NATIONAL CANCER INSTITUTE
NCI Alliance for Nanotechnology in Cancer

NCI Alliance for Nanotechnology in Cancer | **Monthly Feature** | November/December 2006

Nanotechnology-Based Assays for Validating Protein Biomarkers

Biomarkers are molecules that can be measured in blood, other body fluids, and tissues to assess the presence or state of a disease. Most men, if they're old enough, are already familiar with at least one cancer biomarker – prostate-specific antigen, better known as PSA. This protein, produced by the prostate gland, is routinely measured in a clinical test or "assay" for prostate cancer screening. If the PSA concentration is high or increasing, a doctor may perform a biopsy to remove tissue samples from the prostate. A pathologist will then examine these samples, looking at the shapes of individual cells and the patterns they form under a microscope, to make a specific diagnosis. Biopsy has been used for several decades to confirm cancer, even though most researchers now agree that analysis of the specific molecules associated with cancer is not only less invasive, but also provides better diagnostic and predictive information.

Why can't the PSA assay alone confirm prostate cancer? It turns out that another noncancerous condition – benign prostatic hyperplasia (BPH), is also associated with increased levels of PSA. Although it can be used effectively for screening, PSA is thus an imperfect biomarker because its concentration is not correlated with only prostate cancer. Are there other molecules present in the blood that can serve as more specific indicators for prostate and other cancers? And if so, can they be used to develop a medical diagnostic for use in the clinic? The answer to these questions is most likely "yes" if better methods for protein biomarker candidate discovery and validation can be developed. But each presents a slightly different technical challenge.

Discovery Requires Sensitivity, Validation Requires Quantification

First, it's important to make one distinction. Only molecules that are validated through human clinical trials to be indicative of a disease are biomarkers. Proteomics and genomics research does not identify biomarkers, but rather, biomarker candidates that must then go through the validation process.

Protein biomarker candidate discovery often involves identifying proteins found at extremely low concentrations. Often, these dilute proteins are masked by highly abundant proteins, such as the albumin found in blood samples. In fact, researchers have found it challenging to develop ultrasensitive detection methods that have a large dynamic range, which is the capability to measure both very large and very small protein amounts. Also difficult has been the task of "capturing" dilute proteins from a complex mixture – even in the absence of interfering proteins, some candidate biomarkers are just too dilute to be measured by many conventional techniques and therefore go undiscovered. In addition, many of the best techniques for finding low abundance biomarker candidates are not quantitative – that is, they can determine whether the protein is present or not, but not measure its concentration.

Another problem with candidate discovery efforts has been that the methods researchers have used to identify low-abundance proteins have issues relating to accuracy, reliability and standardization. The NCI's Clinical Proteomic Technologies Initiative (CPTI) (http://proteomics.cancer.gov) is designed specifically to build the foundation of technologies, data, reagents and reference materials, analysis systems and infrastructure needed to systematically advance our understanding of protein biology in cancer. The NCI's Alliance for Nanotechnology in Cancer is expected to play a critical role in developing the necessary technologies and reagents needed to achieve this end.

Biomarker validation, on the other hand, depends on accurate and reproducible protein quantitation. For a biomarker to be "validated," researchers must conduct clinical trials to prove that biomarker concentrations are different in patients with cancer versus control patients, and that the differences correlate with the disease. Researchers need new tools that can simply, reproducibly, and accurately measure proteins present at low concentrations. And while nanotechnology can aid in discovery and validation, this month's feature focuses on nanotechnology-based assays for validating existing biomarkers that are found at extremely low levels in biological fluids. As the following examples illustrate, nanotechnology offers some unique capabilities for producing the highly sensitive and selective assays that are required. Researchers have already developed, and in some cases even commercialized, various nano- optical, magnetic, and electrical assays or devices that can be used for biomarker validation.

"The first step toward a new cancer treatment or cure is a good diagnostic."

"We have shifted the focus to a whole new region of the analytical scale in terms of sensitivity. The challenge now to the medical community is to give us the biomarkers for any type of cancer for which early diagnosis leads to new treatment or cure."

—Chad Mirkin, Ph.D.

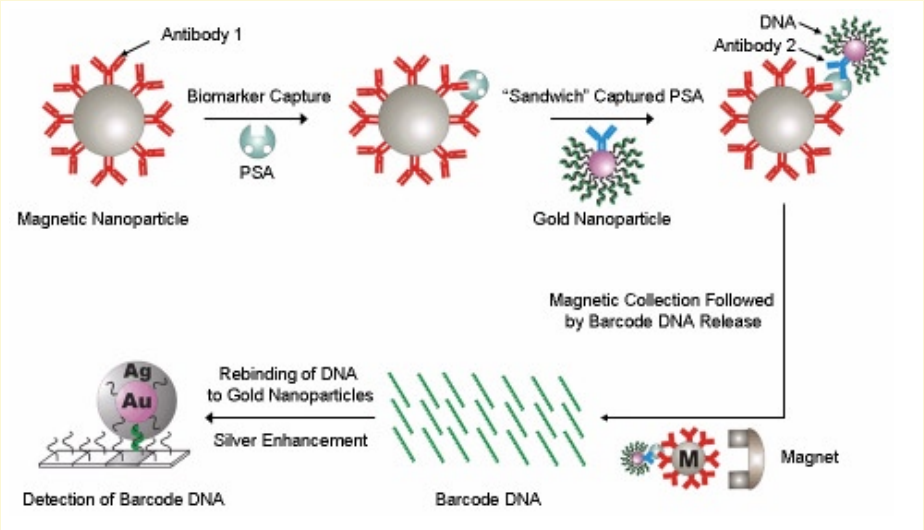

Figure 1. Detection of PSA using the biobarcode assay. *Courtesy: Chad Mirkin, Ph.D., Northwestern University*

A Sensitive Optical Assay

Sensitivity is defined as the smallest amount of the target molecule that an assay can detect. In the case of PSA, sensitivity is not usually the issue. In men, every drop of blood contains billions of molecules of this protein, and conventional assays can easily detect 100 million molecules. But what if the pathologist confirms that the patient has cancer and an urologist removes the prostate gland? After radical prostatectomy, the PSA concentration should be essentially zero. In theory, doctors could monitor the PSA concentration after this procedure to screen for prostate cancer recurrence. The drawback with this approach, of course, is that zero is simply determined by the lowest concentration that the assay can measure. Recurrent cancer would remain undetected until the number of molecules of PSA rose above the 100 million molecule-threshold. New technologies that could measure much lower levels of PSA should let doctors diagnose recurrence much earlier, and bring in lifesaving treatment much sooner.

Researchers at the Nanomaterials for Cancer Diagnostics and Therapeutics Center for Cancer Nanotechnology Excellence (CCNE) at Northwestern University have developed an ultrasensitive method that can detect as few as 100 molecules of PSA in a drop of blood,[1] which is six orders of magnitude higher sensitivity than the conventional assays. Led by Chad Mirkin, Ph.D., principal investigator of the Northwestern CCNE, the research team uses nanoparticles as scaffolds to carry both molecules that bind to biomarkers, and molecules that boost the signal. They call this powerful amplification and detection scheme the biobarcode assay.

The assay takes full advantage of unique physical, chemical, and optical properties of two types of nanoparticles. The first nanoparticle is magnetic and is coated with an antibody that binds specifically to PSA. The second nanoparticle is made of gold and is coated with hundreds of identical pieces of "barcode" DNA. The second nanoparticle is also coated with a different antibody that binds to PSA.

Detection of PSA requires five basic steps (Figure 1). First, antibodies on the magnetic nanoparticles extract and concentrate PSA. Second, antibodies on the gold nanoparticles "sandwich" the biomarker between the two types of nanoparticles. Third, a magnetic field separates out all the magnetic nanoparticles – including those which are tethered to the gold nanoparticles through a PSA "bridge." The fourth step, which is responsible for amplification, heat, or chemical treatment, releases hundreds of pieces of barcode DNA. In the last step, the DNA is again bound by gold nanoparticles, which are later coated with silver to make them larger and easier to detect on the surface of a gene chip. The PSA concentration in the original sample is determined from the light scattered by the nanoparticles anchored to the chip.

What makes the biobarcode assay so much more sensitive and versatile than the more traditional assays? Mirkin says there are two main reasons.

"First, other assays typically use antibodies attached to the bottom of wells on a microarray or titer plate for biomarker capture. In extremely dilute solutions, biomarkers take a long time to find these flat binding surfaces. With antibody-coated nanoparticles, however, you can add large quantities to the sample and stir. The nanoparticles, aided by homogeneous mixing and the high surface-to-volume ratio, will quickly and efficiently probe all the solution volume in search of biomarkers.

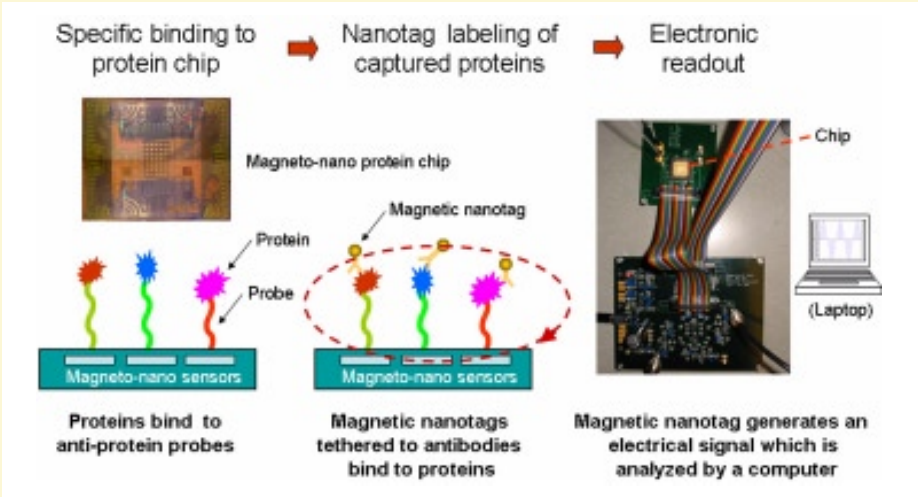

Figure 2. Detection of proteins using the magneto-nano protein chip.
Courtesy: Shan Wang, Ph.D., Stanford University

Second, for every PSA molecule captured, a gold nanoparticle releases hundreds of pieces of barcode DNA. This amplification step is another key to the assay's high sensitivity."

In the PSA example above, DNA only amplified the signal – it didn't "code" for anything. Recently, however, Mirkin's team simultaneously detected three different protein biomarkers in the same sample: PSA, a biomarker for testicular cancer, and a biomarker for liver cancer.[2] To accomplish this feat, three pairs of nanoparticles were used – each containing a different DNA barcode. A commercial instrument uniquely identified and measured each barcode based on its optical signal, which allowed the researchers to measure the biomarker concentrations independently. "A sensitive panel assay built on the biobarcode assay platform might one day improve patient diagnosis for ovarian cancer," says Mirkin.

Scientists once thought the protein CA-125 would be a good biomarker for ovarian cancer, but like PSA, its concentration may be high with benign tumors and other diseases as well. However, simultaneously analyzing a panel of different biomarkers, many occurring at extremely low concentrations in blood, may be the key to distinguishing ovarian cancer from benign diseases.[3,4]

A Selective Magnetic Assay

"Selectivity" refers to how well an assay can detect particular molecules in a complex mixture without interference from other molecules in the mixture. Most assays are not highly selective. That's because they often rely on optical labels that produce or emit light when excited, and most body fluids such as blood contain a host of other compounds that behave similarly. Also, the intensity of the emitted light often varies with sample pH and decreases over time, a result of a chemical process known as photobleaching.

To avoid these problems, researchers at the Center for Cancer Nanotechnology Excellence Focused on Therapy Response (CCNE) at Stanford University have been working on alternative detection methods.[5] Led by Shan Wang, Ph.D., an investigator at the Stanford CCNE, the research team has developed labels based on magnetic nanoparticles they call nanotags. Because all other components in a blood sample solution are essentially non-magnetic, interference effects and background signals are removed. Furthermore, the magnetic properties of nanotags are stable over time.

The hard part of this research entailed designing a tiny device that could correlate the number of magnetic labels on a surface with an electronic signal, while ensuring selective protein binding. That's where the Wang team excelled. They produced an inexpensive microchip with integrated microfluidics pipes to pump multiple samples over a magneto-nano sensor array (Figure 2).[6] Each individual sensor, which is 1.5 millionth of a meter wide, responds to stray magnetic fields by changing its electrical resistance. As more and more nanotags deposit, their stray magnetic fields cause the electrical resistance of the sensor to decrease in proportion to the number present.

There are three basic steps for protein quantitation using the magneto-nano protein chip. First, probes specifically "capture" proteins from the sample and bind them to the sensor surface. Second, nanotag-labeled antibodies bind to these surface-bound proteins. Finally, an external magnetic field is applied to the chip and the stray magnetic field produced by the nanotag labels is measured. The lower the resistance, the more nanotags are present and the higher the concentration of protein in the original sample.

When the researchers measured the concentration of the protein interferon gamma (IFN-γ) with the first-generation sensor, the limit of detection was similar to that seen with traditional assays. They aim to increase the sensitivity by shrinking the sensor area and developing improved nanotag labels and sample delivery fluidics.

"The good news is that our present sensor gives the same signal for a given concentration of IFN-γ in blood as in buffer, without the need for any pre-purification or amplification," says Wang. "The assay platform shows excellent selectivity. Furthermore, the signal per nanotag increases rapidly when the sensor width is reduced.[7] If this increased 'magnetic sensitivity' can be translated into biomolecular sensitivity, magnetic detection will become even more attractive for biomarker validation."

Two Electrical Sensors in One Nanoscale Package

Another strategy for increasing assay selectivity is to use two independent signals to determine protein concentrations. While either signal may be subject to interference from noise or nonspecific protein binding, the tandem signal can be used to minimize or even eliminate false-positive readings.

This approach is being taken by a research team led by Chongwu Zhou, Ph.D., of the University of Southern California (USC). Zhou and his colleagues have incorporated two individual nanosensors into one device that relies on the electrical properties of proteins to determine their concentrations. The device they used is called a field-effect transistor (FET) and it functions by measuring the influence of charged proteins on current flow through channels linking two electrodes.

A FET actually has three electrodes: a source, where electrons are added; a drain, where they are removed; and a gate, which can control the flow of electrons, or

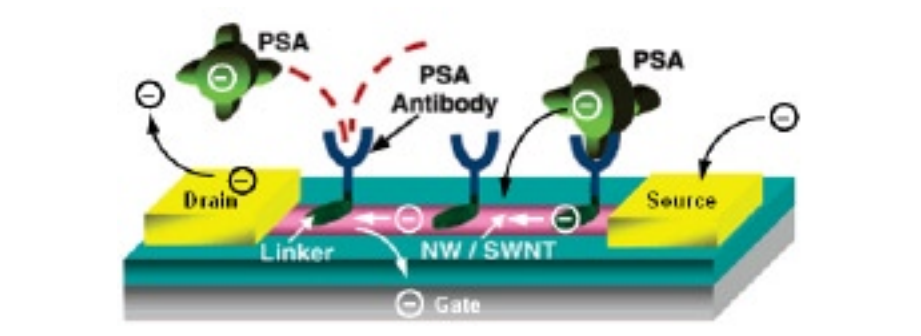

Figure 3. Schematic diagram of field-effect transistor nanosensor. Electrons flow from right to left through the channel formed by nanowires (NWs) and single-walled carbon nanotubes (SWNTs).

Courtesy: Chongwu Zhou, Ph.D., University of Southern California

current, between the source and the drain (Figure 3). If the electric field at the gate is changed, it will be harder (or easier) for electrons to flow through the channel. But charged molecules placed near the channel can also provide a similar gating effect.

This is the phenomenon the Zhou group relied on when they linked PSA-binding antibodies to channels of indium oxide nanowires (NWs) and single-walled carbon nanotubes (SWNTs). [8] Conduction increases in NW channels if the negatively charged PSA molecule is captured by antibodies on the surface, and decreases in SWNT channels upon PSA binding.

Using two materials that produce opposite responses in the same device allowed the team to confirm the signal change was caused only by PSA binding. Nonspecific protein binding or electrical noise would not produce a characteristic complementary electrical response (Figure 4). The USC combination device could measure PSA in buffered solutions at concentrations well within the clinically useful range. The team is now optimizing their nanowire/nanotube device to detect multiple biomarkers simultaneously.

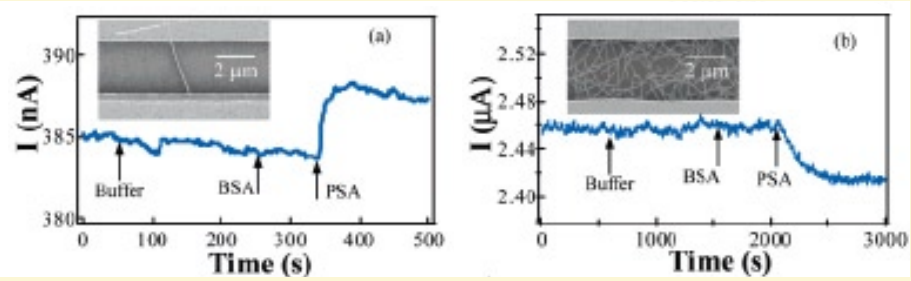

Figure 4. Current recorded over time for a nanowire (NW) device (a) and a single-walled carbon nanotube (SWNT) device (b) when sequentially exposed to buffer only, BSA (a nonspecific protein), and PSA. Note the current increase in (a) and the complementary current decrease in (b) upon addition of PSA. *Courtesy: Chongwa Zhou, Ph.D., University of Southern California*

Biomarker Candidates into the Clinic

Researchers are continually searching for new biomarker candidates because they are one of the keys to personalized or post-genomic medicine. They have the potential to help us detect cancer earlier, determine a tumor's aggressiveness, or predict a patient's response to a particular treatment. But the process of identifying candidates and validating them in order to produce new molecular diagnostics for the clinic has been slow and challenging.

Although more than 1,000 protein or peptide candidates have been discovered, fewer than ten have been approved by the FDA for cancer screening and monitoring. Some scientists see a lack of high-throughput discovery methods as a bottleneck to development. Others cite validation as the slow step. But because nanotechnology offers the potential to produce inexpensive, multiplexed, high-throughput assays with high sensitivities and selectivities, both candidate discovery and validation can benefit. More importantly, these same characteristics are needed in clinical diagnostic devices and sensors.

The challenge now is to transition nanotechnology-based assays from the research laboratory through FDA approval, and finally into a commercial diagnostic product. A substantial investment of time and money will be required to complete this journey, but the reward offered – developing new diagnostic, prognostic, or therapeutic products that can change the lives of cancer patients – is well worth the risk.

—David Conrad

End Notes

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