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Perspectives

Single-Molecule ELISA

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The need for assay methods of sufficient sensitivity to determine the low concentrations of hormones present in body fluids led to the original development of immunoassays and analogous "binding" (or "ligand") assays in the late 1950s and early 1960s. These methods use a binding agent (also commonly referred to by other terms such as "receptor," "binding reagent," and "analyte-specific reagent"), a substance used in a binding assay to bind the target analyte. Typical binding agents include antibodies, antigens, cell receptors, and serum binding proteins. Immunoassays still constitute the most widely used class of binding assays, although microarray-based nucleic acid assays that employ oligonucleotides as binding agents are rapidly increasing in popularity. A principal objective in this field since the development of these assays has been to increase their sensitivity.

Rissin et al. *(1)* recently described their attempts to further improve immunoassay sensitivities. These authors, who used an ELISA-type system, reported that they were able to "detect serum proteins at subfemtomolar concentrations" and to increase the sensitivity of measurements "using a typical ELISA plate reader by a factor of about 68000."

Before we discuss the novel features of the approach reported by Rissin et al., we should briefly examine the concept of sensitivity and the meaning of the term "sensitive" to describe the performance of a binding assay performance—or indeed that of any measurement system. Many workers in this area, Rissin et al. included, identify sensitivity with the lower limit of detection (LoD)[3] of an assay. Others, including the American Chemical Society and the International Union of Pure and Applied Chemistry, define sensitivity as the slope of the dose–response curve (or the response/dose [R/D] ratio)—an intrinsically meaningless concept with which we strongly disagree (see Fig. 1).

In short, the more sensitive of 2 systems has been regarded by scientists since the 1850s as the system that measures the smaller amount of that which the systems are intended to measure, i.e., the system that exhibits the lower LoD.

The fundamental difference between these 2 concepts of sensitivity has not only led to past controversy and debate *(2, 3)* but also has profoundly influenced the design of immunoassays and analogous methods. Equating sensitivity with slope or the R/D ratio has, in practice, led to the use of relatively high antibody concentrations, typically approximately 1/K (where K is the affinity constant governing the binding reaction under the conditions used in the assay) in competitive assays (e.g., RIAs) and approximately 20/K in noncompetitive immunometric methods (e.g., sandwich assays), enabling the capture of approximately 40%–50% and >90%, respectively, of the analyte in a sample. (Immunoassays that rely on the use of labeled antibodies are generally termed IRMAs and are sometimes of competitive design.) But an important factor that affects an assay's LoD is the presence of noise in the system, i.e., the variation in the signal generated by a blank sample containing 0 analyte, generally represented by the SD of the blank measurement.

Thus a key determinant of an assay's LoD (as of any measuring system) is the signal/noise ratio, where "signal" refers to the signal deriving from the target analyte. Clearly the lower the analyte concentration, the lower the signal/noise ratio, it being commonly accepted that a ratio of 3 defines the LoD.

Rissin et al. clearly recognized the importance of maximizing the signal/noise ratio to maximize immunoassay sensitivity. Their approach to achieving this objective (termed digital ELISA) was to count individual target molecules that were captured by antibody on a solid support (which comprised thousands of microscopic beads, each 2.7 μm in diameter) and subsequently bound by a second, enzyme-labeled antibody to form an antigen/antibody sandwich. By using large numbers of beads, the authors ensured that both a high proportion (approximately 70%) of analyte molecules in a sample was captured and that most beads captured only a single analyte molecule.

After the beads were exposed to the sample and to the labeled antibody, they were put into contact with an array of femto-sized wells into which approximately 10%–15% of the beads became trapped. These trapped beads were then exposed to a fluorogenic substrate that was fluorescence generated in the immediate vicinity of the beads and bore enzyme-labeled complexes that

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[3] Nonstandard abbreviations: LoD, limit of detection; R/D, response/dose.

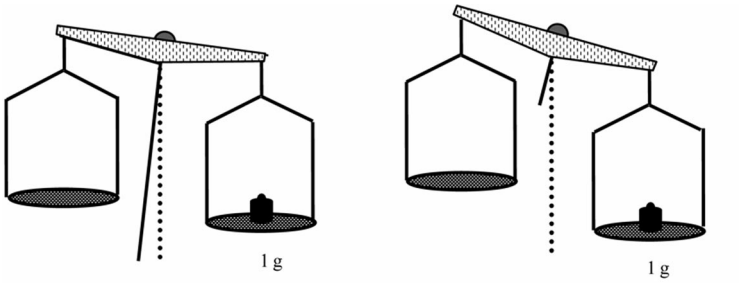

Fig. 1. The response of 2 balances to a weight of 1 g placed on the pan.

This image was placed on a clinical chemistry discussion website in 1999. Of the 120 respondents, approximately 48% opted for balance A, approximately 48% opted for balance B, and approximately 5% were undecided. Note that International Union of Pure and Applied Chemistry defines the sensitivity of a balance as the movement of the pointer across the scale when a weight is placed on the pan; the American Chemical Society as the angular rotation of the balance arm [see *(7)*]. Both are examples of the R/D definition of sensitivity.

were observable as individual—and countable—pinpoints of light.

The strategy of counting target analyte molecules to which labeled antibody is bound (as opposed to measuring the signal generated by an aggregate of target molecules as in conventional ELISAs) has much, in principle, to commend it. For example, in an assay that requires measurement of fluorescent signals, background noise may be created by the instruments used to detect the fluorescence, the support matrix on which captured analyte molecules are located, and the resulting nonspecific binding of labeled antibodies. Counting of molecules to which a fluorogenic enzyme is attached reduces some—though not all—of the noise from these sources.

So, have Rissin et al. succeeded in their aim, and transformed the immunodiagnostics field by developing a method capable of determining subfemtomolar protein concentrations?

First it must be noted that similar sensitivity claims were made for 2 methods described in reports published some 30 years ago *(4, 5)*. Both methods relied on enzymes as signal amplifiers. One of these methods, which used a fluorogenic substrate and fluorescence measurement, was claimed to have an LoD (for mouse IgG) of 24 000 molecules/L, or 40 zmol/L *(4)*. The other method, which used a radioactive substrate ([^{3}H]AMP) and measurement of [^{3}H]adenosine, was reported to have an LoD of 600 molecules/L (or 1 zmol/L) of cholera toxin after an incubation time of 1000 min *(5)*, an LoD that was some 1000-fold lower than the LoDs of both an RIA and of a conventional ELISA that relied on color measurement. In short, both methods yielded sensitivities (as assessed by LoD) considerably superior to that achieved by Rissin et al.

In addition, both of these older assays employed reagents and methods within the compass of a competent biochemist, albeit respectively requiring measuring instruments (i.e., a fluorometer and liquid scintillation counter) not found in every laboratory.

One might therefore ask: why did these reports, and the methods described therein, not attract the attention now accorded to the report by Rissin et al.? Although the actual reasons must remain a subject of conjecture, we believe the principal reason for the lack of attention was that these methods were much ahead of their time. Both labeled antibody and ELISA methods were proposed in the late 1960s, but more than 10 years later the only labeled antibody method in common use was an assay for hepatitis-B antigen; indeed, doubts still persisted regarding the relative sensitivities of RIAs and IRMAs.

Moreover, factors other than sensitivity (e.g., precision, working range, performance time, and cost) are also relevant an assay system's clinical utility. Of increasing importance in this context is the ability to simultaneously assay multiple analytes in the same sample, primarily because knowledge of their concentrations may be of clinical importance. But most clinically important analytes (e.g., hormones such as human chorionic gonadotropin, thyroid-stimulating hormone, and human growth hormone) are of heterogeneous molecular composition, comprising (variable) mixtures of isoforms, each differing in molecular structure and (potentially) biological activity.

However, assays of analytes that differ between samples—termed "comparative" in the 1950s by Gaddum and Finney—are inevitably method dependent and cannot be standardized. Thus, results yielded by different immunoassays of such analytes typically dif-

fer, as do those between immunoassays and bioassays. Hence only determination of the amount and biological activity of each molecular species contained in a heterogeneous analyte can, in principle, provide a valid representation of the amount of the analyte in a sample.

Recognition of this issue led to the establishment of an IFCC project and working group (who have since published reports in *Clinical Chemistry*) on the standardization of human chorionic gonadotropin measurements after a presentation by one of us (R.E.) on immunoassay standardization at the 3rd Bergmeyer Conference on Assay Results in Life Sciences in 1990. This presentation highlighted the clinical need for an ultrasensitive method that would permit simultaneous determination of the multiple isoforms that characterize many polypeptide hormones. Indeed, this need had been an important factor in the original development of microspot array-based binding assays (now termed microarray methods) in the late 1980s *(6)*. These methods are based on the concept of ambient analyte assay (i.e., an assay in which the binding agent concentration employed is $<0.1/K$, and ideally $<0.01/K$, binding $<9.1\%$ and $<1\%$, respectively, of the analyte present in the sample).

Such assays are, among other things, independent of sample volume. Counterintuitively, however, they also require shorter incubation times and can yield higher sensitivities than conventional binding assays. To achieve these objectives, fluorescent microspheres have been used as signal amplifiers (note that these microspheres must be nonsticky, and that Boehringer Mannheim synthesized its own spheres to achieve this end) and binding agents located at high surface density within micropots on solid supports as molecular monolayers, thereby minimizing background noise. In practice (by using antibodies of an affinity of 10^{11} L/mol and confocal microscopy to scan arrays) sensitivities of approximately 1000 molecules/mL, i.e., approximately 1.6 amol/L, have been claimed by Boehringer Mannheim researchers. Again, this sensitivity is equal to or greater than that yielded by Rissin et al.'s approach.

Thus, in summary—and notwithstanding its admirable ingenuity, novelty, and high sensitivity—the method of Rissin et al. *(1)* has yet to match the advantages of existing microarray methods, particularly their ability to simultaneously determine a multiplicity of target analytes in a sample.

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