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SENSITIVE MACROMOLECULAR QUANTITATION VIA FLUOROMETRIC METHODS

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ABSTRACT: A sensitive method for quantitation of macromolecular concentrations in liquid is being developed by combining the techniques of fluorescence immunoassays utilizing probes that amplify the fluorescence signal and time-resolved fluorescence spectroscopy. A model system is used in which a horseradish peroxidase-rabbit antibody complex serves as antigen-antibody system. An assay sensitivity greater than 0.1 nM has been possible to reach. Time-resolved fluorescence spectroscopy measurements indicate that this limit may be improved by a factor of 10 to 100 depending on the fluorescence probe used.

INTRODUCTION

Increased demand in modern laboratory work for sensitive detection methods of chemical compounds has prompted various fluorometric methods to be explored. Often a fluorescence measurement is more complicated to perform than many other popular assays in use but its potential for high sensitivity may outweigh such drawbacks. Thus, for instance, fluorimetry is typically a factor of a thousand more sensitive than spectrophotometry and has a potential to be several orders of magnitude more sensitive than radioactive tracer methods (Yallow and Berson, 1960) where assay sensitivity of the order of 100 picomolar is possible. The sensitivity limit for radioactive tracer methods is partly set by radiation safety requirements, a restriction which, of course, is not applicable to the fluorescence method.

In this paper we discuss a method which is being developed for quantitation of macromolecular concentrations in liquid utilizing the technique of fluorescence immunoassays with probes that amplify the fluorescence signal. Preliminary measurements indicate that an assay sensitivity below picomolar range is possible to reach, a limit which may be improved by use of a time-resolved fluorescence spectroscopy technique.

METHOD

A model system was used in which a horseradish peroxidase-rabbit antibody complex served as antigen-antibody system. The horseradish peroxidase molecules were ionically attached to submicron-size (0.1 μm diam.) polystyrene spherical particles (SSF). To amplify the fluorescence signal and thus enhance detectability and sensitivity these small spheres were doped with a large number of easily detectable fluorophores with excitation wavelength 440 nm and emission wavelength 480 nm (Fluoresbrite microspheres Coumarin Dye, Polysciences Inc.; Warrington, Pa). Non-fluorescent poly-

styrene particles (LS) 100x larger than SSF were ionically attached to rabbit anti-HRP. Bovine Serum Albumin or no protein attached to SSF were used as controls. All proteins not incorporated onto the spheres were removed by centrifugation. Protein-bound SSF and control SSF were then incubated separately with LS in coating buffer at room temperature overnight. Theoretically, LS-Ab bind to SSF-HRP by conventional antigen-antibody interaction, thus labelling the larger spheres with a fluorescent marker. Un-incorporated SSF were separated from the complexed spheres by sucrose-density gradient centrifugation. Polystyrene particles are used in various immunoassay situations (see e.g. Bangs,1987; Lentrichia et al., 1987; Saunder et al.,1987) and their properties are therefore reasonably well-known and thus suitable for this investigation.

The fluorescing probes were detected using a variety of fluorescence techniques. Before discussing the results it is of interest to calculate a detection limit using parameter values that are reasonably easy to reach.

The number of photons, N, registered by the detector in a fluorometer is given by

$$N = S \phi k n Q e V$$

where the following symbols are used (numerical numbers applicable to the case at hand are given in parentheses):

N: Number of photons registered by the detector ($>=$ 100/s)
S: Cross section ($<= 10^{-16}$ cm^2)
ϕ: Light intensity (10^{16} photons/cm^2/s; this corresponds to approximately 10 mJ HeCd laser output)
k: Number of fluorophores in each small spheres ($<=$ 100)
n: Concentration of small spheres with fluorophores (number of spheres/cm^3)
e: Detection efficiency ($<=$ 0.1)
Q: Quantum yield ($<=$ 0.1)
V: Sample volume (1 cm^3)

With the numerical values indicated above we find for the concentration of small spheres, n

$$n >= 100 \text{ spheres/cm}^3$$

This value corresponds to approximately 0.01 femtomolar. In principle, therefore, there is no difficulty in reaching extremely low detection limits. The practical application of the technique, however, is often limited by instrumental difficulties to separate excitation light from emission light paired with the natural large linewidth of the emitted light. A development of the fluorescence technique must therefore be directed towards improvements of light separation methods. This is considered in the next section.

MEASUREMENTS AND RESULTS

In this report we will concentrate the discussion on aspects of detection of the small spheres, since that forms the bases for the detection method. Details of the chemical separation techniques as well as results from the antibodyantigen complex experiments will be published elsewhere.

Table 1 Detection sensitivity limits (order of magnitude) of concentrations of small spheres (SSF) and Antibody-Antigen complexes. Values within parentheses are estimated numbers.

Experimental Condition	Concentration of Small Spheres		Concentration of Antibody-Antigen
	(numbers/ml)	(M)	(M)
Fluorometry, Conventional	10^{7}	10^{-13}	$>= 10^{-12}$
Fluorometry, Laser Excitation	10^{4}	10^{-16}	$(>= 10^{-15})$
Time Resolved Fluorescence Spectroscopy, Conventional	10^{6}	10^{-14}	$(>= 10^{-13})$
Time Resolved Fluorescence Spectroscopy, Laser Excitation	(10^{3})	(10^{-17})	$(>= 10^{-16})$

Table 1 shows some results obtained in this investigation. As indicated in the first column of the table four different experimental conditions were considered: Conventional fluorometry; Laser excitation fluorometry; Conventional time-resolved fluorescence spectroscopy and Laser time-resolved fluorescence spectroscopy. We discuss briefly these four techniques.

Conventional fluorometry: For most of the measurements a fluorometer of type GREG PC photon counting fluorescence spectrofluorometer was used. It was available at the Laboratory for Fluorescence Dynamics at the University of Illinois, Urbana, Ill. The detection limit listed in the table is mainly set by the light source spectral output which even after passage through monochromator is far from monochromatic and thus can not completely separate excitation light from emission light.

Laser excitation fluorometry: The basic fluorometer was the same as the conventional fluorometer listed above, but with a HeCd laser (Liconix Helium Cadmium Laser) producing excitation light of wavelength 440 nm instead of the conventional incandescent light source. Due to the laser's high monochromaticity a considerable better separation of excitation and emission light is possible with corresponding improvement in detection sensitivity.

Conventional time-resolved fluorescence spectroscopy: Most of the measurements in this category were performed with an air spark source (Ortec 9352) with single photon counting (see e.g Alvager and Balcavage, 1978). The advantage of this method is based on the fact that the fluorescence light is delayed in relation to the scattered (=excitation) light and by a suitable time delay a certain enhancement in the detected signal can be achieved. The amount of the enhancement depends on the particular fluorophore involved.

Laser time-resolved fluorescence spectroscopy: By combining a laser with the above timing technique an improved sensitivity can be obtained. This has not been demonstrated in this investigation but is known from previous experiments (Alvager and Davis, 1987).

In Table 1 only order of magnitude numbers are included since this discussion is mainly about principles not a technical description of any particular experiment. It is seen that the experimental values for detection sensitivity of small spheres are approaching the theoretical limit calculated in the previous section. Fewer data are available

for the antibody-antigen complex system but in preliminary experiments we have shown that the detection efficiency is somewhat less sensitive than for small spheres alone but still high. This situation is mainly attributed to a larger degree of scattering in the complex system.

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