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Detection of biomarkers using recombinant antibodies coupled to nanostructured platforms

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Abstract

The utility of biomarker detection in tomorrow's personalized health care field will mean early and accurate diagnosis of many types of human physiological conditions and diseases. In the search for biomarkers, recombinant affinity reagents can be generated to candidate proteins or post-translational modifications that differ qualitatively or quantitatively between normal and diseased tissues. The use of display technologies, such as phage-display, allows for manageable selection and optimization of affinity reagents for use in biomarker detection. Here we review the use of recombinant antibody fragments, such as scFvs and Fabs, which can be affinity-selected from phage-display libraries, to bind with both high specificity and affinity to biomarkers of cancer, such as Human Epidermal growth factor Receptor 2 (HER2) and Carcinoembryonic antigen (CEA). We discuss how these recombinant antibodies can be fabricated into nanostructures, such as carbon nanotubes, nanowires, and quantum dots, for the purpose of enhancing detection of biomarkers at low concentrations (pg/mL) within complex mixtures such as serum or tissue extracts. Other sensing technologies, which take advantage of 'Surface Enhanced Raman Scattering' (gold nanoshells), frequency changes in piezoelectric crystals (quartz crystal microbalance), or electrical current generation and sensing during electrochemical reactions (electrochemical detection), can effectively provide multiplexed platforms for detection of cancer and injury biomarkers. Such devices may soon replace the traditional time consuming ELISAs and Western blots, and deliver rapid, point-of-care diagnostics to market.

Keywords: *phage-display*; *scFv*; *Fab*; *therapeutic antibody*; *affinity maturation*; *mutagenesis*; *nanotechnology*; *carbon nanotube*; *nanoshell*; *electrochemical detection*

Michael Kierny and Thomas Cunningham contributed equally

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Thomas Cunningham graduated with his Bachelor's degree in Immunology from the department of Molecular and Cell Biology at the University of California-Berkeley. After receiving his PhD in Biochemistry at the University of Illinois Urbana-Champaign he joined Brian Kay's lab at the University of Illinois at Chicago to isolate affinity reagents using recombinant antibody techniques, like bacterial phage-display. He currently manages operations at a large antibody manufacturer in the California biotech community and maintains an avid interest in the antibody engineering field.

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One of the driving forces in basic and applied science has been the search for biomarkers, such as proteins, post-translational modifications, peptides, or metabolites, which are unique and representative of a particular cell type or disease state. In the ideal case, these would be biomarkers that are released from the source in sufficient amounts to be easily detected in complex mixtures, like serum or saliva. (Fig. 1a). Protein biomarkers are the most common type of biomarker used in medical diagnostics. The discovery process (Fig. 1b) for protein biomarkers has been dominated by the use of liquid chromatography fractionation in conjunction with tandem mass spectrometry (LC/MS/MS) (1–3). This method has been successful because it can separate and identify thousands of proteins from a single complex sample that has been digested with a protease to yield short, traceable peptides (Fig. 1c). Following a comparison of a diseased to a healthy subject's profile, one can identify the proteins that are correlated to the diseased state, which in most cases for biomarkers have a concentration in the nanogram per milliliter range (4). The proteins or peptides are then used as targets in antibody selection schemes (Fig. 1d) to generate detection reagents. After these biomarkers are

Department of Energy Ames Laboratory. In 2007 he joined the Biological Sciences PhD program at the University of Illinois at Chicago. He is currently in Brian Kay's lab conducting research related to the detection of biomarkers for retinal injury using scFv recombinant antibodies generated using phage-display.

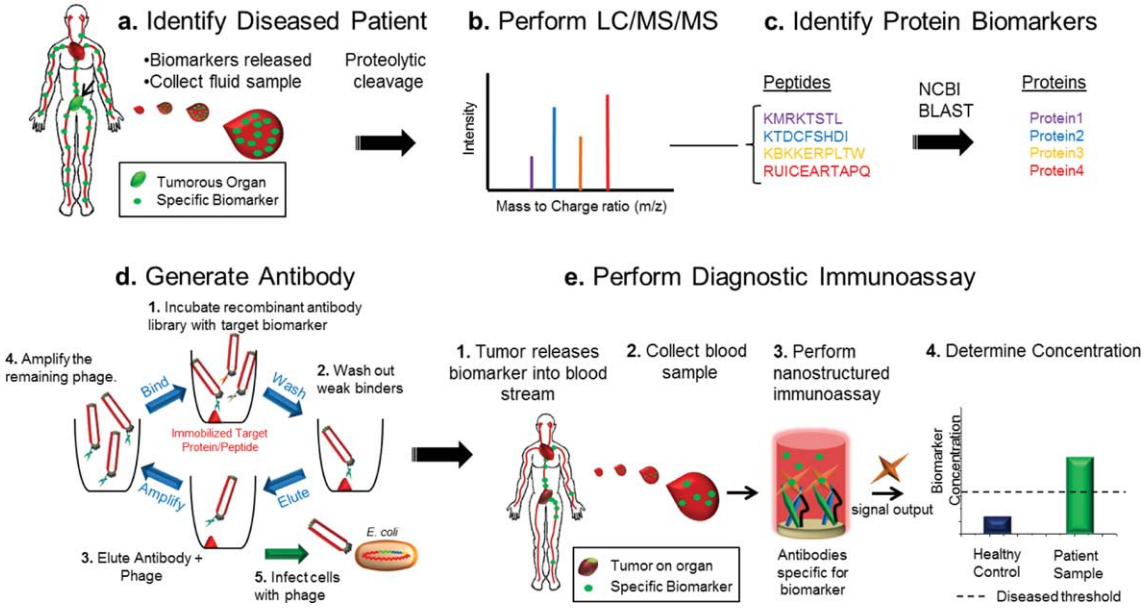

Fig. 1. From biomarker discovery to disease diagnosis. (a) Specific proteins are released into circulation in response to developing disease or injury. Shown here is cancer growth on an organ (arrow), such as the prostate gland, releasing biomarkers (green spheres) that enter the blood stream, where they circulate before eventually being cleared. To determine the biomarkers present, the serum is collected from a diseased individual and subjected to trypsin digest to generate many short peptides. (b) The sample is then separated using liquid chromatography, and fractions are analyzed by tandem mass spectrometry where fragment ions of the peptides result from collision-induced dissociation. The mass-to-charge ratio of the fragments are used to determine the amino acid sequence of a charged peptide (3). (c) A database, such as GenBank at the National Center for Biotechnology Information (NCBI), is searched with a bioinformatics program like BLAST (Basic Local Alignment Search Tool) using the amino acid sequence to identify the full length protein. Comparison of the diseased serum protein profile to a healthy profile will elucidate the biomarker protein. (d) The full protein or a synthesized peptide is used in a phage-display selection to generate specific recombinant antibodies. **1**. The phage-display library is incubated with the target protein or peptide. **2**. The weak binding antibodies are washed away. **3**. The tight binding antibodies are retained and eluted. **4**. The antibody-displaying phage are amplified through infection of bacteria. The process is then repeated under more stringent conditions. **5**. Single colonies of phage infected *Escherichia coli* are sequenced and the antibodies characterized. (e) A diagnostic immunoassay is performed on serum from a patient with suspected early onset of disease. **1**. The tumor is releasing low amounts of biomarker protein into the blood stream. **2**. A sample of blood is collected and prepared. **3**. Using the previously generated recombinant antibodies, the biomarker is recognized when the blood serum sample is introduced into a nanostructured immunoassay. Depending on the platform, a signal is produced that is proportional to the concentration of the biomarker. **4**. The concentration is determined, compared to healthy controls, and a diagnosis made. Certain concentrations of a particular biomarker may be indicative of a disease or injury. This can lead to additional tests that are more invasive to the the patient.

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validated to be indicative of a particular disease, an accurate diagnosis can typically be made immunologically on a nanostructured platform, or once again through mass spectrometry (Fig. 1e). While one would ideally prefer to find a single biomarker that is representative of a cell state or disease, it is likely that combinations of biomarkers will be needed to make a confident assessment.

Currently, there are a number of biomarkers known to be associated with certain states of human health and disease. These include human chorionic gonadotropin (hCG) in urine and serum (for monitoring pregnancy) (5), alpha-fetoprotein (AFP) in amniotic fluid (as an indication of neural tube defects) (6), cardiac troponin I (cTnI) in blood (to monitor acute myocardial infarction in patients) (7), and a panel of five biomarkers including alpha-II Spectrin Breakdown Product (SBDP) (to diagnose traumatic brain injury) (8, 9). Many types of cancer are evaluated to some degree by the occurrence of biomarkers. These include Carcinoembryonic antigen (CEA) for colon and rectal cancers (10), cancer antigen 125 (CA125) for ovarian cancer (11), Prostate-Specific Antigen/Kallikrein 3 (PSA/K3) for prostate cancer (12), and Human Epidermal growth factor Receptor 2 (HER2/Neu) for breast cancer (13, 14).

Antibodies are commonly used to locate and quantify individual proteins in complex mixtures and tissues. Often the amount, activity, or location of a particular protein changes over time with genetic mutation, infection, or disease, thus antibodies are extremely useful in monitoring individual proteins. In the past, antibody production was triggered upon presentation of an antigen to a host's immune system. Polyclonal antibodies, purified from the serum of an immunized animal (i.e. mouse, rabbit, goat, etc.), or monoclonal antibodies, secreted by immortalized B cells from the spleen of an immunized animal (15) are commonly used in immunological assays. This is in contrast to newer methods that involve the generation of large libraries of recombinant antibodies or scaffolds that are engineered to behave like antibodies, and then screening said libraries *in vitro* for the desired binding properties. The advantages of using recombinant antibodies will be discussed below.

Recombinant antibodies

Recombinant antibodies have many attractive attributes compared to the traditional polyclonal antisera and monoclonal immunoglobulin antibodies. First, they can be overexpressed and easily purified in a range of common eukaryotic and prokaryotic hosts. Second, the genotype and phenotype of the antibody can be linked through various display technologies, allowing for the easy recovery of the coding regions of recombinant antibodies. Third, by depositing the sequence information in GenBank any researcher can renew clones through gene synthesis. Third, the sequence information allows for subcloning and bioengineering to generate fusions with fluorescent proteins or enzymes. Fourth, they can be tagged with epitopes or short peptide sequences, without interfering with their binding properties. Fifth, unlike antibody generation through immunization, researchers can select for recombinant affinity reagents that work in the presence of certain salts, buffers, detergents, at a particular pH, etc. Furthermore, through the process of subtraction, one can develop antibodies that recognize specific epitopes (regions of the protein), post-translational modifications, and conformations. The increased throughput (16) over rabbit or mouse monoclonal antibody generation has made it technically feasible to attempt to analyze the $>$20,000 proteins that comprise the human proteome (17). Finally, with recombinant antibodies, the knowledge of the DNA sequence and the ease at which it can be manipulated allows for unique studies. For example, antibodies have been expressed in cell lines, where they can interact with their cell target (18, 19), their affinity can be improved through directed evolution under increased selection pressures (20, 21), they can be used to inhibit signaling pathways to study organism development (22), they can serve as the basis of complex biosensors (23), and they can be designed to incorporate unnatural amino acids, thereby increasing the range of molecular interactions by which they may bind their targets (24).

Many of these attributes make recombinant antibodies especially well suited for incorporation and fabrication into nanostructured devices. They can be engineered to include short peptide extensions, such as the AviTag, which serves as a substrate for a biotin ligase to add a single biotin molecule (25). This form of post-translational modification does not interfere with the antibody's function, as sometimes is the case with chemical biotinylation. The attached biotin allows for orientation-specific attachment of the antibodies to nanostructures through the streptavidin linkage, and guarantees that 100% of the antibody molecules can bind to antigen, which allows lower detection limits to be reached, and yields more reliable assays. Other methods for orientation-specific immobilization can be made through amino acid replacement or addition of a cysteine residue and subsequent attachment to gold-coated surfaces (26).

Introduction of free cysteine residues or biotin can also serve as the starting point for the attachment of DNA oligonucleotides, which permits Proximity Ligation Assay (PLA) (27), an assay that combines the protein recognition capabilities of an immunoassay with the amplification power of the Polymerase Chain Reaction (PCR). Two antibodies against the same antigen can be tagged with unique oligonucleotides, where upon binding, the oligonucleotides are brought in close proximity to one another where they can be ligated together through

the presence of a splint oligonucleotide. The ligated DNA molecule can then serve as a template for PCR or Rolling Circle Amplification (RCA), thereby allowing *in situ* detection of antigen recognition in cells or tissues (28). This method can also be used for highly sensitive detection of biomarker proteins in solution (29, 30).

Another advantage of a recombinant antibody is that its binding can be altered through point mutations to either eliminate binding to an antigen (for use as a negative control) or to fine tune its binding or specificity. It has been shown that tumor penetrating efficiency is reduced for scFv antibodies that have a very high affinity (K_d values of 1 nM to 10 pM), due to their long off rate kinetics for the initial antigen interacton occuring at shallow depths. Antibody penetration depth actually increases when affinities are lowered to 100 nM through mutation (31). The success of tumor targeting of antibody-coupled nanostructures will depend on the ability of complexes to diffuse through the tissue to reach its antigen.

Types of recombinant antibodies

Immunoglobulin isotype G (IgG) antibodies are composed of two heavy and two light chains (Fig. 2a). Diversity in antibody–antigen interactions is achieved through the interaction of six short (3–25 amino acids) complementarity determining regions (CDRs), within the variable domains of the antibody heavy and light chains. Fragments of antigen binding (Fabs) (Fig. 2b) and single chain fragments of variation (scFv) (Fig. 2c) represent structurally minimized versions of full-length human antibodies and, as such, are popular formats in recombinant antibody technology. The scFv consists of the variable domains (i.e. V_H, V_L) of both heavy and light chains that have been combined into a single polypeptide (32, 33). ScFvs are well suited for molecular biology because they can be cloned and manipulated as individual polypeptides, and can be displayed on the surface of bacteriophage (phage), while still retaining selective binding to antigens. As shown in Fig. 2c, the antigen-binding surfaces of the heavy and light chains can be connected *via* a 15 amino acid linker. In addition, such scFv molecules can be tagged at their C-termini with the c-myc epitope and six histidines, permitting detection and purification, respectively. Even though these antibody fragments are significantly smaller (25 vs. 150 kDa) than IgGs, they can still bind their respective antigens tightly (i.e. with dissociation constants of 5 μM to 10 nM), and are amenable to directed evolution (34) for improvement of their affinity. Fab antibodies (Fig. 2b) are ~50 kDa, which is twice the size of scFvs, and are composed of two

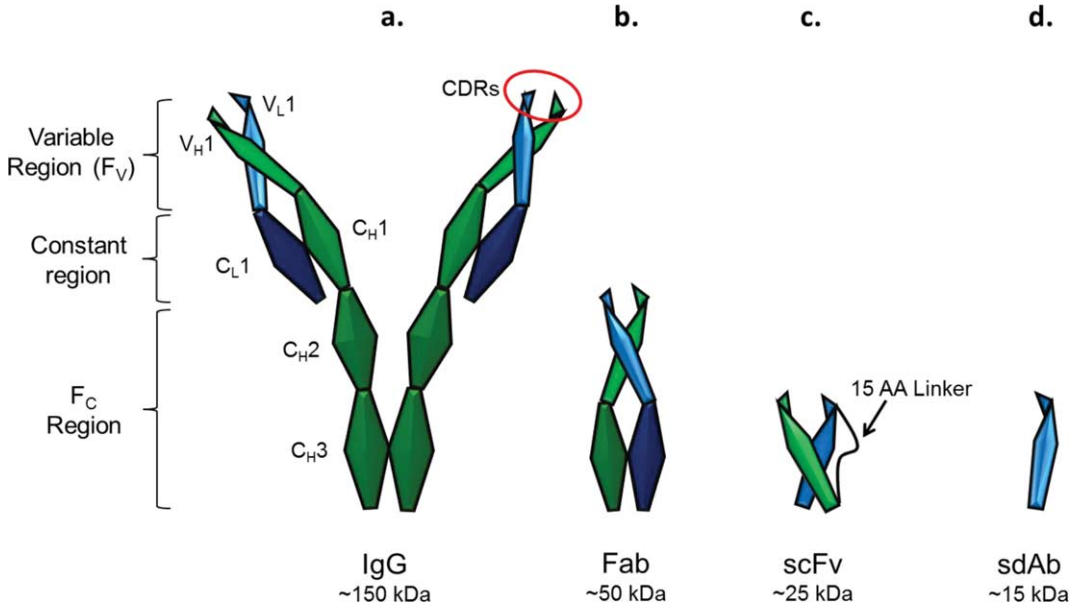

Fig. 2. Molecular formats of antibodies. (a) Immunoglobulin subtype G (IgG): full size antibody molecule, which is secreted by B cells of the mammalian immune system. The variable domains (V_L and V_H) contain six complementarity determining regions (CDRs), which contact the antigen. Constant regions (C_H, C_L) provide stable interactions between the light and heavy chains. The Fragment of the constant region (Fc) triggers a physiological response by interacting with Fc receptors of cells. The IgG molecule is bivalent and can simultaneously recognize two identical antigen molecules. (b) Fragment of antigen binding (Fab): Composed of the light chain and half of the heavy chain; the two chains are held together *via* disulfide bonds. The Fab is monovalent, and can recognize only one antigen molecule at a time. (c) Single-chain Fragment of variation (scFv): The variable domains of the heavy and light chains can be joined through a flexible 15 amino acid long linker. The scFv is monovalent, recognizing one antigen molecule at a time. (d) The single-domain antibody (sdAb) is devoid of light chains and can be derived from camelids V_{HH} antibodies.

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polypeptides with constant regions linked through disulfide bonds. Fabs are monovalent and, in contrast to scFvs, generally resistant to aggregation. Affinity maturation and selection techniques are similar to that of scFvs, but conversion of a Fab to and from an IgG is straight forward since the highly conserved constant region allows the layout of the variable domain to remain unchanged (35). Finally, single-domain antibodies (sdAb) (Fig. 2d), such as those derived from V_{HH} immunoglobulins present in camelids, lack the V_L domain and consist just of the V_H domain (36). Such antibody fragments are advantageous because of their small size, ~15 kDa, they are readily expressed in bacteria, and they penetrate deep into tissues after being introduced into patients (37).

Other types of scaffold proteins

In contrast to the use of antibody fragments for generating affinity reagents, one can use alternative scaffolds. One such example is the fibronectin type III domain of the glycoprotein fibronectin. It is an extracellular matrix ligand binding protein that has six loops of which three have been randomized and displayed on phage to generate a library (38). DARPins are another set of repurposed scaffolds designed from the ankyrin repeats (39, 40) that are normally involved in mediating protein interactions. Here, repeats of two alpha-helices joined by a loop are linked by beta-hairpins to one another in a modular fashion to form a 2–3 repeat binding pocket with N- and C-terminus ankyrin caps. Affibodies also originate from a protein unrelated to antibodies or antibody fragments. These are composed of the Z-domain in the cell wall protein A, of the *Staphylococcus aureus* bacterium, that has two randomized alpha-helices for interaction with a target protein (41). Libraries of scaffold variants can be constructed through molecular biology techniques, and then screened through multiple rounds of affinity to yield tight, binding clones. This procedure yields a renewable, *in vitro* generated affinity reagent that has high specificity and affinity to a given antigen (42–45).

Display technologies

In vitro screening methodologies benefit from the development of faster and more efficient ways to 'test' candidate reagents when presented to a given 'target'. DNA and RNA based affinity reagent selection systems like the evolution of single-stranded DNA or RNA aptamers, by SELEX (Systematic Evolution of Ligands by Exponential enrichment), select for nucleic acids with a tertiary structure that bind specifically to analytes (46–48). For display technologies, affinity reagents often are antibodies, antibody fragments, peptides and even DNA and RNA. While DNA (49) and RNA (50) based affinity reagents do not require a host, antibody and peptide based affinity reagents require a system to express or display prior to selection. These include ribosome and mRNA-display (51), yeast-display (52), bacterial-display (53) and phage-display (54), where antibodies, and fragments of antibodies (scFv, Fab, $F(ab)_2$) are expressed on the surface of cells or, as in phage-display, where they are synthesized on the envelope of the viral phage (55). Ribosome and mRNA-display, as *in vitro* display techniques, are capable of selecting extremely high affinity reagents by incorporating mutations in each round of selection (56). Phage-display requires a secondary mutagenic library to be constructed after the initial rounds of selection from the naïve library, to obtain the highest affinity reagents (57). For the sake of brevity, this review will focus on the utility of scFv antibodies that have been generated by phage-display.

Phage-display

In phage-display, antibody fragments, cDNA segments (complementary DNA), or combinatorial peptides are expressed as fusions to a capsid protein present on the surface of viral particles. While bacteriophage M13 is the most commonly used vector for phage-display, λ, T7, retrovirus, and baculovirus have also been used. Phage-display offers the following conveniences: [1] the peptide or proteins, which are expressed on the surface of the viral particles, are accessible for interactions with their targets; [2] the recombinant viral particles are stable; [3] the viruses can be amplified, and [4] each viral particle contains the DNA encoding the recombinant library member on the surface of that viral particle, thereby providing a physical linkage between the genotype and phenotype. Thus, phage libraries are conveniently screened by affinity selecting viral particles that bind to targets, propagating the recovered phage particles, and sequencing the DNA inserts of clonal isolates. Usually three rounds of selection are sufficient to screen a phage-display library, with binding clones confirmed in an enzyme-linked immunosorbent assay (ELISA) (58).

Typically, scFvs are displayed as fusions to the N-termini of coat proteins pIII or pVIII of bacteriophage M13. Phage particles are composed of circular, single-stranded DNA, which is surrounded by a cylinder of coat proteins (Fig. 3). Most of the viral capsid consists of the major coat protein pVIII, of which there are ~2,700 copies per phage. At one end of the phage particle, there are five copies each of pIII and pVI that are involved in host-cell binding and in the termination of the assembly process, respectively, whereas the other end contains five copies each of pVII and pIX, which are required for the initiation of assembly and for maintenance of virion stability, respectively (59). Traditionally, both pIII and pVIII have been used to display peptide and antibody fragments. Large, non-immune or 'naïve'

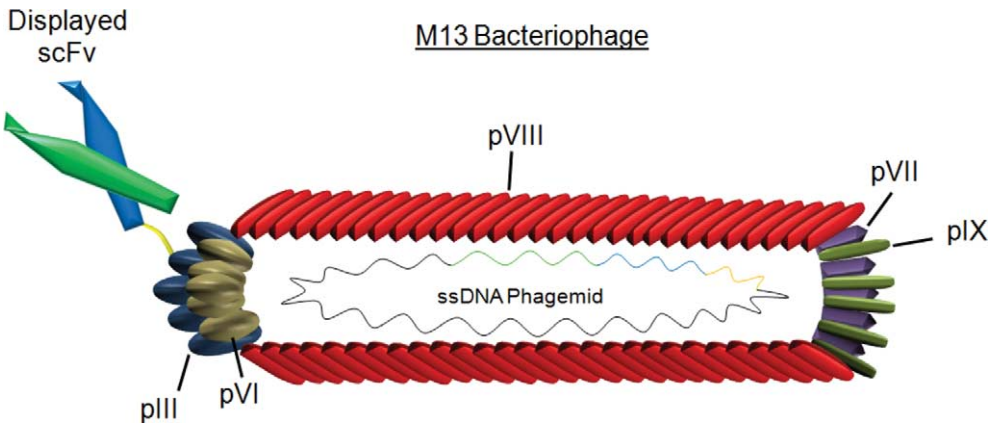

Fig. 3. Phage-display using M13 bacteriophage. As shown here, a phage particle displays an scFv fused to the N-terminus of the minor coat protein, pIII, through a short peptide linker. The major coat protein, pVIII, encapsulates the single-stranded DNA (ssDNA) viral genome that encodes the genes for scFv and pIII. While five molecules of pIII are expressed per phage particle, typically zero or one copy of the scFv is actually displayed. When using a phagemid genome for display of scFvs, proteins pVI, pVII, pVIII, and pIX are supplied by a helper phage genome that is present inside the *Escherichia coli* cell.

antibody libraries displayed on pIII, have proven to be a general method to readily isolate high affinity and specific human antibodies against a variety of target antigens (60, 61). In addition, pVII and pIX can be used to display V_H and V_L domains, respectively; this form of heterodimeric presentation yields a viable fragment of variation (Fv) with fully functional binding and catalytic activities (62). It is also possible to display human scFvs (63) and Fabs (64) fused to the N-terminus of pIX.

Phage-display selection procedures

The key procedural advantage of phage-display is the linkage between an antibody's genotype (DNA sequence encapsulated in the phage particle) to its phenotype (specificity and affinity of the phage-displayed antibody). The selection procedure is fast and operates on sequential rounds of display and enrichment of target specific antibody binders (Fig. 1d). Three rounds of selection are sufficient to isolate and amplify the strongest binders while removing non-specific, non-binding clones. The first round of selection is most important, as the entire library of affinity reagents is introduced to the target. A subset of target specific binders stay bound to their respective epitopes while the majority of the phage library containing nonspecific binders is washed away. Recovered phage particles are then eluted to infect bacteria, which are amplified in selective media. Elution of specific phage can be performed different ways, with extreme pH (i.e., pH 2 or 12) and protease cleavage (i.e., trypsin) (65, 66) being the most common. It is also possible to introduce a protease cleavage site in the target (67) so only target-bound phage particles are released. With an intact target and naïve library in hand, three rounds of selection can be carried out in a little over 1 week.

Target presentation is critical to finding the best binder in a library. Selection methods fall into two categories of antigen presentation; solid support and in solution. Solid phase selections, or biopanning, immbolize the target to a solid support, for example, by adsorption to the well of a plate or the attachement to a column (68). In-solution selections depend upon affixing the target to a freely moving particle in solution, like paramagnetic beads or live cells (69, 70). Protein and non-protein targets can be biotinylated *in vitro* or *in vivo* for presentation on neutravidin or streptavidin coated beads or plates (71). Specific enzymatic biotinylation *in vivo*, through the use of the AviTag, is a common strategy for optimal target presentation (72, 73).

Affinity maturation using phage-display

It is important to note that many antibody properties (specificity, thermostability, protein expression, etc.) can also be selected through variant or affinity matured library generation, although increased affinity is often the initial primary characteristic. The affinity of antibodies generated by animal immunization generally ranges between 50 nM and 100 pM (74, 75). However, *via* directed evolution, it is now possible to lower antibody binding constants to the low picomolar and mid-femtomolar range (20). The affinity of an antibody isolated from an initial screen can be increased two to three orders of magnitude to that of the original sequence using affinity maturation techniques based on display technology (76–79).

The focus of recombinant antibody affinity maturation strategy involves pairing optimum heavy and light chain variable regions. Display technology allows one to quickly create large libraries of mutant clones by PCR (random or directed). These mutated sequences are then displayed on filamentous phage and screened for improved binding. Recently, the size of a matured library has been correlated with the optimum K_d of the best

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binder from that library (80). Two types of antibody diversification techniques predominate; chain shuffling and mutagenesis. Chain shuffling involves fixing the DNA sequence of one of the two chains (VH or VL) and combining with a library, or repertoire, of complementary chains to yield a library from which an optimum chain combination can then be selected from. Chain shuffling can be performed using a fixed V_H or V_L, although a fixed V_H is often shuffled with a library of V_Ls due to the fact that the V_H of an antibody contributes the bulk of the binding energy (81). Through chain shuffling, large improvements in affinity have been observed (82–84). Mutagenesis of the antibody DNA sequence yields mutant antibody variants, which when displayed on phage, can be selected for depending on the desired characteristic (increased affinity, thermostability (85, 86), increased expression, etc.). This is commonly achieved by error-prone PCR (87) or through the misincorporation of nucleotides (88).

From an affinity standpoint, this kind of approach can be used to select for mutations across the entire length of the scFv (not just the crucial CDR's) that enhance the affinity of an antibody for its antigen. This increased affinity may result from the relief of steric hindrance, the promotion of a more compact structure, etc. Systematically directing the mutagenesis towards the variable regions (CDR's) to increase an antibody's affinity is also an option (89). Direct mutagenesis, in conjunction with alanine-scanning and epitope modeling, can help pinpoint the residues most critical for antigen binding.

Examples of phage-display derived therapeutic antibodies

Since emerging in the early 1990s (90), phage-display has proven to be a powerful tool for therapeutic antibody development. There are several examples of phage-display derived antibodies currently used as therapeutics. The US Food and Drug Administration (FDA) has thus far approved two and, as of 2010, there are 25 therapeutic antibodies in clinical trials for human therapeutic use. It is interesting to note that of the 131 monoclonal antibodies developed for therapeutic use between 2001 and 2008, nearly 27% were phage-display derived recombinants (91, 92).

Adalimumab (Humira®, Abbott Laboratories) (93) is the first therapeutic antibody derived from phage-display technology. Approved by the FDA for rheumatoid arthritis in 2003, it is now poised to become 2012s best selling drug in the world (94). This human antibody antagonizes the pro-inflammatory activity of the cytokine, tumor necrosis factor alpha (TNF-α). Adalimumab binds specifically, and with high-affinity (K_d=100 pM), to TNF-α and neutralizes its activity (IC_{50}=130 pM) (95). Adalimumab was created through phage-display, using guided selection from a mouse monoclonal antibody. This method involves transitioning the mouse sequences to human *via* various chimeric forms (96, 97). The heavy and light variable chains of the mouse antibody were converted to scFv format and split to pair with a complementary human light or heavy variable region. These human heavy and light variable chain sequences were then combined and continually selected for with TNF-α. After transitioning the scFv to an IgG, Adalimumab exhibits low immunogenicity and has a serum half-life of 10–20 days (98). Currently, Adalimumab is prescribed to treat rheumatoid arthritis, Crohn's disease, psoriasis, arthritis and ankylosing spondylitis.

The second therapeutic to be generated from a phage-display library of scFvs is the monoclonal antibody Belimumab (Benlysta®), a B-lymphocyte stimulator (BLyS) inhibitor (99, 100). Belimumab inhibits the function of BLyS, tumor necrosis factor superfamily member 13b (TALL1), which is a cytokine known to regulate B cell proliferation and differentiation, by binding to its three receptors TACI, BCMA, and BAFFR (101). Inhibition of this cytokine cell signaling causes an increased number of autoreactive B-cells to undergo programmed cell death, thereby reducing the formation of auto-antibodies and providing relief for patients suffering from autoimmune diseases (102). Recent FDA approval of Belimumab for the treatment of Systemic Lupus Erythematosus (SLE) is the first new therapy for this autoimmune disease in 56 years (103). Phase III clinical trials of the drug showed efficacy and safety by reducing SLE disease activity and, in some cases, decreased the frequency of flares, as compared to placebo (104). The antibody, known as LymphoStatB, was selected through a screen of a naïve phage-displayed scFv library with a diversity of 10^{10}–10^{11}. Affinity maturation, using random mutagenesis by PCR, and then subsequent conversion into a full IgG, yielded an antibody that bound tightly to and inhibited BLyS in an immobilized assay, with an effective concentration at half maximum (EC_{50}) value of 0.02 nM and, an in solution inhibitory concentration at half maximum (IC_{50}) value of 8 nM. In addition, cross reactivity with TNF-α or any other closely related protein was minimal. The specificity, binding strength, long half-life, and cytokine neutralizing ability of the antibody lend to a potentially successful therapeutic for many autoimmune disorders (105).

Given the contributions to therapeutic antibody development over its short history, phage-display has been shown to be a future source of therapeutic affinity reagents. Phage-display offers the advantage of working with human antibody sequences, as well as a quick, yet powerful tool to affinity mature first generation binders, and thus has become an accepted source for human antibody therapeutics.

Microarray technology

Microarray ('chip') technologies were commercialized in the mid to late 1990s by companies (106, 107) for the detection and quantitation of disease and biomarker genes. These nucleic acid-based micro- and nano-scale arrays allow researchers to quickly and quantitatively measure the entire expression profile of a disease state, or a targeted subset of fully customizable target genes. Due to issues like reagent stability and advances in DNA synthesis technology, nucleic acid-based arrays have matured faster than their protein-based counterparts. Like nucleic acid-based arrays, protein- or antibody-based arrays hold the promise of sensing targets of interest on a systems level, but are inherently more complex due to the increased variability of the proteome compared to the genome. Nevertheless, antibody-based arrays have the exciting potential to further identify biomarkers and aide in diagnosing disease (108, 109).

Nanostructured biosensor platform technologies

In the second half of this review, we highlight several recent publications that utilize engineered antibodies in nanostructured sensors to detect biomarkers of disease or injury (Table 1, Fig. 4). However, as both the fields of nanotechnology and synthetic antibody generation are relatively new, there have not been enough studies to completely adhere to these criteria. Therefore, when necessary, we have discussed nanotechnologies that have not yet implemented engineered antibodies or been used to detected biomarkers of disease, but show an obvious connection to this end. We also describe some novel micro-scale technologies that have been adopted for sensing of protein biomarkers (Fig. 5). In the past, biomarkers were detected by ELISA (110), Western blotting (111), and mass spectrometry (112), which are labor intensive and time consuming techniques. Nanostructured platforms offer

Table 1. Nanotechnologies used to detect protein biomarkers of disease. A list of published nanotechnologies and signal outputs that use monoclonal or recombinant antibodies to detect biomarkers in solution or in tissue

Technology	Antibody type	Signal output	Analyte	Detection details	References
SWCNT	IgG	Electrical conductivity	Chromogranin A (CgA)	100 pM–1 nM (5 ng/mL–50 ng/mL)	(123)
Ni coated SWCNT	scFv	Electrical conductivity	Carcinoembryonic antigen (CEA)	10 ng/mL	(122)
CNT	scFv	Electrical conductivity	Osteopontin (OPN)	1 pg/mL	(124)
SWCNT forest	IgG	Electrochemical redox	Interleukin-6 (IL-6)	30 pg/mL	(115)
MWCNT-HRP	IgG	Electrochemical redox	Interleukin-6 (IL-6)	0.5 pg/mL	(117)
Gold nanosphere	IgG	SERS	Carcinoembryonic antigen (CEA)	1 pg/mL–100 ng/mL	(135)
Gold nanosphere	IgG	SERS	Angiogenin (ANG)	0.1 pg/mL	(136)
Gold nanosphere	IgG	SERS	Alpha-fetoprotein (AFP)	1.0 pg/mL	(136)
Gold nanoparticle	scFv	SERS	Endothelial growth factor receptor (EGFR)	30 mm^3 tumor detection	(138)
Gold nanoshell	Fab	SERS	Calcium Channel Voltage-Dependent α-1F (CACNA1F)	~20 ng (10 ng/μL)	(137)
QCM	IgG	Δ Resonance frequency	Prostate Specific Antigen (PSA)	290 pg/mL in serum	(157)
QCM	scFv	Δ Resonance frequency	Cytochrome P450 1B1 (CYP1B1)	130 ng/mL	(160)
CdSe/ZnS quantum dot	scFv	Photoluminescence	Human Epidermal growth factor Receptor 2 (HER2)	Imaged breast cancer cell line MCF7	(150)
CdSe/ZnS quantum dot	scFv	Photoluminescence	Prostate Specific Cancer Antigen (PSCA)	Imaged breast cancer cell line MCF7	(150)
CdSe/ZnS quantum dot	SdAb	Photoluminescence	Endothelial growth factor receptor (EGFR)	Imaged breast cancer cell line SK-BR3	(154)
Quantum dot	scFv	Diffuse Fluorescence Tomography (DFT)	Human Epidermal growth factor Receptor 2 (HER2)	Whole-body tumor imaging	(155)
Silicon nanowires	IgG	Electrical conductivity	PSA; CEA; Mucin-1	75 fg/mL (2 fM); 100 fg/mL (0.55 fM), 75 fg/mL (0.49 fM)	(144)
Si_3N_4 microcantilever	IgG	Cantilever deflection	PSA	200 pg/mL–60 μg/ml in serum	(168)
Microcantilever	IgG	Resonance frequency	PSA	10 pg/mL	(169)
Microcantilever	scFv	Cantilever deflection	Peptide	20 ng/mL (1 nM)	(26)
Microcantilever	Fab	Cantilever deflection	HER2	2 ng/mL	(170)

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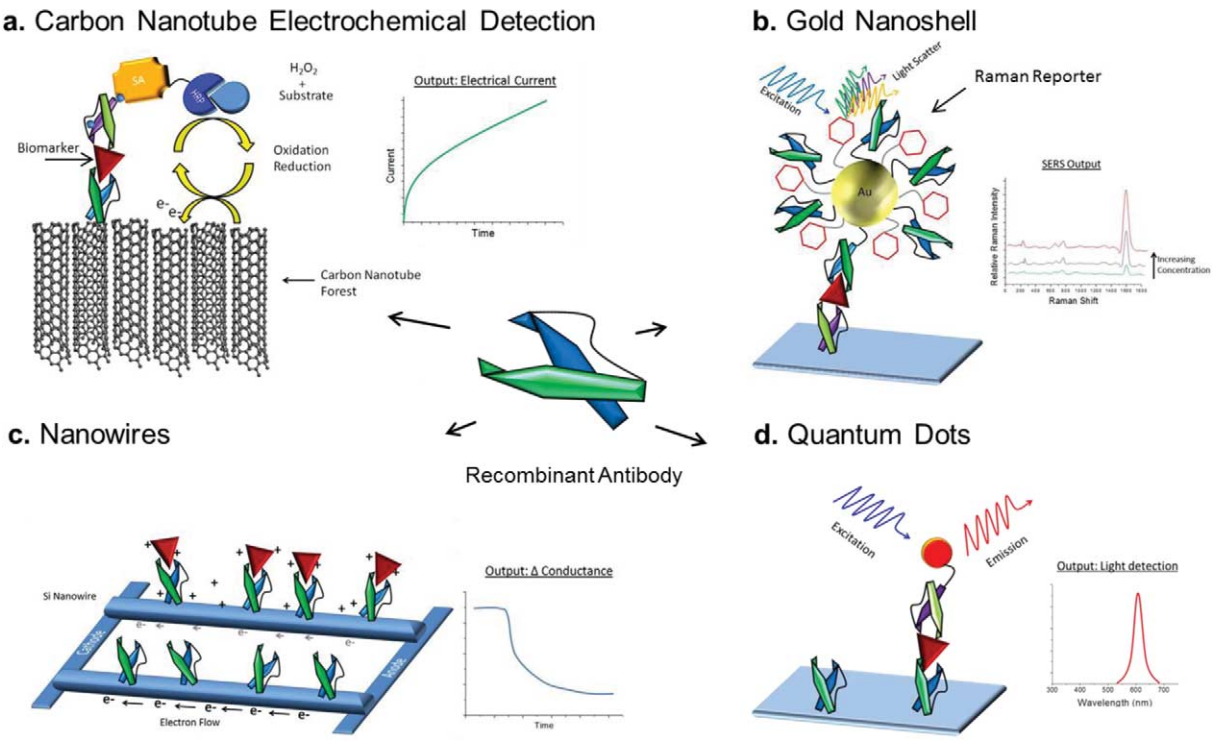

Fig. 4. Engineered antibodies coupled by nanotechnology for biosensing. Four different assay formats and readouts (insets) are shown here. (a) Carbon nanotubes in electrochemical detection: sandwich assay followed by oxidation of substrate by Horseradish peroxidase (HRP) results in an electrical current. (b) Gold nanoshell: changes in peak intensity in a Raman spectrum are detected when an antigen binds. (c) Nanowires: changes in current, upon analyte binding, are detected in a label-free manner. (d) Quantum dots: light output is detected at specific emission wavelengths.

the promise of simpler, quicker, and more sensitive assays. As nanostructures become cheaper and easier to fabricate, we anticipate the fields of biology and nanomaterials coming together to yield handheld portable devices for point-of-care diagnostics.

Carbon nanotubes

Carbon nanotubes (CNTs) (Fig. 4a) are hollow cylindrical nanostructures made up of carbons in a hexagonal construction, which have electrically and structurally interesting properties. They can be either single or multi-walled, with diameters between 0.3 and 100 nm (113). Carbon nanotubes can be utilized in two ways: one is through usage as an electrode in an oxidation-reduction electrochemical reaction, and the other as a Field Effect Transistor (FET), sensitive to changes in charge on the surface of the nanostructure.

First, we will discuss carbon nanotubes used in a system producing an electrochemical signal. This is assumed to be the most specific because of the requirement for dual antibody recognition of an antigen to generate a signal. An antigen can be detected in an immunosandwich assay (Fig. 6c), where the capture antibody is first immobilized to the surface of the CNT. The antigen is then added and allowed to bind to the capture antibody. The detection antibody, conjugated to an HRP (Horseradish Peroxidase) enzyme, recognizes a secondary epitope of the antigen. Upon addition of a HRP substrate, an electrochemical redox reaction occurs to generate an electrical signal that is proportional to the amount of bound antigen (114).

Many current studies are using nanotube 'forests' (Fig. 6). These 'forests' are typically single-walled carbon nanotubes (SWCNT) arrayed in a vertical manner that are electrically conductive and allow for high-density antibody attachment and increased sensitivity. In one study (115), capture antibodies (Fig. 6b) are chemically attached to carbon nanotube forests (Fig. 6a) through primary amine coupling. In an example of bionanotechnology, Interleukin-6 (IL-6), a biomarker for Head and Neck Squamous Cell Carcinoma as well as for other cancers that cause inflammation, can be detected at lower limits of 30 pg/mL in serum. While patient blood analysis would be the eventual application for this nanotechnology, the ability to sensitively detect the tumor biomarker in serum demonstrates its relevance to potential usage in medical diagnostics. (Fetal calf serum is often used to simulate the assay conditions of a highly complex sample.) To be biologically relevant, the detection limit must be lowered at least 2-fold to the diseased target concentration of 19.5 pg/mL, and even lower for normal levels present at less than 6 pg/mL (116). To increase

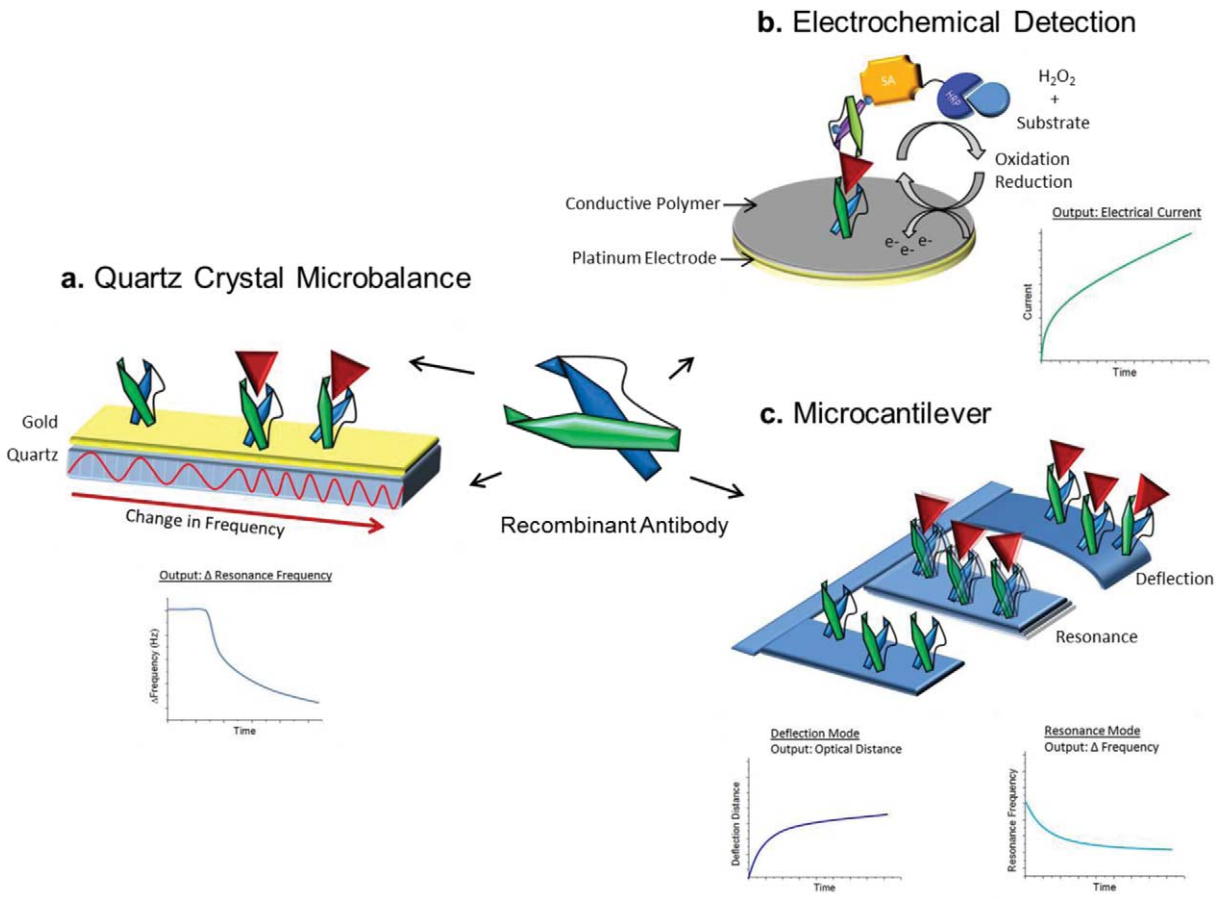

Fig. 5. Microscale technology biosensors. (a) Quartz crystal microbalance (QCM): changes in resonance frequency are detected in a label-free manner, upon analyte binding. (b) Electrochemical detection: platinum electrodes carry changes in current due to the oxidation–reduction reaction of HRP and its substrate. (c) Microcantilever: In Deflection mode, changes in bending of the cantilever are measured, whereas in Resonance mode, changes in resonance frequency are detected.

sensitivity, the same research group attached a multi-walled carbon nanotube (MWCNT), containing over 100 HRP molecules (Fig. 6d), to the detection antibody that provides amplification through current generation (Fig. 6e). With this enhanced system, the authors lowered the detection limit below healthy levels, with a 60-fold increase in sensitivity to 0.5 pg/mL, in fetal calf serum (117). This assay can be improved by multiplexing, so that a panel of biomarkers can be detected in a single sample, thereby increasing the test's diagnostic confidence. For diagnosing prostate cancer, four relevant biomarkers (PSA, PSMA, IL-6, and PF-4) can be tested with nanotube forests arrayed across four individual electrodes (118).

Carbon nanotubes can also be used as FETs (Fig. 7), where binding of an analyte to an anchored antibody causes a change in the environment near the surface of the nanotube, thereby altering the electrical conductance (119, 120). CNT-FET is expedient because only one antibody is required to capture the antigen and report a signal, as opposed to the dual antibody system described above. This was first shown in biomarker detection using an IgG against the tumor marker, Carcinoembryonic antigen (CEA) (121). Later, engineered scFv antibodies were immobilized on Nickel nanoparticle coated nanotubes through the polyhistidine–Ni interaction. This resulted in a highly dense and orientation specific attachment and a subsequent 10-fold decrease in the detection limit of CEA (i.e. 10 ng/mL) (122).

In another study (123), an IgG antibody against the neurodegenerative disease and neuroendocrine tumor biomarker, Chromogranin A (CgA), is attached to a 2 nm thick single-walled carbon nanotube, through a 1-pyrenebutanoic acid succinimidyl ester linkage. Here, charge transfer from molecules adsorbing or binding to the nanotube surface causes a detectable change in resistivity. Because this biomarker protein is negatively charged at neutral pH, the binding of it near the nanotube causes an increase in electrical conductivity. The experimenters were able to detect 100 pM to 1 nM CgA, in a complex solution of fetal calf serum.

Finally, in a recent report implementing recombinant antibodies, a research group attached an anti-Osteopontin scFv antibody to a carbon nanotube FET

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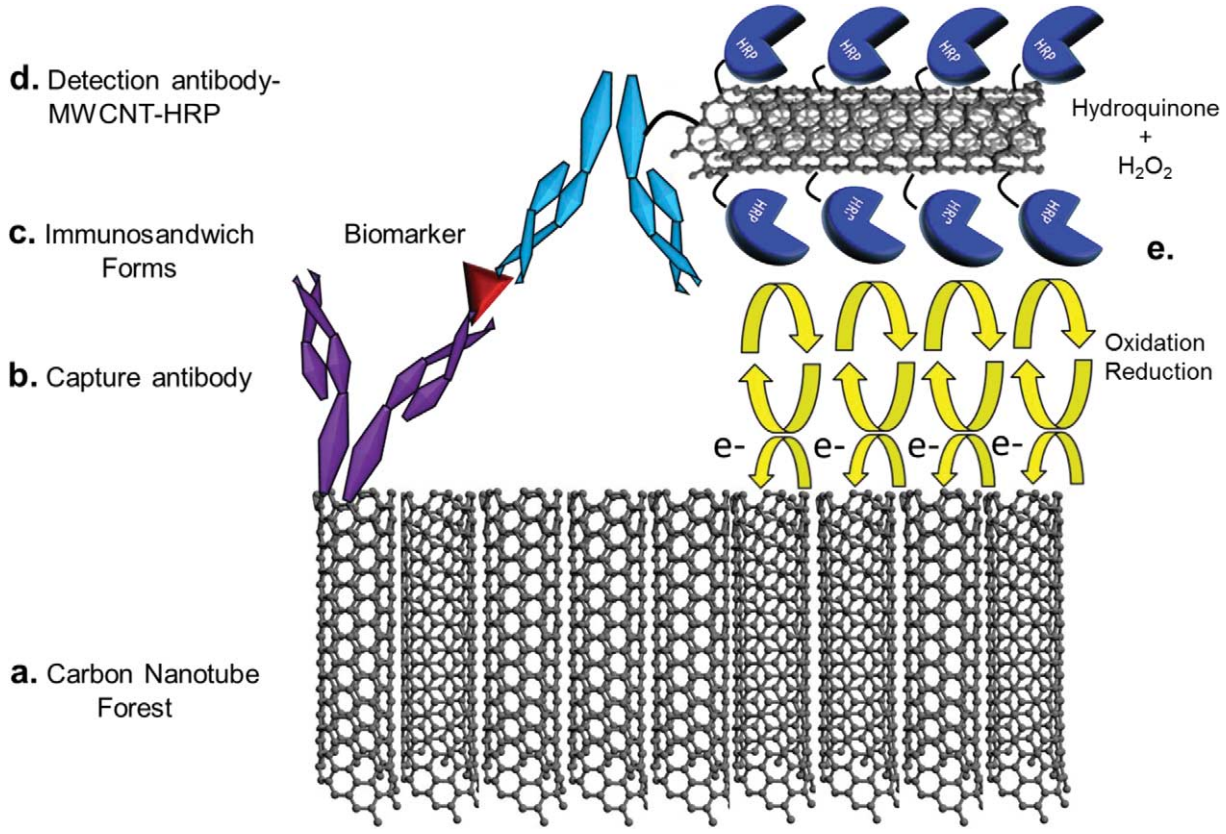

Fig. 6. Nanotube forest using multi-HRP carbon nanotube conjugated detection antibody for signal amplification. A capture antibody (b) is linked to a carbon nanotube 'forest' (a), through coupling by 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide and *N*-hydroxysulfosuccinimide (EDC–NHSS) chemistry, to create an immunosensor. The sample containing the biomarker protein is then incubated with the immunosensor, and bound antigen is then detected with a second antibody (d) is attached to a multi-walled carbon nanotube in parallel with over 100 HRP molecules and incubated with the complex forming an immunosandwich (c). The sensor is then immersed in buffer with (e) hydroquinone and hydrogen peroxide where an oxidation of hydroquinone occurs. Upon application of voltage, an amperiometric response is recorded.

(124). Osteopontin is a biomarker for tumor progression and an indicator of patient survival for many different cancers (125). Using the scFv-decorated carbon nanotube as a FET, the biomarker could be detected at physiologically relevant concentrations of 1 pg/mL.

Gold nanoshells, nanoparticles, and SERS

When light strikes a surface, the majority of photons are elastically scattered at the same energy of the incident photons (photons before striking the surface). However, a small number are scattered at a different energy. This inelastic scattering is known as Raman scattering (126–128). Fleischman et al., and others (129–131) discovered that pyridine, adsorbed to a roughened silver electrode surface, greatly enhanced the scattering effect. This was termed 'Surface Enhanced Raman Scattering' (SERS). Gold nanoshells (Fig. 4b) have been utilized as a scatter enhancing substrate for the attachment of antibodies and detection of binding events (132, 133). With inclusion of an attached molecule with a characteristic vibrational signal known as a Raman reporter, 10–100 different antibody–antigen interactions can potentially be monitored simultaneously, as the Raman vibrations of reporter molecules are narrow and do not overlap (134).

Using these principles, the tumor biomarker CEA was detected in an immunosandwich complex formed between a polyclonal antibody immobilized on a gold nanosphere and a monoclonal antibody attached to a magnetic particle. In this study (135), the gold nanosphere contained a Raman reporter, 4,4′-dipyridyl (DP), as well as the anti-CEA polyclonal IgG, which was attached through a primary amine linkage (Fig. 8a). A monoclonal antibody was attached to the magnetic particle in the same manner (Fig. 8b). Since both sets of antibodies are in solution, the constraint of a slow diffusion across a solid surface is eliminated. Upon mixing the CEA antigen with the antibody-linked particles, a concentration dependent shift of the Raman spectra peak intensity is observed (Fig. 8c and d). With the assay taking less than an hour to complete, a linear response can be detected in an antigen range of 1–100 pg/mL.

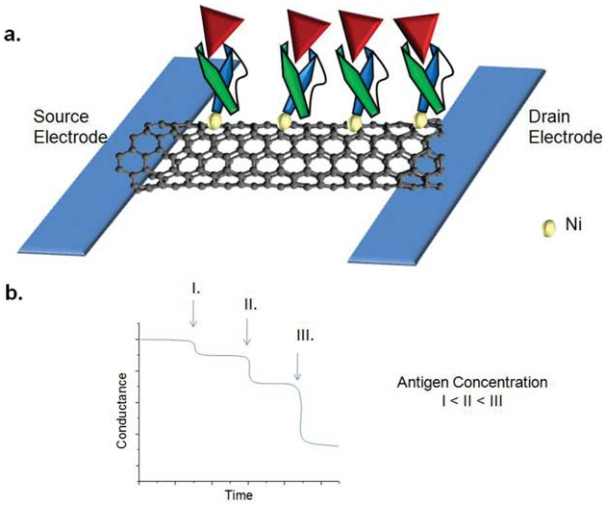

Fig. 7. Carbon nanotubes as a field effect transistor. (a) Single-walled carbon nanotubes (CNT) are connected, using photolithography, between source and drain electrodes. Nanotubes are then decorated with Nickel nanoparticles that have captured scFv molecules through the His_6 tag present at their C-termini. Upon addition of antigen to this nanofabricated structure, a change in the electrical conductance of the CNT is detected. (b) As antigen is added at increasing concentration (I–III), the conductance decreases over time.

In another report (136) showing the greatly enhanced sensitivity of nanostructures over traditional immunoassays, a monoclonal antibody was immobilized onto a gold electrode arrayed surface, while a polyclonal was attached to the nanosphere along with the Raman reporter Malachite Green isothiocyanate. Here, an attempt was made to detect a tumor growth biomarker, Angiogenin (ANG), as well as alpha-fetoprotein (AFP), a biomarker of hepatocellular carcinoma. When compared to standard ELISA, the SERS gold nanoshell immunoassay was reproducibly 1,000 times more sensitive in detecting the ANG at 0.1 pg/mL and 10,000 times more sensitive detecting the AFP at 1.0 pg/mL. This technique also provides a wider dynamic range than ELISA.

In a recent publication (137), recombinant antibodies of biomarkers for retinal injury have also been attached to nanoshells containing Raman reporters. Recombinant Fab antibodies selected against synthesized peptides of the photoreceptor cell specific calcium-ion channel protein, CACNA1F, were able to detect a response in 200 ng of total retinal lysate protein spotted on a nitrocellulose membrane, in a 'half sandwich' assay. The authors note that the CACNA1F protein composes less than 1% of the total protein and, therefore, the system is at a detection limit of $\sim$2 ng.

In a final example showing the application in the study of living systems, Qian et al. (138) used pegylated gold nanoparticles decorated with scFvs against the tumor biomarker epidermal growth factor receptor (EGFR), to detect the biomarker expressed on the surface of human carcinoma cells in culture. Going further, when injected into mice, the nanoparticle-linked scFvs were shown to accumulate at the site of a xenografted tumor (human head and neck squamous cell carcinoma overexpressing EGFR) and subsequently allowed SERS detection through the skin of the animal. These results demonstrate the low toxicity and compatibility of the gold nanoparticles in living systems, as well as the tumor targeting capacity of the scFv *in vivo*.

Nanowires

A nanowire (Fig. 4c) is a wire-like nanostructure that has a diameter in nanometers, but a length in micro to millimeters. These structures are considered one-dimensional because they have a length to width ratio of more than 1,000 and are confined in two dimensions (139). Nanowires can be made of conducting, insulating, or semi-conducting metals or metal alloys, but because these nanowires are so small in size, they have different physical properties than a wire of the same composition on the macro-scale (140). Silicon, for example, is generally used in the microchip industry as a semiconductor material. However, when silicon is grown into a nanowire, it can become an excellent conductor of electricity (141). Since the nanowires are so small in diameter, there are very few channels for an electron to travel as it moves through the material. The electron travels in a wave and has a quantum dimension known as the de Broglie wavelength of the electron. If a material has a dimension that is the same size or smaller than the de Broglie wavelength, then the electron cannot travel through and thus is confined to move through the other two dimensions. However, if two dimensions are confined, then the electron can travel in only one direction through the material. This confinement is what allows detection of changes in conductivity when charged molecules bind to the surface, altering the flow of electrons through the nanowire (142, 143).

Although nanowires have been used to detect various types of biological molecules, no one has yet implemented recombinant or engineered antibodies. However, it is easy to imagine the replacement of a traditional immunoglobulin with an antibody fragment, as we have seen previously in other nanostructured sensors. As a biosensor, Zheng and co-workers (144) use an array of nanowires to detect low concentrations of three cancer biomarker proteins. They do this by attaching monoclonal antibodies to electrically addressable nanowires and then recording any changes in conductance. They first coated silicon nanowires with Aldehyde Propyltrimethoxysilane (APTMS). Monoclonal antibodies raised against cancer biomarkers were applied to the surface by contact printing, where the aldehyde in the

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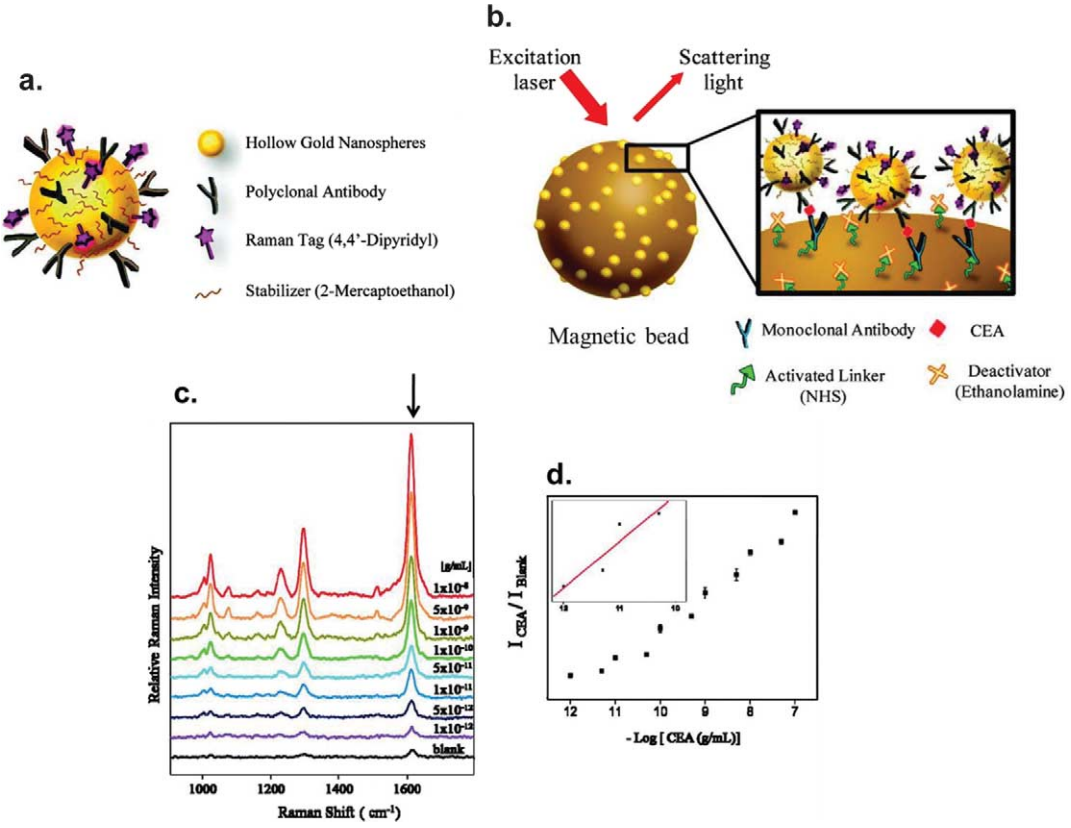

Fig. 8. SERS immunosandwich assay. (a) The gold nanoshell is functionalized with the anti-Carcinoembryonic antigen (CEA) polyclonal antibody along with the Raman reporter tag DP. (b) The larger magnetic bead is functionalized with a monoclonal antibody against the CEA antigen through a primary amine, by reacting them with *N*-hydroxysuccinimidyl (NHS). An immunosandwich forms when the polyclonal antibody from the nanoshell and the monoclonal antibody from the magnetic bead recognize the same CEA molecule simultaneously. When the complex is excited with a laser, a characteristic scattering spectrum is obtained. (c) Several surface enhanced Raman scattering (SERS) spectra are shown, with the wavelength along the *x*-axis and the relative intensity along the *y*-axis. The intensity shift is measured at the peak wavelength of 1612 cm^{-1} (arrow). The bottom black spectrum is the blank with no antigen, and, therefore, no immunosandwich forms. Moving from bottom to top shows the spectra at increasing concentrations of the CEA antigen and subsequent increases in number of immunosandwich formation that is detected by the increasing intensity at 1612 cm^{-1}. (d) The CEA concentration is plotted versus the intensity of the signal at 1612 cm^{-1}. The increase in intensity is concentration dependent.
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APTMS forms bonds to free amines provided by lysine residues of the protein. This device, with less than 1 cm^2 area, was fabricated to include 200 nanowires. Potentially a different monoclonal antibody can be attached to each nanowire. A microfluidic channel running perpendicular to the array delivers sample to the nanowires. P-type or Positive-type (Boron Doped) silicon nanowires will give an increase in conductance when the antibody recognizes a negatively charged protein because a negative charge causes an accumulation of electrons. A positively charged protein binding event will give a decrease in conductance because it depletes the electrons, creating vacancies in the nanowire and therefore an increase in resistance.

To test their ability to detect proteins of disease, varied amounts of the cancer biomarker PSA are flowed over a single P-type nanowire. The change in conductance is proportional to the log of the concentration of the negatively charged PSA and can be detected between an incredibly low 75 fg/mL (~2 fM) and a physiological relevant high of 5 ng/mL (~160 pM). The nanowire array device could also detect low concentrations of other cancer biomarkers, such as the tumor biomarker CEA and the colon cancer marker Mucin-1. The future success of such a device could be furthered with the use of many of the arrayed nanowires to perform assays on panels of biomarkers for a multitude of diseases using a single sample. With this in mind, the authors demonstrate that detection can be done in a multiplexed format amongst high concentrations of unrelated proteins in the form of human serum (144). As these devices continue to advance and become more robust sensing platforms, the application of recombinant antibodies will undoubtedly emerge.

Quantum dots

Semiconductor quantum dots (QDs) (Fig. 4d) have emerged as popular nanostructures for imaging, tracking, and sensing biomarkers in tissues and within cells (145). They are semiconducting nanocrystals with diameters of 1–10 nm, composed of a core and an outer shell layer where a CdSe core and an outer layer of ZnS form one of the most popular such QDs. QDs display unique properties because of the phenomenon termed 'quantum confinement'. When the particle size has a radius smaller than the electron orbit of the semiconductor, confinement occurs in three dimensions. This causes a shift to a higher energy state, releasing a higher energy light emission upon absorption of a photon (reviewed in (146, 147)). The size of the crystal dictates the emission peak and can be tailored to a wide range of wavelengths with narrow emission peaks. This gives QDs an advantage over traditional fluorophores by permitting increased options for multiplexing (148). More importantly, QDs also are more photostable and more resistant to photobleaching than most fluorophores (149). Since QDs have been reported to have difficulty crossing membranes (150), many studies have utilized QDs to probe cell surface targets of cell and tissues (151). However, through the use of peptide translocation domains attached to QDs, internalization into the cell occurs readily (152).

In one interesting study involving these technologies, researchers attached dimers of scFvs, called cys-diabodies, to pegylated QDs. The cys-diabody selected against the breast cancer cell surface antigen HER2 was used with a CdSe/ZnS QD emitting a peak wavelength at 655 nm. A second diabody against the prostate cancer antigen PSCA was coupled to a QD emitting at 800 nm. When combined together, it is now possible to image the two surface antigens simultaneously on cultured tumor cells expressing both PSCA and HER2. (150). An even smaller engineered antibody, the single domain antibody (sdAb)(Fig. 2d) of camelids (36) was utilized in another study. These are heavy chain single domain antibodies with a size half that of an scFv, and dissociation constants in the mid pM to high nM range (153). The sdAbs were engineered to complex with a CdSe/ZnS QD to detect the tumor marker EGFR in breast cancer cells (154).

Recent experiments have been able to identify tumors in whole organism body scans using injected scFv linked QDs (155). An anti-HER2 scFv conjugated to a QD was injected into immunodeficient mice carrying a xenografted human breast cancer. The technique, Diffuse Fluorescence Tomography (DFT), was then used to image the entire mouse (Fig. 9). The scFv specifically targeted the cell surface antigens in the tumor and the brightness of QDs provided a contrasting agent for visualization of the cancer through the skin of the animal using tomography.

Quartz crystal microbalance sensing

Quartz crystal microbalance (QCM) (Fig. 5a) sensing is based on the direct linear relationship between mass accumulated on the surface and a change in the resonance frequency of a piezoelectric crystal, as first described by Sauerbrey (156). Since resonance frequency depends on mass, data recording is performed in real-time, where multiple binding events can be measured in series. Recently, QCMs have been used for detection of biomarkers in a label-free manner. Uludag et al. (157) utilized SAMs to attach monoclonal IgG antibodies, generated against PSA, to the surface of the crystal. They could detect PSA down to 18.8 ng/mL in phosphate buffered saline (PBS), but found that using a second detection antibody brought the detection limit to 4.7 ng/mL. The second antibody created an immunosandwich, which boosted the change in resonance frequency because of the increase in accumulated mass. The sandwich was required when probing a complex solution with high concentrations of proteins, like human serum, to achieve sensitive detection (i.e. 9.4 ng/mL) of PSA. Further, modification of the detection antibody with 40 nm gold nanoparticles again increased the mass, and permitted a 30-fold increase in sensitivity down to 0.29 ng/mL. This sensitive detection in the complex sample shows the relevance of the technique in a more plausible clinical scenario (157).

Shen et al. (158) first showed the possibility of using scFv engineered antibodies, coupled to a gold-coated QCM, to detect a protein in solution. By replacing two amino acid residues with histidines in the linker region of the antibody, they took advantage of the strong and orientation specific interaction that histidine is known to have with gold (159). As a proof of concept, they used a scFv selected against rabbit IgG to detect 350 ng/mL of the antibody in PBS. The same group later translated their method from conceptual to a more medically relevant application by detecting the breast cancer biomarker Cytochrome P450 1B1 (CYP1B1). Here they synthesized peptides 13–14 amino acids in length, which correspond to a region of the CYP1B1 that distinguishes it from other closely related members of the Cytochrome P450 family. ScFv antibodies were then generated from a phage-display library selection against these peptides. These recombinant antibodies also recognize the peptide when it is part of the full-length biomarker. The scFvs were attached to the gold-coated crystal through a biotin–neutravidin linkage, allowing for a high-density packing of the antibody (Fig. 10a). Using cancer cell lysates and breast cancer microsomes, the group was able to detect CYP1B1 down to 130 ng/mL (2.2 nM) *via* the scFv-QCM biosensor (Fig. 10b and c). Finally, they used the QCM biosensor to quantify the protein biomarker in various lysates of cancerous cell lines versus healthy cell lysates,

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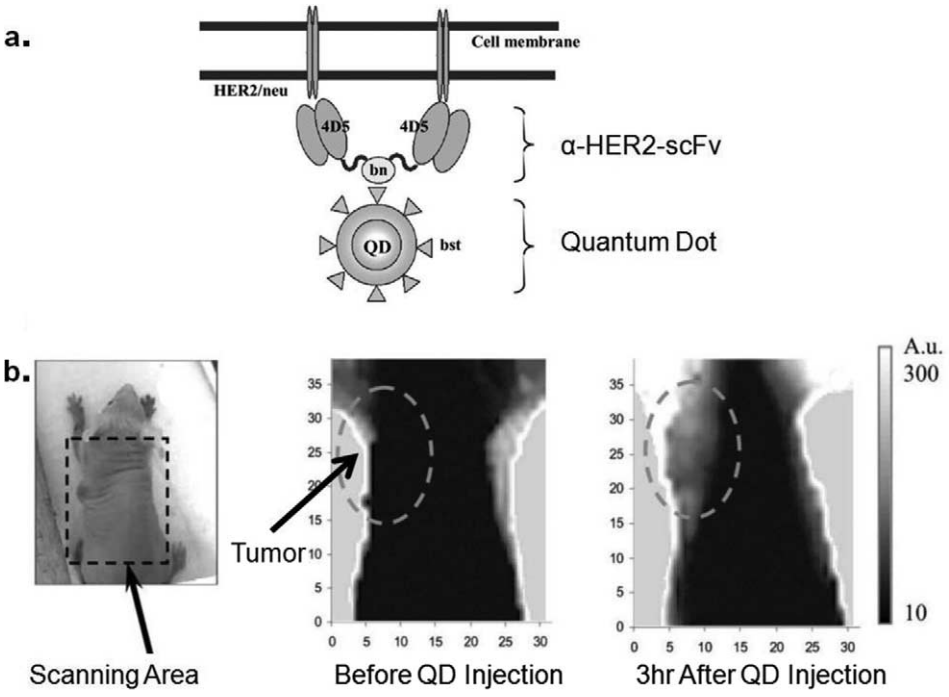

Fig. 9. ***In vivo*** imaging using Quantum Dots (QD) that are conjugated to scFvs. (a) Two anti-HER2-scFvs are conjugated to a 705 nm emitting QD, using the Barnase(bn)–Barstar(bst) system, which is similar in binding strength to biotin–streptavidin. The antibody targets the HER2 (Erb2-Human Epidermal growth factor Receptor 2). (b) A mouse bearing a human breast cancer tumor is imaged using diffuse fluorescence tomography, before and after intravenous injection of 150 pM of scFv-QD conjugates. Signal increase was observed from 0.5 to 9 h after injection. Reproduced with permission from Balalaeva et al., 2012 (155).

demonstrating the diagnostic and disease progression monitoring capabilities of their system (160).

Electrochemical detection

Electrochemistry (Fig. 5b) has been an attractive analytical technique because of the sensitive and wide dynamic range of detection, the ability to adapt into many miniature formats, and in large part to the success of the blood glucose meter, which comprises 85% of biosensor sales (161). These devices either sense a change in voltage (potentiometric) or a change in current (amperiometric) due to binding of antigens within the biosensor. Potentiometric devices sense the production of an ion from a reaction between an enzyme and a substrate, while amperiometric devices detect changes in current due to an oxidation–reduction of an electrochemical reaction. In the case of an immunoassay, usually a two-antibody sandwich recognition system is required. A capture antibody is immobilized to a transducer such as an electrode, conductive polymer, carbon nanotube, or gold nanoshell. The sample containing the antigen is then applied. A second detection antibody specific for a different epitope of the same antigen carries an enzyme that provides an electrical signal upon addition of a substrate (162, 163).

In a commercial application of an electrochemical detection assay, CombiMatrix Corporation (now CustomArray Incorporated), developed a multiplexed sandwich immunoassay that can be performed on thousands of individually addressable platinum electrodes that fit in a square centimeter of a complementary metal oxide semiconductor (CMOS) microchip (164). Antibodies can be specifically attached to a desired location by first synthesizing a unique DNA oligonucleotide directly on an electrode. Hybridization to a unique complementary oligonucleotide conjugated to the capture antibody, immobilizes the antibody to the electrode. After antigen addition, a biotinylated detection antibody is incubated followed by a streptavidin–HRP (SA–HRP). With the addition of an HRP substrate, an oxidation-reduction reaction occurs, creating an electron flow detected by the electrode below the sandwich complex. Using this scheme, the liver inflammation biomarker, α-1-acid glycoprotein (AGP), was detected at a lower detection limit of 5 pg/mL, in a sample volume of 50 μL (161). While this ability for multiplexing and immobilizing of antibody in a parallel manner by a DNA barcode system is advantageous, which has also been used in other platforms in what is known as DNA-encoded antibody libraries (DEAL) (165), the sandwich ELISA scheme requires multiple antibodies for each analyte, followed by an amplification step before reading.

Alternatively, the assay could be designed to utilize only one antibody and no amplification step. In a recent study (162), a sensitive, label-free electrochemical biosensor was

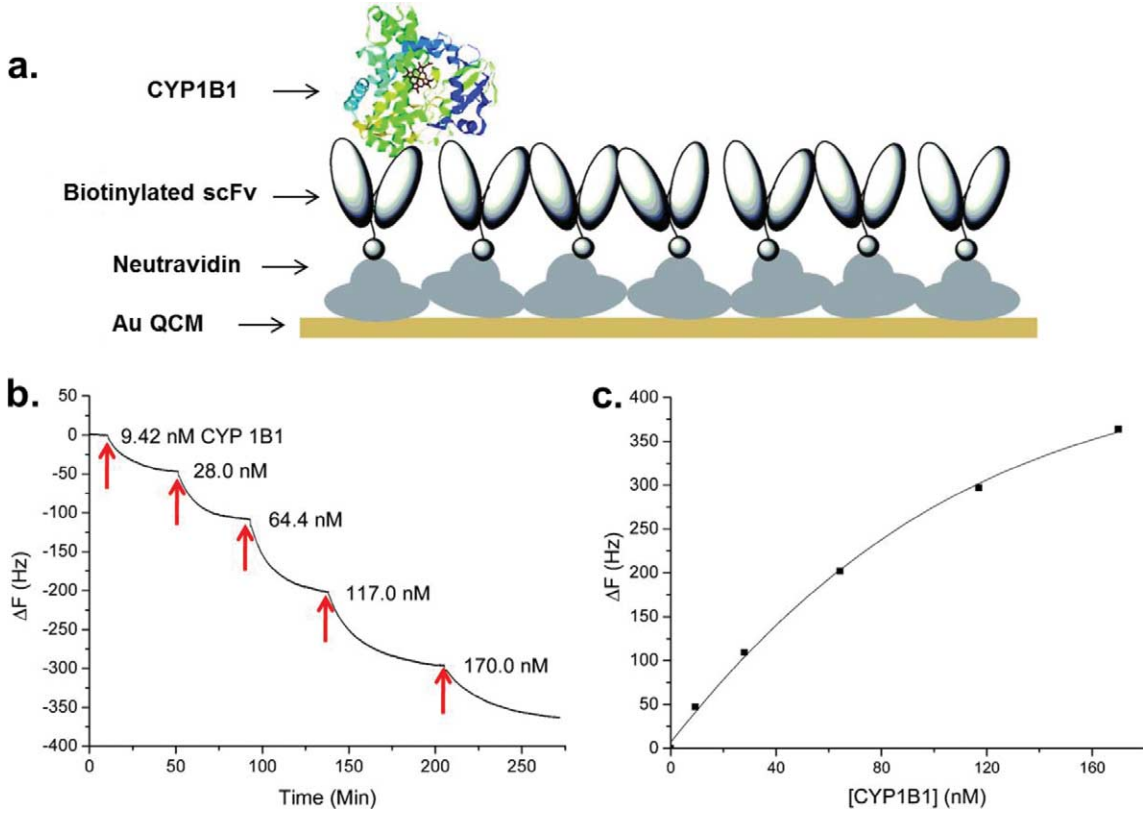

Fig. 10. Detection of Cytochrome P450 1B1 using scFvs on QCM. (a) NeutrAvidin®, an engineered version of avidin, is immobilized on a gold-coated quartz surface (QCM). Chemically biotinylated scFv antibody against CYP1B1 was allowed to tightly pack on the surface. (Orientation is more disordered than as shown.) (b) A dose response is shown with the addition of CYP1B1 (ribbon representation of its three-dimensional structure) to the antibody-immobilized surface. Arrows show addition of different concentrations of antigen over time: the resulting change in frequency can be observed over time, where the greater the concentration of antigen, the greater the change in frequency. (c) The calibration curve is plotted with the change in frequency against concentration of antigen. The curve is concentration dependent.
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developed by first incorporating scFvs onto an electrically conductive polymer, polypyrrole, deposited on a gold electrode. The differential pulse voltammetry (DPV) of electrodes coated with antibody is then recorded and used as a baseline reading. Subsequent binding of an antigen causes a reduction in electrical current from baseline by blocking charge transport of ions to the surface and therefore inhibiting the electron transfer. The decrease in current is proportional to the amount bound to the antibody. Here, they report a linear, concentration dependent response in current from antigen binding at concentrations from 1 pg/mL down to 100 ng/mL.

Microcantilevers

Microcantilevers (Fig. 5c) are micrometer thin projections that are sensitive to changes in surface mass. Immobilization of antibodies to the cantilever surface allows one to probe sample mixtures in two modes. The Resonance mode detects the mechanical bending and, therefore, a change in resonance frequency upon accumulation of material on the cantilever (166). In Deflection mode, the antibody is deposited on one side of the cantilever and upon binding of the analyte, a mechanical stress and physical bending of the cantilever is measured. To perform the deflection measurements, there are optical methods that monitor the interference in a reference laser by the reflection of a laser focused on the surface of a cantilever. In addition, there are piezoresistive methods that measure the change in resistance upon deflection, and there are also microelectronic methods that measure changes in capacitance. In the Resonance mode, the changes in the frequency of natural oscillations of the microcantilever can be measured to detect antibody–antigen recognition events. The most common way to do this is through the use of piezoelectric materials that convert the resonance frequency to an electrical signal whereby changes can be sensitively detected. A more sensitive method is to integrate sealed microfluidic channels into the cantilevers where the sample is passed through. Deposited antibody in the channels

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captures the analyte resulting in a detectable change in frequency (167).

In the very first use of a microcantilever device for the detection of a biomarker, Wu et al. (168) immobilized polyclonal anti-PSA antibodies on gold-coated silicon nitride cantilevers. The method here was to attach the antibodies to one side of the cantilever and record the deflection caused by antibody–antigen binding. In a background of human plasma, the device could detect PSA at concentrations between 60 and 200 pg/mL. More recently, using mechanical resonance frequency shifts of a piezoelectric cantilever coated with antibody, another group was able to detect PSA at concentrations as low as 10 pg/mL (169).

The first application of scFvs to microcantilevers involved the functionalization of anti-peptide scFvs on single-sided, gold-coated silicon (26). With the capability to engineer the antibody fragment through simple molecular biology techniques, a cysteine amino acid was added to the C-terminus for directing the attachment of the antibody fragment to the gold-coated cantilever. By monitoring the deflections of the cantilever in solution for 30–60 min, the device was able to detect the peptide fragment at 20 ng/mL (i.e. 1 nM). Very recently,

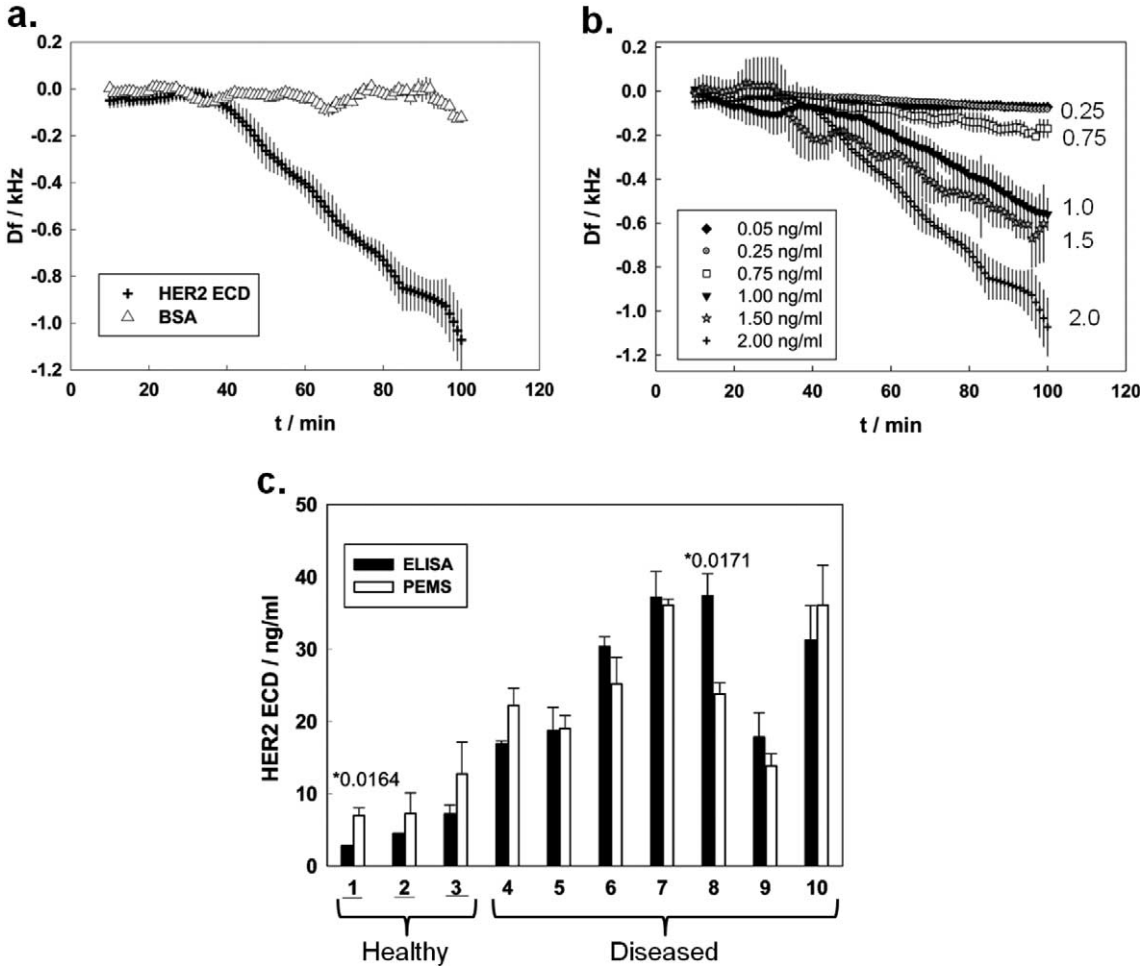

Fig. 11. Detection of breast cancer biomarker using piezoelectric microbalance. The anti-HER2 antibody (trastuzumab, trade name 'Herceptin') is first functionalized to a Piezoelectric Microbalance Sensor (PEMS) through an amine-sulfhydryl linkage, and the sensor is then placed in a flow cell. (a) Serum-spiked recombinant HER2–Extracellular Domain (ECD) is administered to the flow cell, and a change in vibrational frequency (kHz) is shown. Addition of the negative control protein, Bovine Serum Albumin (BSA), shows no change in frequency. (b) Additions of increasing concentration of the HER2–ECD give increasing changes in frequency. The sensitivity is adequate to detect healthy levels in the low nanogram per milliliter range. (c) PEMS detecting concentrations of HER2 in serum from confirmed breast cancer patients is shown in samples 4 through 10, and is also compared to ELISA results in the adjacent bar. Patient samples 1–3 are healthy, negative controls. In general, the PEMS results are consistent with the ELISA data, with the exception of patient #1 and #8 that show significant differences between the two. This was attributed to the prototype apparatus, but the difference in signals between healthy vs. diseased is apparent.
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a group functionalized and attached the Fab, trastuzumab (Herceptin®), to a piezoelectric cantilever, in an attempt to detect the breast cancer marker HER2 (170). In spiked human serum (Fig. 11a and b), detection limits of the trastuzumab sensor were shown to be less than 2 ng/mL, which is within the normal range of concentration of HER2 present in the serum of healthy individuals, and, therefore, is well below the threshold level of disease. In a stunning clinical application of the instrument, the authors used serum from confirmed breast cancer patients to show the assay is capable of distinguishing between healthy controls and diseased patients (Fig. 11c). Through the correct identification of patients with tumors, future diagnosis of breast cancer by this assay looks promising.

Photonic crystal binding assays

Although photonic crystals are relatively new to the arena of nanostructured biosensors, they are worth mentioning briefly (171). These biosensors are label-free, high throughput, and detect protein binding to a nanostructured surface in real-time (172). The photonic crystals are composed of a layer of low refractive index material and a layer of high refractive index dielectric material. This optical resonator allows most wavelengths from a light source to pass, but reflects a narrow range of wavelengths that are collected by a spectrometer and recorded as the peak wavelength value. As proteins accumulate on the surface or bind to molecules deposited on the surface, the wavelengths allowed to pass begin to change, which causes the reflected light to increase in wavelength. This change in the peak wavelength is directly proportional to the adsorbed mass and, therefore, can quantify antibody-antigen binding events. Recently, these principles were used to enhance fluorescence signals by 1,000-fold or more from traditional fluorophore conjugated antibodies in protein microarrays. Eighteen cancer biomarkers were detected in the array with lower limits nearing the single pg/mL range (173). As the sensitivity of these biosensors improves, the potential for its application in medical diagnostics will become more apparent and practical.

Conclusion

Employing the powerful technology of phage-displayed library selection to obtain specific antibodies for the detection of biomarkers, combined with the extremely small size, multiplexing ability, and high sensitivity of nanostructures, one can imagine the impact of on the spot diagnosis of disease and injury, on public health. These antibody-coupled nanodevices have many advantages over traditional immunoassays of ELISA or Western blotting. The small size of the devices, and the ability to integrate nanomaterials and microelectronics into microfluidic sample delivery systems, greatly simplifies the assay, making it convenient, sensitive, robust, and low-cost. Furthermore, many of the laborious and time consuming steps of traditional immunoassays can be replaced with a nanodevice, which would require a minimum amount of sample preparation and processing time, thereby yielding a rapid, yet accurate, result. Many devices also provide for multiplexing detection of panels of biomarkers for a more confident diagnosis. Finally, the unique physical properties like quantum confinement and Raman scattering allow for label-free, real-time sensing of the presence of molecules and also, in other cases, as a spatial locater of molecules within cells or tissues.

Emerging recently is the first of the personalized 'omics' (proteomics, genomics, and metabolomics) studies that can track thousands of biochemical changes in the body to an extent where onset of disease can actually be reversed (174). We envision that one day this powerful diagnostic will be in the hands of the general public, where nanostructured immunoassays will be tailored for the individual based on their sequenced genome, their occupation, and their lifestyle. This will allow for the earliest detection of malignant tumors, onset of inherited diseases, or injury to internal organs, and subsequently allow for proper treatment. We can also speculate further to include the detection and identification of environmental biological agents, bacterially contaminated food, tainted water, or polluted air, available for immediate analysis in a sensitive, easy to use, hand-held instrument.

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There is no conflict of interest in the present study for any of the authors.

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