter independent experiments in which the simple random 60 mer array was first purified through a spin column (10 kD cutoff) then electrophoresed in polyacrylamide to yield more stable baseline fluorescence. (a) Three independent experiments using different gel strips in which a purified CARS was scanned before and after exposure to the *B. anthracis* capsular antigen gene probe 1. (b) Three other independent experiments in which gene probe 6 was used.

DNA arrays appear to primarily dampen fluorescence emissions (i.e., energy absorption by the array-bound analyte). However, some instances of minor fluorescence enhancements also appear to exist along the array after analytes bind, suggesting that molecular conformation, size, or aggregation may play a part in determining the degree of absorbance or fluorescence emission of the analyte and the DNA array at any given point along the array.

The present work illustrates that detection, whether highly reproducible or not, of analyte binding to a spatially ordered combinatorial DNA array is possible for both protein and nucleic acid targets. The nature of the nucleic acid interactions is unknown and may involve a complex set of conventional base-pairing, other types of hydrogen bonding or strong and weak forces, but addition of oligonucleotides (e.g., BACA gene probes, G10 mers, or N6–N20 mers in Figs. 4–6) to the DNA arrays, clearly alters the fluorescence scan patterns even after the excess oligonucleotides are washed away with neat buffer.

Future work will focus on three areas of array development: 1) liberating the array from its gel matrix and affixing it onto a membrane or substrate (Fig. 1) to allow direct interaction of the DNA array with analytes (although work by Charles *et al.* [26] suggests that the 3-D nature of the gel may amplify signal intensity) and 2) further exploration of DALM as an underlying photonically and electronically active transducer material, and 3) exploration of generating 2-D arrays by rotating electrophoretically CARS and applying a second electric field perpendicular to the first [27].

With regard to using DALM as a photonic transducing or electrically conductive coupling material for a DNA-based "universal sensor," several interesting and potentially advantageous properties of DALM should be noted. DALM is a fluorescent, chemiluminescent, slow luminescent, and electrochemiluminescent material [22], [23], [25], [28] that appears to conduct electrons [23]. Hence, DALM may be quite useful as a photonically or electrically excitable, slow decaying

light emitter, which could amplify some analyte-DNA array interactions, if used as the underlying transducer layer for a CARS-based or other DNA array-based biosensor. Naturally, derivatives of DALM and other potential enhancing materials such as the silver surface described by Lakowicz *et al.* [20] will also be considered.

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