Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 8, pp. 803–809, 1987
Printed in Great Britain

0731-7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

Nephelometric immunoassay*

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Abstract: The theory of the scattering of light by particles is summarised and the principles underlying instrumentation for nephelometry in immunoassays are outlined. Consideration is given to the various factors that influence nephelometric assays and methods for increasing sensitivity are discussed.

Keywords: *Nephelometric immunoassay; turbidimetric immunoassay; nephelometric inhibition; particle enhanced immunoassay; light scattering.*

Introduction

The use of light-scattering techniques in assessing antigen–antibody reactions began in the late nineteenth century more or less in parallel with the development of the theory of light scattering by Rayleigh and others. However, instrumentation at that time was extremely crude by present standards [1]. Occasional reports of the application of light scattering appeared over the ensuing decades, but significant interest did not arise until the late 1940s and the 1950s, coincident with the development of instruments which had relatively stable and high-intensity light sources (e.g. mercury arc lamps), improved photosensors (photomultiplier cells and photodiodes), and the capability for automation.

This paper represents a brief summary of light scattering theory and of nephelometric immunoassay. For further information, the reader should consult the specific references listed or one of the review articles on theory [2], nephelometry [3–5], and particle-enhanced assays [6].

Theory

The scattering of light by particles in a liquid is affected by several factors including the refractive index of the solution or suspension, the wavelength of the incident light beam, the angle of measurement, and the size, shape, and concentration of the particles [7, 8]. For practical purposes in a given quantitative immunoassay method, all these factors are considered to be constant except for the concentration of the antigen (and therefore of the "particles" or complexes).

The measurement of light scattered at an angle from the incident beam is known as *nephelometry* whereas the measurement of the change in light transmitted at 0° to the

*Presented at the "International Symposium on Immunoassays", November 1986, Arlanda, Sweden.

incident beam is *turbidimetry*. Both types of measurements may be used for assessing immunological reactions. Light sources in common use for immunochemical assays have wavelengths (λ) from 340 to 650 nm. For particle sizes up to one-twentieth of the wavelength ("Rayleigh scatter" range), scatter is symmetrical (back scatter equals forward scatter at corresponding angles) and there is substantial scatter at 90° from the incident beam (Fig. 1). As shown by Debye [9] and Mie [10], scatter becomes progressively asymmetric towards 0° (forward) as the particle size increases to and beyond the wavelength of the incident beam. In addition, the total measurable light scattered decreases because of phase interference (Fig. 2).

In light-scattering immunochemical assays, the early complexes or "particles" are comparatively small (one or two molecules each of antigen and antibody). With time, much larger aggregates, or lattices, are formed. The rate of formation of larger particles is dependent upon the antigen–antibody system but is usually quite rapid for polyclonal antisera. For IgG : anti-IgG complexes, apparent molecular weights may exceed 10^8 daltons and are directly proportional to antigen concentration as long as antibody is

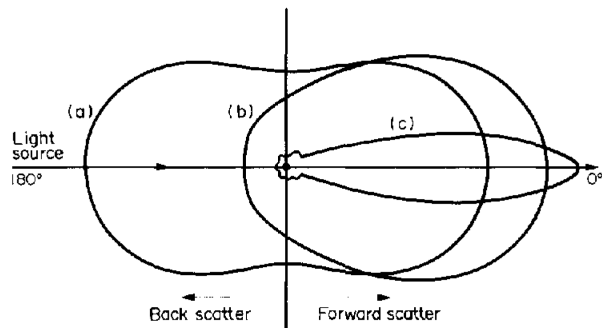

Figure 1
Relative directional light scatter for particle size (a) less than λ/20 (Rayleigh scatter), (b) approximately equal to λ (Rayleigh–Debye scatter), and (c) greater than λ (Mie scatter). (Revised from reference 2).

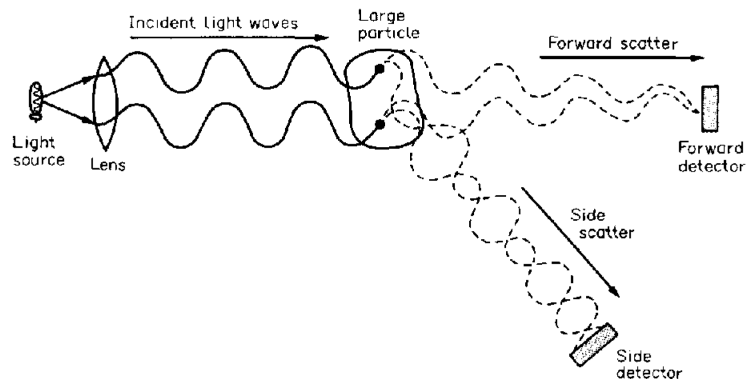

Figure 2
With light scatter from relatively large particles measured at forward (or low) angles, the phase concordance results in relative enhancement (a_1 and b_1); at lateral (or high) angles, there is phase interference (a_2 and b_2) and a reduction in apparent scatter. (Revised from reference 2).

present in excess [11]. Surprisingly, the effective radii of gyration for light scatter are relatively unaffected by concentration and the apparent molecular weight of the complexes formed, with mean radii of about 700 nm and ranges of 400–1400 nm in "endpoint" measurements (30–60 min incubation). For comparison purposes, a single IgG molecule has an effective radius of 20 nm, whereas small immune complexes are 30–50 nm and chylomicrons 200–450 nm [2, 3].

Clinical Instrumentation

The first instrument for the routine clinical application of light scattering was the Technicon AIP, a nephelometric system which wedded a fluorimeter with the Technicon Autoanalyzer system [12]. Nephelometry was chosen rather than turbidimetry because of the relative merits of the instruments available and because of comparative research at the time. The demonstration in 1947 by Boyden *et al.* [13] that the amount of immunological precipitate was directly proportional to the light scattered, up to equivalence, and the report in 1948 by Bolton *et al.* [14] that moderate change in particle size had little effect on nephelometric determinations, was in contrast to turbidimetric assays. This suggested that assays of proteins with varying molecular size, such as immunoglobulin M and haptoglobin, would give more accurate values by nephelometry (see under Other Considerations).

Reaction rates at this time were very slow; completion of a single assay took from minutes to hours. The Technicon AIP system permitted incubation as long as needed because of its flow design. However, prolonged incubation increased the likelihood of sample cross-contamination and, in some cases, causes formation of large, flocculent precipitates which interfered with measurements. With the introduction of enhancing agents, especially polyethylene glycol (PEG) by Hellsing in 1972 [15], reaction rates were greatly accelerated, sensitivity was increased and assay ranges were significantly extended.

PEG enhancement was also important for the development and introduction of the rate nephelometer, the Beckman ICS, in 1977 [16]. Combining very rapid, multiple readings with continuous cuvette blanking (to quantify nonspecific scatter) and microprocessor curve smoothing, the ICS used the maximal rate of increase in light scatter rather than endpoint scatter as was the case with the Technicon AIP. Both speed and precision of nephelometric assays were increased by this method. A hypothesis was proposed that the use of rate neutralized the "blank effect" thus increasing sensitivity. However, the sensitivity of nephelometric assays is in part proportional to the sample-to-blank (or sample-to-noise) ratio, since the photodetector "sees" the noise even if it is electronically subtracted from the signal. The practical detection limit, assuming high-precision measurements, is approximately equal to the mean blank reading plus 3 standard deviations [3].

Both Hyland and Behringwerke had previously introduced laser nephelometers which measured forward-angle light scatter (Table 1). Buffone and co-workers [17] had shown that "laser-induced near front surface light scattering", using a modified centrifugal analyzer, was a viable method for immunoassay. Because of size, cost and safety, the helium-neon laser was used. Although the intensity of the incident light is much greater with the laser, the higher wavelength partly cancels the advantage; for a given particle size and the same light intensity, the scatter of light at 632.8 nm is less than one-sixth that at 400 nm. However, as predicted by Rayleigh theory, this loss in sensitivity is recovered

Table 1
Characteristics of nephelometric immunoassay instruments

	Technicon AIP	Beckman ICS/Array	Hyland Laser PDQ	Behring Laser Nephelometer
Light source	mercury arc lamp	tungsten lamp with monochromator	helium–neon laser	helium–neon
Wavelength	355 nm	400–550 nm	632.8 nm	632.8 nm
Angle of measurement	90°	70°	31°	0–12°
Time of measurement	endpoint	kinetic	endpoint	timed/endpoint
Calculation method	test minus blank	maximum rate	test minus blank	test minus blank
Antigen excess detection	double peaks	addition*	addition*	addition*

* Addition of further antigen (or antibody) and evaluation of the subsequent reaction.

in part for endpoint measurements since relatively large particles (antigen–antibody complexes) are formed as the reaction nears completion [11].

Automation of nephelometric assays is important in increasing the precision not only of transfer by pipette but also of timing. Precise timing is critical particularly for rate or timed (non-endpoint) assays. In addition, automation and computerization improve data reduction. Beckman has marketed an automated version of the ICS since 1981; both Beckman and Behringwerke have recently introduced new automated instruments.

Other Considerations

In addition to the instruments, many other factors influence nephelometric assays. For maximum sensitivity and precision, background light scatter of both the sample and the antiserum should be as low as possible to minimize the "noise" level. For antisera, clarification can be accomplished by the use of IgG fractions, PEG treatment followed by filtration, water dialysis and other techniques. For some analytes with concentrations at or near the lower end of the assay range for direct nephelometry, such as C-reactive protein, pretreatment of samples with PEG is now routine. Clarification of samples is not always recommended, however, since any procedure may result in reduction in concentration of the analyte under consideration, such as the apolipoproteins.

Antiserum affinity and avidity are also important. High affinity — the initial attraction of antibodies to their respective antigenic epitopes — is essential for rate reactions, whereas high avidity, which influences lattice formation, is important for late timed or endpoint measurements. The two characteristics do not necessarily occur in parallel, as reported by Hudson *et al.* [18]. Flocculation may occur in some assay systems if affinity and avidity are too high, as was reported for PEG-enhanced assays of albumin and transferrin in the Technicon AIP [5]. This problem can be alleviated by removing PEG or by using another antiserum. Very rapid reaction rates with high affinity antisera may also be a problem in automated turbidimetric systems. For example, up to 30 or 40% of the reaction (and the resulting difference in measured light transmitted) may be missed if IgG is assayed on the COBAS BIO in assay type 7.5 (blank reading taken after mixing of antigen and antibody solutions) using very high affinity antisera (R. Liedtke, personal communication). This may be avoided by taking the blank reading just before addition of the antiserum (assay type 7.6).

As noted earlier, Bolton and coworkers [14] reported that moderate variation in particle size had relatively little effect on nephelometric measurements. However, it has become increasingly apparent that size may be important. Valette *et al.* reported in 1979 [19] that haptoglobin phenotype influenced quantification by nephelometry; haptoglobin 1-1, with a molecular weight of 85,000 Da, gives measurements about 35% higher than those of haptoglobin 2-1 (polymers with varying molecular weight) at 70–90° (Beckman and Technicon instruments); measurements were about 20% higher with forward scatter (Behring nephelometer). They demonstrated that this was not due to differences in antigenic epitopes. Delacroix and Vaerman [20] showed that concentrations of dimers, trimers, and tetramers of IgA and of secretory IgA are underestimated by 16–24% in the Technicon AIP system, compared with measurements of monomeric IgA. Ritchie reported similarly that Fc fragments or free gamma chains give readings about 30% higher than those of intact IgG molecules using antiserum to the Fc fragment and right angle scatter [5]. In each of these cases, as predicted by Rayleigh–Debye theory, smaller particles result in relatively higher scatter as the angle

of measurement increases up to 90° whereas lower angles give higher readings for large particles. Thus, the accuracy of measurement of antigens with varying size must be questioned in all light scattering techniques.

In general, very small antigens, such as haptens and small peptides, cannot be measured by direct light scattering immunoassay because of the lack of significant lattice formation. Inhibition methods for their assay are discussed in the next section.

Methods for Increasing Sensitivity

Direct nephelometry has a practical sensitivity limit for plasma proteins of about 1–20 mg/l (actual cuvette concentration), depending upon the analyte. The first practical method for increasing sensitivity was nephelometric inhibition using the Technicon AIP [21] or the Beckman ICS [22]. In this procedure, antigen solution is incubated with antiserum and a blank reading is taken. A fixed amount of "developing antigen" is then added and the difference in light scatter is determined. If low antiserum concentrations are combined with relatively high concentrations of developing antigen, the reaction occurs on the antigen-excess portion of the Heidelberger curve and the sample antigen "consumes" the high-affinity fraction of the antibodies. Sensitivity is thus increased by a factor of at least 10, compared with direct methods [22]. The introduction of nephelometric inhibition also permitted the quantification of haptens [21, 22]. Here the developer antigen (hapten) is conjugated to a carrier such as a protein; the reaction between the developer and the antiserum is inhibited by the presence of free hapten, just as with protein antigens.

Further increases in sensitivity can be obtained by particle enhancement. Antibody, developer antigen (with inhibition techniques) or both may be conjugated to any of several types of particles. For practical purposes, latex particles are most commonly used. As expected from light-scattering theory, forward-scatter methods work best with particle enhancement; the particles should be relatively small (0.05–0.02 μm) and of as nearly the same size as possible. In addition, measurement of particle-enhanced assays at 60–90° is deleteriously affected by the increase in background scatter by particles and by non-particle reactions in the solution. It is thus not surprising that Behring was the first company to introduce particle-enhanced direct (i.e. not inhibition) kits for the quantification of several proteins with their automated, forward-scatter nephelometer.

Several other particle-enhanced nephelometric techniques have also been reported. Quasi-elastic light scattering (QLS) is based in part upon the Doppler effect [23]. The wavelength of the light scattered changes in proportion to the size of the molecules scattering the light, as a reflection of changes in diffusion rates. In addition, larger particles with multiple scattering centres produce more phase interference in the scattered light (Fig. 2). In QLS, the fluctuation of the scatter about the mean is measured and analyzed as a reflection of these parameters. Inhibition methods are also applicable to QLS [24].

A second method, anisotropic light scatter, uses computer analysis of the changes in scatter at two angles (e.g. 90° and 10° from forward) to characterize particle aggregation. This is reported to be the most sensitive of all nephelometric assays to date, with a lower assay limit of about 0.1 ng/ml for hCG [25].

Because of the rapid development of automated, high-precision instruments for turbidimetric assay and the relatively greater influence of particle size on turbidimetric assays, it appears at present that turbidimetry may be preferable to nephelometry for

particle-enhanced assays. The application of this technique is discussed in detail in the chapter by Christopher Price. Turbidimetry is not without its problems, however, for routine (non-enhanced) assays. Major problems which must be addressed include the detection of and correction for differences in antigen size and the development of simple but effective methods of detecting antigen excess.

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[*Received for review 21 January 1987*]