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Heightened sense for sensing: recent advances in pathogen immunoassay sensing platforms

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Introduction

As part of its own defense mechanism, most bacteria have developed an innate ability to enable toxic secretion to ward off potential predators or invaders. However, this naturally occurring process has been abused since over production of the bacteria's toxin molecules could render them as potential bioweapons.[1] As these processes (also known as "black biology") can be clandestinely performed in a laboratory, the threat of inflicting enormous potential damage to a nation's security and economy is invariably clear and present.[2] Thus, efficient detection of these biothreat agents in a timely and accurate manner is highly desirable.

A wealth of publications describing various pathogen immuno-sensing advances has appeared over the last few years,[3-6] and it is not the intent of this review article to detail each reported approach. Instead, we aim to survey a few recent highlights in hopes of providing the reader an overall sense of the breath of these sensing systems and platforms. Antigen targets are diverse and complex as they encompass proteins, whole viruses, and bacterial spores. The signaling processes for these reported immunoassays are usually based on colorimetric, optical, or electrochemical changes. Of equal interest is the type of platform in which the immunoassay can be performed. A few platforms suitable for pathogen detection are described.

Nanowire-based immunoassay sensing system

Metallic multi-striped nanowires as potential platforms for immunoassays were first reported by Nicewarner-Pena and co-workers, in which the nanowires can be fabricated to contain various combinations of metallic segments.[7] Using a controlled pore-sized template, metals can sequentially be deposited in the desired permutations, thus enabling rapid fabrication of addressable "barcodes". The multi-striped nanowires, when coated with specific antibodies, have been reported by Tok and co-workers to efficiently and accurately allow multiplex detection of biowarfare simulants (**Figs 1a & b**).[8] In

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addition, the metallic multi-striped nanowires have also been used by Sha and co-workers to detect and differentiate nucleic acid strands which contain single nucleotide polymorphisms.[9]

The ability to fabricate addressable nanowire arrays as sensing platforms in either micro- or nano-scale has achieved tremendous progress over the last few years. Lieber and co-workers have pioneered the development of nanowires for protein and virus sensing.[10-12] The nanowires are configured as field-effect transistors (FETs), whereby slight variations at the nanowire's surface elicit a change in conductivity (Fig 1c). By first coating the p-type Si nanowire with influenza-A virus-binding antibody, the nanowire is able to detect a single positively-charged influenza virus particle. The observation of an individual conductance change corresponds to a discrete biomolecule binding event. The nanowire-FET sensing system, functionalized with the appropriate antibodies, has also been able to differentiate both influenza A and adenovirus.[11]

Heath and co-workers have recently reported the fabrication of an ultrahigh-density nanowire circuit.[13] The electric field effect-based demultiplexing architecture (a demultiplexer is an electronic circuit designed to separate two or more combined signals) allows each of the nanowires to be individually addressed. Due to the small scale and efficiency of the platform, only minimal amounts of sensing ligands, e.g. antibodies, need to be immobilized for sufficient signal generation.[14] It is anticipated that such a high density nanowire-circuit array geared towards pathogen sensing can be fabricated *en mass* and be used for biosecurity surveillance.

Microbead-based immunoassay sensing system

In recent years, micrometer-sized beads have proven their utility in biosensing applications due to their tunable physical, chemical, and optical properties.[15] The beads can be engineered such that they are easily manipulated by either magnetic forces or electric field, and the myriad of available surface chemistries enable facile ligand conjugation to the microbeads. These two features ensure that biomolecules can be appropriately captured by the functionalized beads and subsequently manipulated for downstream analysis. Advances in manipulating optical properties have further enhanced the utility of microbeads by providing a means for their multiplexed analysis. For example, Luminex Corp (Austin, TX; *www. luminex.com*) has manufactured polystyrene microbeads which are internally dyed with different ratios of red and infrared fluorophores, hence conferring a unique spectral signature and/or fingerprint to each type of bead. Flow cytometry is then used to both detect antigen binding and decode the mixture of unique beads, allowing multiplex interrogation.

As each of the dye-encoded polystyrene microbeads can be decorated with a specified sensing ligand, the beads can thus collectively be used for efficient pathogen detection. To demonstrate this application, Luminex beads have been conjugated with various antibodies against biowarfare pathogenic

simulants, e.g. plague and anthrax (it sounds like these are simulants) (**Fig 2a**).[16-18] In the presence of the target antigens, fluorescently-labeled reporter antibodies are able to complete the sandwich formation. Subsequently, the fluorescent beads can be efficiently monitored via flow cytometry. An example in which the Luminex® beads are used to enable an integrated and portable pathogen sensing instrument has recently been demonstrated in a system known as Autonomous Pathogen Detection System (APDS).[16-19] Subsequent improvements to the APDS system enabled rapid PCR genetic analysis of the antigen and wireless transmission of the obtained flow cytometry and PCR data to a remote/centralized location.[17, 19]

As illustrated in **Fig 2b**, the APDS system contains an aerosol collector to constantly "inhale" particles from its surrounding environment for analysis. It is essential that samples be appropriately pre-filtered and pre-concentrated to enable sensitive and accurate pathogen detection. To achieve sample pre-concentration in the aerosol collector, the APDS system uses a virtual impactor to capture 1-10 μM particles.[16-18]

Another recent approach to pre-concentration includes the development of magnetic silica microparticles which, when coated with didecyldimethylammonium bromide, have been utilized by Yitzhaki and co-workers to efficiently capture and concentrate bacillus spores.[20] As few as 100 bacillus spores can be captured with 90% efficiency. This highlights the usage of simple, yet efficient, analytical methods for pre-concentration and even detection of targeted pathogens.

In addition to organic dyes, chemical coding using short oligonucleotides has been described in recent years by Mirkin and co-workers (**Fig 2c**).[21-23] In this approach, termed "bio-bar-code", reporter beads are decorated with specific coding DNA sequences that function as identifiers for specific biomolecular targets. After a sandwich capture event, the DNA tags are released and can be directly amplified via PCR or amplified either by a silver-staining process or a Au-mediated FET to enable their ultrasensitive detection process.[24, 25] In this manner, pathogens are identified by the amplification of their corresponding coding oligonucleotides. Additionally, reporter beads containing DNA conjugated with raman dyes have also been reported for efficient multiplex detection.[26, 27]

Microarray-based immunoassay sensing system

(a) Protein/antibody microarray. Recent concerns of spinach contamination by pathogenic *E-coli* underscore the need of a rapid and accurate way for their identification and detection. Traditional methods to accomplish the aforementioned task have ranged from plate culture to ELISA. A promising approach for multiplexed bacterial detection, and subsequent accurate differentiation of pathogenic from harmless bacteria, is the use of planar microarray detection systems. Gehring and co-workers have recently optimized the fabrication of an antibody microarray for the detection of O157:H7 *E. coli* pathogenic bacteria.[28] For example, it was observed that direct contact printing of biotinylated anti-O157:H7 *E. coli*

antibody on streptavidin coated microarray slides, as opposed to an indirect attachment via biotinylated protein G-bound captured antibodies, produced a readout signal which is ~5-times higher.

Another promising technology recently described by Endo and co-workers involves the development of localized surface plasmon resonance (LSPR) for the analysis of the nanoscale microarray chip (**Fig 3**).[29] This reagentless analytical method, which operates the same way as the widely used surface plasmon resonance approach, eliminates the need for tedious labeling processes while also requiring only limited sample. Thus nanochips, consisting of core-shell structured nanoparticles layer, capable of providing 300 "nanospots" on its sensing surface can be fabricated. By spotting various antibodies on the nanochip, proteins such as immunoglobins, C-reactive proteins, and fibrinogen can be detected down to a concentration of 100 pg/mL.

(b) Aptamer microarray. Short single-stranded oligonucleotides have been developed to efficiently bind target molecules by folding into complex tertiary structures. Such target-binding oligonucleotides are termed aptamers and are generated through the iterative selection process known as Systematic Evolution of Ligands by EXponential enrichment (SELEX).[30, 31] Certain aptamers, such as thrombin-binding ssDNA, have been shown to undergo structure switching ability (or fold) upon target binding. Plaxco and co-workers have recently described an electronic aptamer-based (EAB) sensing system consisting of an immobilized aptamer functionalized with a redox probe (methylene blue, Fig 4a).[32-35] Upon introduction of target, the aptamer folds to bind the protein, which in turn effects electron transfer between the redox probe and gold electrode surface. With the ability to rapidly fabricate aptamers in an array format (**Figs 4b & c**),[36-38] pathogen sensing aptamer-based microarray chips can be envisioned for rapid pathogen identification and characterization. Using either reagentless electrochemical or optical readout, the aptamer-based microarray chip could be readily employed for multiplex detection of targeted pathogens.

Whole cell-based immunoassay sensing system

An intriguing and promising pathogen sensing system recently reported is the whole-cell sensing system.[39] Rider and co-workers have demonstrated the successful engineering of B lymphocyte cells in which both pathogen sensing membrane-bound antibodies and an associated light-emitting reporting system are all conveniently expressed *in vivo*. The B lymphocyte cell-based sensing system, termed CANARY, hinges on an expressed calcium-sensitive bioluminescent protein (cytosolic aequorin) from the *Aequoria victoria* jellyfish. When exposed to targeted biowarfare pathogenic compounds (*Y. pestis, F. tularensis, B. anthracis*) an increase in photons was observed within the B lymphocyte cells in a matter of seconds. The photon changes can then easily be detected using an inexpensive optical system.

In addition to eukaryotic cells, sensors have also been developed using whole virus particles. Sapsford and co-workers utilize the cowpea mosaic virus (CPMV) to afford a "programmable" biological nanoscaffold.[40, 41] As CPMV can be modified at addressable sites on its surface, specific antibodies or fluorescent organic dyes can thus be selectively attached. As a result, CPMV can be utilized as a potential immunoassay tracer. In addition, the possibility to attach multiple dyes to the viral capsid surfaces should potentially also result in improved pathogen detection sensitivity.

Other virus-based sensors have been derived from bacteriophages routinely used for phage display, an iterative selection process used to generate target binding peptides.[42, 43] Target binding peptides are usually displayed together with either the tip (pIII) or the coat (pVIII) proteins of the bacteriophages. Short peptides which bind to pathogenic targets such as *B. anthracis* spores have been successfully generated through this technique.[44-46] By immobilizing viruses that express pathogen-binding peptides onto electrodes, "virus-based" pathogen sensing chips can be fabricated. This approach has recently been demonstrated by Yang and co-workers against cancer biomarkers, in which the prostate-specific membrane antigen (PSA) binding M13 bacteriophage was covalently attached onto gold electrode surfaces.[47] Electrochemical impendence spectroscopy was used to measure specific PSA binding, resulting in a reliable signal-to-noise ratio of ~10 for PSA concentrations down to 100 nM. The "virus-based" sensing chips represent an improvement in both the capacitive impedance and frequencies over other previously reported electrochemical biosensors.

In another report, specific antigen detection and simultaneous readout were accomplished using tailored hydrogels comprised of M13 bacteriophage and gold nanoparticles (AuNP) associated by non-covalent interactions (**Fig 5**).[48] While target binding is directed by the expressed peptides on the engineered phage surface, the phage-associated AuNPs can be used for a variety of detection schemes, including enhanced fluorescence and dark-field microscopy, surface-enhanced raman detection, and near-infrared photon-to-heat conversion. Imidazole-mediated AuNP aggregation can further enhance the detection. This reporting system was used by Souza and co-workers to target α_v integrins expressed on the surface of melanoma cells. It is anticipated that this technique should be adaptable for pathogen detection.

Challenges and Future Outlook

Advances in nanotechnology-inspired sensing systems have taken significant strides over the last few years. Coupled with the tremendous progress in both micro- and nano-electromechanical systems (MEMS and NEMS, respectively),[49-52] our traditional conception of how immunoassays can be performed have been drastically altered. It is anticipated that further advances in microfluidic design and development will play an integral role for both biodefense and medical applications. The ability to

miniaturize and adapt traditional bench-top immunoassay protocols to a fully automated micro- or nano-fluidic chip holds tremendous promise to enable multiplex, efficient, cost-effective, and accurate pathogen sensing systems.

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References

[1] B. Durodie, *Curr Opin Biotechnol*, 2004, **15**, 264.
[2] J. Bohannon, *Science*, 2003, **300**, 414.
[3] M. M. Cheng, G. Cuda, Y. L. Bunimovich, M. Gaspari, J. R. Heath, H. D. Hill, C. A. Mirkin, A. J. Nijdam, R. Terracciano, T. Thundat, and M. Ferrari, *Curr Opin Chem Biol*, 2006, **10**, 11.
[4] S. S. Iqbal, M. W. Mayo, J. G. Bruno, B. V. Bronk, C. A. Batt, and J. P. Chambers, *Biosens Bioelectron*, 2000, **15**, 549.
[5] B. Pejcic, R. D. Marco, and G. Parkinson, *Analyst*, 2006, **131**, 1079.
[6] D. V. Lim, J. M. Simpson, E. A. Kearns, and M. F. Kramer, *Clin Microbiol Rev*, 2005, **18**, 583.
[7] S. R. Nicewarner-Pena, R. G. Freeman, B. D. Reiss, L. He, D. J. Pena, I. D. Walton, R. Cromer, C. D. Keating, and M. J. Natan, *Science*, 2001, **294**, 137.
[8] J. B. Tok, F. Y. Chuang, M. C. Kao, K. A. Rose, S. S. Pannu, M. Y. Sha, G. Chakarova, S. G. Penn, and G. M. Dougherty, *Angew Chem Int Ed Engl*, 2006, **45**, 6900.
[9] M. Y. Sha, I. D. Walton, S. M. Norton, M. Taylor, M. Yamanaka, M. J. Natan, C. Xu, S. Drmanac, S. Huang, A. Borcherding, R. Drmanac, and S. G. Penn, *Anal Bioanal Chem*, 2006, **384**, 658.
[10] F. Patolsky, G. Zheng, O. Hayden, M. Lakadamyali, X. Zhuang, and C. M. Lieber, *Proc Natl Acad Sci U S A*, 2004, **101**, 14017.
[11] F. Patolsky, G. Zheng, and C. M. Lieber, *Anal Chem*, 2006, **78**, 4260.
[12] G. Zheng, F. Patolsky, Y. Cui, W. U. Wang, and C. M. Lieber, *Nat Biotechnol*, 2005, **23**, 1294.
[13] R. Beckman, E. Johnston-Halperin, Y. Luo, J. E. Green, and J. R. Heath, *Science*, 2005, **310**, 465.
[14] Y. L. Bunimovich, G. Ge, K. C. Beverly, R. S. Ries, L. Hood, and J. R. Heath, *Langmuir*, 2004, **20**, 10630.
[15] R. Wilson, A. R. Cossins, and D. G. Spiller, *Angew Chem Int Ed Engl*, 2006, **45**, 6104.
[16] B. J. Hindson, S. B. Brown, G. D. Marshall, M. T. McBride, A. J. Makarewicz, D. M. Gutierrez, D. K. Wolcott, T. R. Metz, R. S. Madabhushi, J. M. Dzenitis, and B. W. Colston, Jr., *Anal Chem*, 2004, **76**, 3492.
[17] B. J. Hindson, M. T. McBride, A. J. Makarewicz, B. D. Henderer, U. S. Setlur, S. M. Smith, D. M. Gutierrez, T. R. Metz, S. L. Nasarabadi, K. S. Venkateswaran, S. W. Farrow, B. W. Colston, Jr., and J. M. Dzenitis, *Anal Chem*, 2005, **77**, 284.
[18] M. T. McBride, D. Masquelier, B. J. Hindson, A. J. Makarewicz, S. Brown, K. Burris, T. Metz, R. G. Langlois, K. W. Tsang, R. Bryan, D. A. Anderson, K. S. Venkateswaran, F. P. Milanovich, and B. W. Colston, Jr., *Anal Chem*, 2003, **75**, 5293.
[19] B. J. Hindson, A. J. Makarewicz, U. S. Setlur, B. D. Henderer, M. T. McBride, and J. M. Dzenitis, *Biosens Bioelectron*, 2005, **20**, 1925.

[20] S. Yitzhaki, E. Zahavy, C. Oron, M. Fisher, and A. Keysary, *Anal Chem*, 2006, **78**, 6670.
[21] J. M. Nam, S. I. Stoeva, and C. A. Mirkin, *J Am Chem Soc*, 2004, **126**, 5932.
[22] B. K. Oh, J.-M. Nam, S. W. Lee, and C. A. Mirkin, *Small*, 2006, **2**, 103.
[23] M. S. Han, A. K. R. Lytton-Jean, and C. A. Mirkin, *Angew. Chem Int. Ed.*, 2006, **45**, 1807.
[24] T. A. Taton, C. A. Mirkin, and R. L. Letsinger, *Science*, 2000, **289**, 1757.
[25] S. J. Park, T. A. Taton, and C. A. Mirkin, *Science*, 2002, **295**, 1503.
[26] Y. C. Cao, R. Jin, J. M. Nam, C. S. Thaxton, and C. A. Mirkin, *J Am Chem Soc*, 2003, **125**, 14676.
[27] Y. C. Cao, R. Jin, and C. A. Mirkin, *Science*, 2002, **297**, 1536.
[28] A. G. Gehring, D. M. Albin, A. K. Bhunia, S. A. Reed, S. I. Tu, and J. Uknalis, *Anal Chem*, 2006, **78**, 6601.
[29] T. Endo, K. Kerman, N. Nagatani, H. M. Hiepa, D. K. Kim, Y. Yonezawa, K. Nakano, and E. Tamiya, *Anal Chem*, 2006, **78**, 6465.
[30] C. Tuerk and L. Gold, *Science*, 1990, **249**, 505.
[31] A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818.
[32] B. R. Baker, R. Y. Lai, M. S. Wood, E. H. Doctor, A. J. Heeger, and K. W. Plaxco, *J Am Chem Soc*, 2006, **128**, 3138.
[33] A. A. Lubin, R. Y. Lai, B. R. Baker, A. J. Heeger, and K. W. Plaxco, *Anal Chem*, 2006, **78**, 5671.
[34] R. Y. Lai, E. T. Lagally, S. H. Lee, H. T. Soh, K. W. Plaxco, and A. J. Heeger, *Proc Natl Acad Sci U S A*, 2006, **103**, 4017.
[35] Y. Xiao, A. A. Lubin, A. J. Heeger, and K. W. Plaxco, *Angew Chem Int Ed Engl*, 2005, **44**, 5456.
[36] K. Stadtherr, H. Wolf, and P. Lindner, *Anal Chem*, 2005, **77**, 3437.
[37] J. R. Collett, E. J. Cho, J. F. Lee, M. Levy, A. J. Hood, C. Wan, and A. D. Ellington, *Anal Biochem*, 2005, **338**, 113.
[38] J. R. Collett, E. J. Cho, and A. D. Ellington, *Methods*, 2005, **37**, 4.
[39] T. H. Rider, M. S. Petrovick, F. E. Nargi, J. D. Harper, E. D. Schwoebel, R. H. Mathews, D. J. Blanchard, L. T. Bortolin, A. M. Young, J. Chen, and M. A. Hollis, *Science*, 2003, **301**, 213.
[40] K. E. Sapsford, C. M. Soto, A. S. Blum, A. Chatterji, T. Lin, J. E. Johnson, F. S. Ligler, and B. R. Ratna, *Biosens Bioelectron*, 2006, **21**, 1668.
[41] B. D. Martin, C. M. Soto, A. S. Blum, K. E. Sapsford, J. L. Whitley, J. E. Johnson, A. Chatterji, and B. R. Ratna, *J Nanosci Nanotechnol*, 2006, **6**, 2451.
[42] J. K. Scott and G. P. Smith, *Science*, 1990, **249**, 386.
[43] V. A. Petrenko and G. P. Smith, *Protein Eng*, 2000, **13**, 589.
[44] J. Knurr, O. Benedek, J. Heslop, R. B. Vinson, J. A. Boydston, J. McAndrew, J. F. Kearney, and C. L. Turnbough, Jr., *Appl Environ Microbiol*, 2003, **69**, 6841.
[45] D. D. Williams, O. Benedek, and C. L. Turnbough, Jr., *Appl Environ Microbiol*, 2003, **69**, 6288.
[46] C. L. Turnbough, Jr., *J Microbiol Methods*, 2003, **53**, 263.
[47] L. M. Yang, P. Y. Tam, B. J. Murray, T. M. McIntire, C. M. Overstreet, G. A. Weiss, and R. M. Penner, *Anal Chem*, 2006, **78**, 3265.
[48] G. R. Souza, D. R. Christianson, F. I. Staquicini, M. G. Ozawa, E. Y. Snyder, R. L. Sidman, J. H. Miller, W. Arap, and R. Pasqualini, *Proc Natl Acad Sci U S A*, 2006, **103**, 1215.
[49] E. P. Kartalov, J. F. Zhong, A. Scherer, S. R. Quake, C. R. Taylor, and W. F. Anderson, *Biotechniques*, 2006, **40**, 85.
[50] J. W. Hong, V. Studer, G. Hang, W. F. Anderson, and S. R. Quake, *Nat Biotechnol*, 2004, **22**, 435.
[51] J. S. Marcus, W. F. Anderson, and S. R. Quake, *Anal Chem*, 2006, **78**, 3084.
[52] K. A. Shaikh, K. S. Ryu, E. D. Goluch, J. M. Nam, J. Liu, C. S. Thaxton, T. N. Chiesl, A. E. Barron, Y. Lu, C. A. Mirkin, and C. Liu, *Proc Natl Acad Sci U S A*, 2005, **102**, 9745.

Figure 1. [a] Analogy between a conventional barcode and a metallic-stripe encoded nanowire. The nanowire is ~250 nm in diameter and ~6 µm in length.[8] **[b]** An image of a post-assay reflectance and fluorescence readout of metallic-stripe encoded nanowires. The identity of the antigen present can be easily identified from the nanowires striping pattern (0=Au; 1=Ag) and their corresponding fluorescence images.[8] **[c]** Schematic of a nanowires-FET sensing device.[10] The nanowires are fabricated to enable multiplexed single-virus detection. The conductance versus time is recorded simultaneously for the various nanowires to provide real-time monitoring of the virus interaction events.

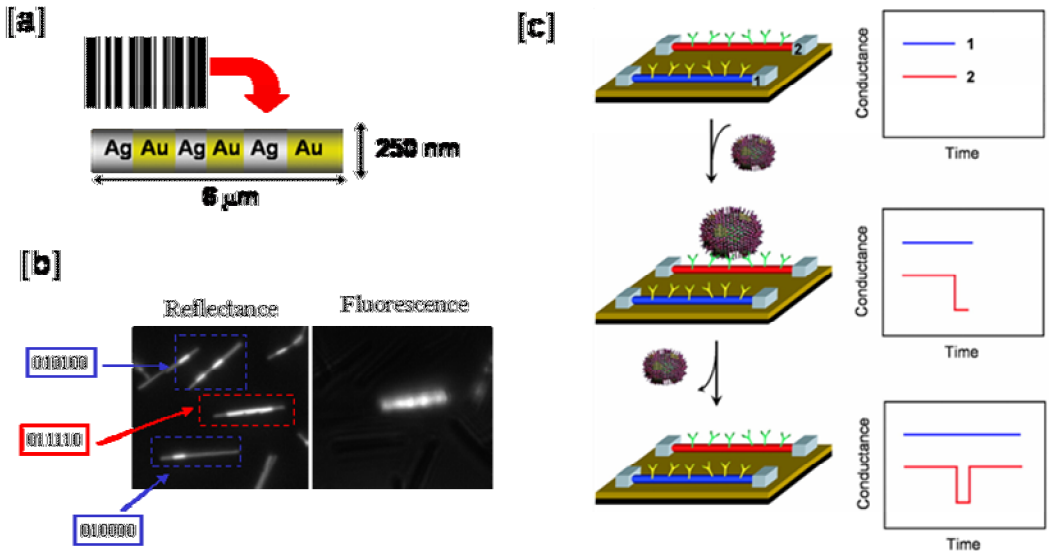

Figure 2. [a] Application of the Luminex® beads as immunoassay platforms.[18] A 100-plex bead array is generated by intercalating varying ratios of red and infrared dyes into polystyrene latex microbeads. After completion of the "sandwich" capture, the beads are then individually analyzed in a flow cytometer. **[b]** In the fully automated APDS system, Luminex® beads are housed in the Luminex® 100.[16, 17] **[c]** Schematic of the "bio-bar-code" system to detect target anthrax DNA sequences.[21]

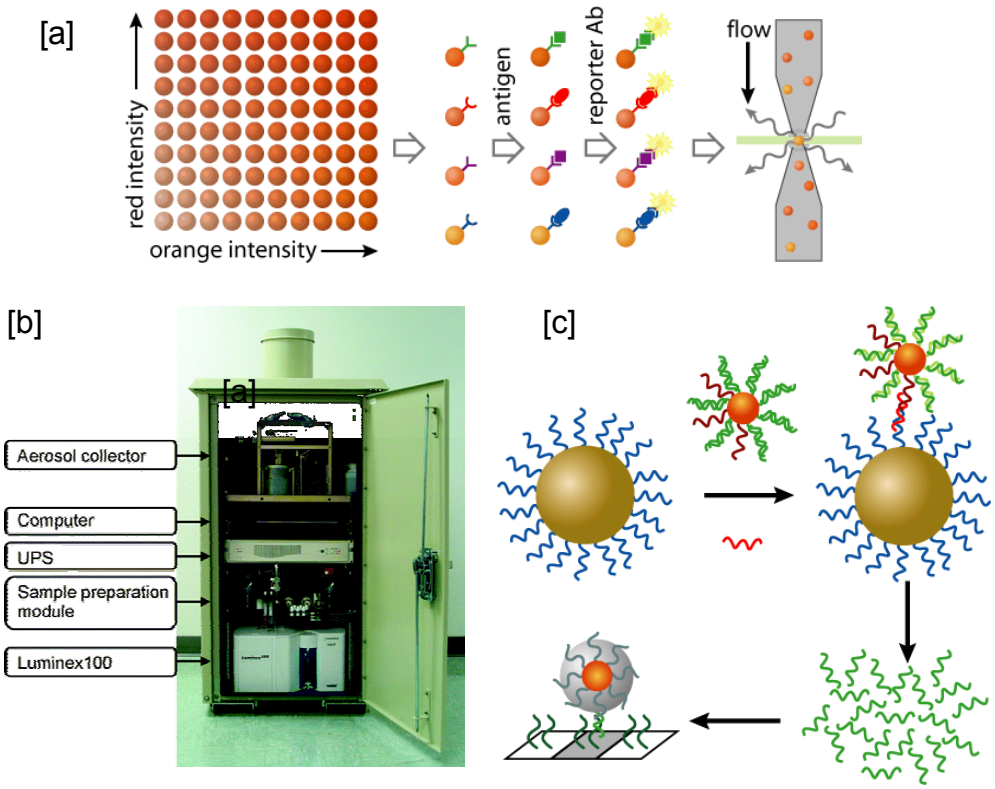

Figure 3. [a] Construction of the multiarray LSPR-based nanochip.[29] **[b]** Spotting of proteins to create a LSPR-based nanochip.[29] **[c]** LSPR-based nanochip detection experimental setup.[29]

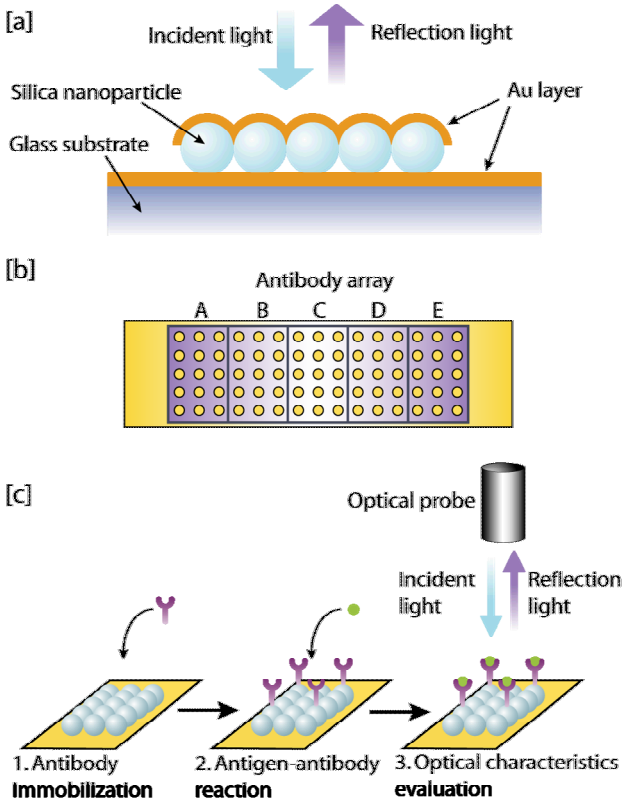

Figure 4. Schematic of the reagentless electronic aptamer-based (EAB) sensing system.[35]

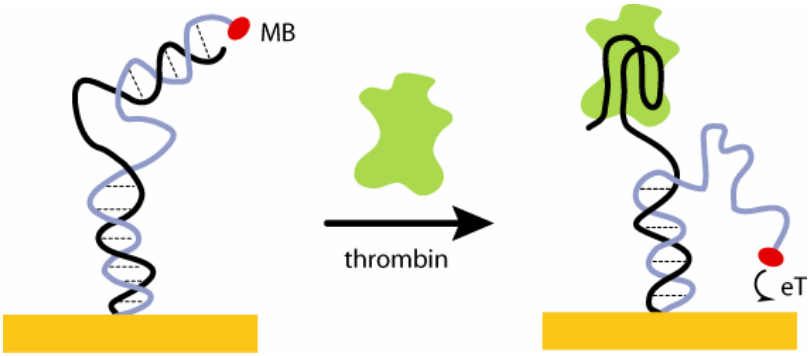

Figure 5. [a] Illustration of a dense Au-phage-imidazole sensing network with AuNP (yellow spheres), M13 phage [elongated structures (not drawn to scale)], and imidazole.[48] **[b]** Cartoon depiction of the electrostatic interaction of AuNP with phage. Target binding peptides are expressed on both the pVIII major capsid and pIII minor capsid proteins.[48] **[c]** Targeted cell-suspension detection scheme through the Au-phage sensing network by SERS.[48]