Signal Amplification in Time-resolved Fluorometry

A frequent question among clinical biochemists is how sensitive analytical techniques should be in the context of clinical diagnostics. Currently, the analyte concentrations usually encountered in clinical chemistry range from 10^{-3} to 10^{-12} mol/L. By far, the most sensitive nonamplification techniques used in the clinical laboratory are based on noncompetitive immunological assays. Is there any need for measuring analytes at even lower concentrations? The answer is likely yes. Once the methodologies for measuring even lower concentrations of analytes are developed and our knowledge of the many new candidate biological markers that likely will be discovered through the Human Genome Project is more complete, we may be interested in or need to measure analyte concentrations that are 1/10th to 1/100th of those currently measured. Hence, we should continually pursue the development of methodologies that can reach the ultimate sensitivity, i.e., detection of single molecules.

In other areas of laboratory medicine, e.g., microbiology, single pathogen particles (e.g., viruses and bacteria) have diagnostic significance. We should not forget that the measurement of a single molecule in a very small fraction of the total blood volume may mean that the whole organism could contain relatively large numbers of such pathogenic or abnormal constituents. When the analytes are nucleic acids (DNA or RNA), PCR and other exponential amplification techniques provide a convenient, yet very powerful, way of amplifying specifically these molecules before final quantification. Unfortunately, proteins are not replicable, and their sensitive quantification usually relies on their interaction with specific reagents, mainly antibodies. An alternative approach to target amplification is the concept of signal amplification, which means that after the analyte interacts with a specific binding reagent, the signal is magnified many-fold to be more easily detected. Various forms of signal amplification have been published, including the use of enzymes, macromolecular carriers, branching reagents, and particles.

In their review of the theoretical and practical aspects of immunological assays, Jackson and Ekins *(1)* analyzed the factors contributing to high sensitivity. Two key components are associated with the final sensitivity of a binding assay: (*a*) our ability to detect the labeling molecule (e.g., radioisotopic, chemiluminescent, or fluorescent label); and (*b*) the quality of the binding reagents and the experimental conditions used. A frequent misconception among clinical chemists is that the use of extremely sensitive detection technologies, such as chemiluminescence and time-resolved fluorescence, should lead to highly sensitive assays. This is not true if the reagents and conditions that complement such assays (e.g., the affinity and specificity of the antibodies, the nature of the solid phase, and the effectiveness of washing) are not optimal. To reach ultimate sensitivity, we need a highly sensitive labeling technology, high-affinity binding reagents, and optimized assay conditions that will minimize the nonspecific binding of the reagents. Under such conditions, immunological assays can approach the theoretical sensitivity of 10^{-16} mol/L *(1)*.

Time-resolved fluorometry is already a mature technology, and its advantages over conventional fluorometry have been reviewed repeatedly *(2–4)*. One of the attractive features of lanthanide fluorescent chelates is that quenching effects from multiple labeling are not usually observed. This has led many investigators to explore multiple labeling to improve the signal and sensitivity of immunochemical and other assays. For example, antibodies and streptavidin have been linked to either proteins or other polymeric compounds to create conjugates that carry hundreds of europium chelates per reagent *(5, 6)*. Time-resolved fluorometry has also been combined with enzymatic amplification *(7, 8)*. More recently, polyvinylamine complexes of streptavidin have been explored to develop extremely sensitive immunoassays and immunohistochemical procedures as well as model microarrays with measurement of the fluorescence directly from the solid phase *(6, 9, 10)*.

In this issue of *Clinical Chemistry*, Harma et al. *(11)* explore the use of polystyrene nanoparticles containing thousands of fluorescent europium chelates as multiply labeled reagents for time-resolved fluorescence immunoassays and other applications. These nanoparticles were conjugated to streptavidin to create a universal detection reagent. With this technology, they report impressive sensitivity of an immunological assay for prostate-specific antigen (PSA) and describe microscopic visualization of single nanoparticles. This report provides further credence to previous publications suggesting that multiple labeling is an efficient method for further improving the sensitivity of time-resolved fluorometry with lanthanide chelates *(5, 12)*.

There are a few issues that need discussion in relation to this report. Nanoparticles have already been used with other immunoassay technologies, and they are also effective for homogeneous multiparametric analysis *(13)*. Although Harma et al. *(11)* report detection limits of a single or a few nanoparticles in solution, these are not actual immunoassay results. When the reagents were used in real immunological assays, the detection limit for PSA was ~1–2 ng/L, similar to the detection limits of immunoassays that use enzymatically amplified time-resolved fluorometry *(14)*. The explanation for not achieving lower detection limits with this system is likely related either to the affinity of the antibodies used or to nonspecific binding of the immunological reagents (e.g., the detection antibody) to the streptavidin label. Other issues that we should consider in multiple labeling strategies include steric hindrance of macromolecular compounds and slow reaction kinetics. Indeed, in the model assay for biotinylated PSA reported by Harma et al. *(11)*, the streptavidin incubation used was 4 h, compared with the 10–15 min

usually used with directly labeled streptavidin reagents *(14)*.

In conclusion, the report by Harma et al. *(11)* further contributes to the literature of time-resolved fluorometry and promotes the following ideas, which have been explored previously by other investigators:

- Time-resolved fluorometry with measurement of the fluorescence directly from a solid phase allows highly sensitive detection and miniaturization, including spatial resolution, which means that the technique can be used in microarray and microspot analysis as well as in immunohistochemistry and other applications *(2, 5, 6, 9, 10, 12, 15)*.
- Multiple fluorescence labeling is a powerful approach to further enhance detection reagent signal, which in turn, under optimized conditions, could improve the final sensitivity of immunological and other assays *(5, 12)*.
- Nanoparticles can be used to develop reagents with high amplification ratios and extremely low detection limits. Such reagents may be useful in new areas of biological analysis, including microspots, microarrays, and immunohistochemistry.

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