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Nephelometric immunoassay with shell/core particles

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Abstract - Nephelometry has become a very reliable and convenient method for immunoassays. Recent innovations in polymer chemistry have led to the design of highly sensitive and reproducible immunoassays. Key advances include the preparation and use of particles with polystyrene cores covered by thin shells with chemically reactive groups. The shells allow a covalent binding of the immunochemicals of interest. Using these particles, a method for the determination of C-reactive protein, immunoglobulin E and myoglobin has been worked out.

INTRODUCTION

New analytical methods had been developed during the last years and were improved for routine application. One of these is the nephelometry. A precipitation reaction in solution with a short incubation time had already been published in 1935 by Heidelberger and Kendall (ref. 1), but could not be adapted for routine use until 1970, when Killingsworth and Savory (ref. 2) reported on a commercial nephelometer. The demand increased to measure parameters at the lower concentration as well. To achieve a higher sensitivity reaction enhancers like PEG (ref. 3) or dextran were used. With these new techniques the immunoprecipitation reaction was enhanced and measuring ranges as low as one to five mg/l could be reached.

It was finally the particle enhancement technology which brought the breakthrough in sensitivity. It brought an increase in sensitivity by a factor 1 000 and allowed nephelometry to compete with ELISA and RIA. This fast increase in sensitivity can be easily explained looking at the physical laws behind this. The intensity of scattered light is highly dependent on the diameter of the scattering particles. This is described in the light scattering laws of Rayleigh and Mie. During the particle enhanced immunoreaction particles aggregate with increasing diameter which resemble as regard to light scattering very large particles.

Consideral work has been performed on particle enhanced immunoassays, but only a few papers report advances in the design of latex particles (ref. 4). Most of these assays use polystyrene particles with either antigens or antibodies adsorbed on the particle surface. Polystyrene particles have a very high optical density and by thus allow the development of very sensitive light scattering tests. Covalent binding of antigens and antibodies was reported first by Grange in 1977 (ref. 5). This technique was improved later by Masson et al. (ref. 6) and Kapmeyer et al. (ref. 7). Covalent binding allows the design of a test with very high sensitivity as no bleeding can occur and of high shelf life. Detergents and chaotropic salts may be used in the test to prevent unspecific reactions.

METHODS

To combine the advantages of both systems, core/shell particles with covalent binding (ref. 8) had been invented. The core is made from polystyrene using well known emulsion polymerisation techniques. This process allows the preparation of particles with a very small size range. So polymer particles with a definite size are created. Polymere cores with a diameter of 180 nm or 250 nm were prepared. The particles can be examined by electron microscopy. They are perfect spheres with a narrow particle size range.

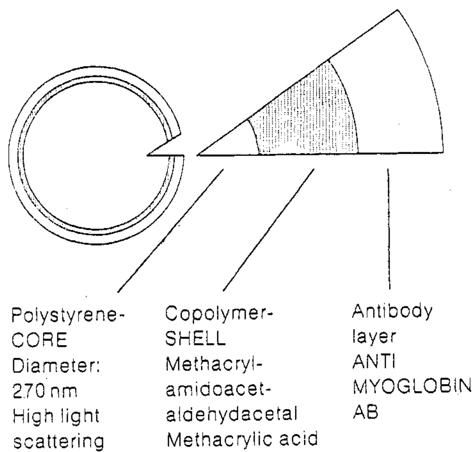

Fig. 1 Composition of N-Myoglobin Latex

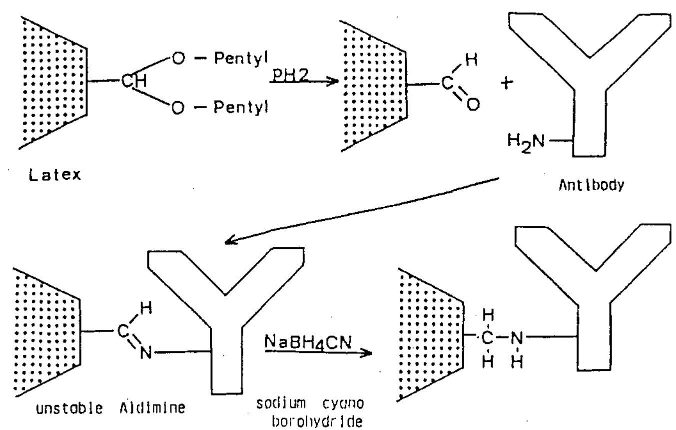

Fig. 2 Coupling of antibodies to latex particles

Around this core a shell is polymerized, some few nanometers thick (Fig. 1). For this process a mixture of several monomers is used. The shell polymer contains acetal groups derived from an acrylamidoacetaldehyde compound. The blocked aldehydes are utilized as linker groups because an acetal does not interfere in polymerisation. Methacrylic acid is used to stabilize the particles by charge. Hydroxypropyl-methacrylate is used to form a less hydrophobic particle surface. Hydrophylic particles are created, stabilized by the charged groups and containing the linker for covalent protein binding. For the preparation of highly sensitive plasmaprotein tests antibodies have to be fixed to the particle surface. For some tests a gammaglobulin fraction purified by DEAE cellulose chromatography can be utilized. In other cases the antibody has to be purified by immunoadsorption techniques. We prefer to use polyclonal antibodies from rabbit, but monoclonal antibodies can be used as well. For the covalent binding antibodies are mixed with a latex preparation in a buffered solution at pH 6 or 7. A detergent is added to prevent a non-specific agglutination of the reagent. Then the pH is lowered to 2 to set free the aldehyde groups. Between these and aminogroups of the antibody an imine is proposed as first reaction product. By reduction a stable alkyl-amine bond is formed (Fig. 2).

To analyze this binding process we interrupted at different steps of the procedure. When the binding was performed without the initial pH 2 step, but included the reduction process no covalent linkage should build up and thus an adsorptive binding is supposed to happen. When the cyanoborohydrid was not added but all other steps had been performed, the labile iminogroup was not stabilized, so a nonstable covalent binding has occured. We added increasing amounts of antibody to the latex preparations to make the binding experiments and determined the unbound protein after a centrifugation step. By this experiment we could determine the amount of fixed antibody (Fig. 3). By these sets of experiments we prooved that only the complete procedure ensures a high content of gammaglobulin bound to the particle surface. The unstable binding without cyanoborohydrid reduction gave a much lower antibody coating. The adsorptive binding procedure did not show anti-body content on the surface. This is not a contradiction to the well known adsorptive binding procedures because our latex has a less hydrophobic surface and the reagent is stabilized in a buffer solution with a high detergent concentration.

So only adsorptively bound antibodies were removed during the following centrifugation and resuspension step. The latex preparations of the binding experiment were checked for antigen binding by an incubation with a series of antigen concentrations (Fig. 4). After the appropriate incubation time light scattering was measured by nephelometry. Only a complete covalent binding gives a proper reaction curve. The incomplete binding with the instable imino linkage gives a much lower agglutination curve. The adsorptive binding gives nearly no agglutination reaction.

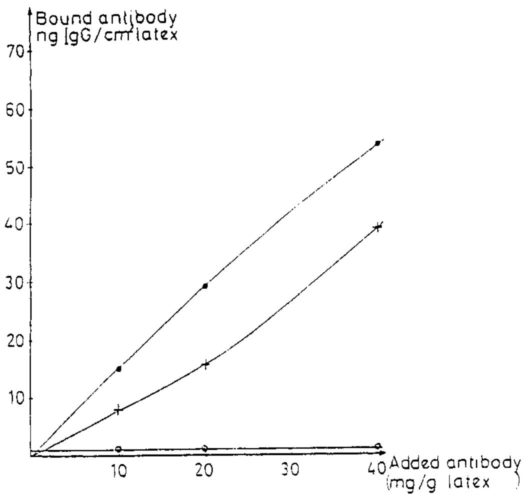

Fig. 3 Binding of anti-CRP antibody to latex in the presence of detergent. The final step of the procedure was the resuspension of reagent in 0.1 mol glycin buffer, containing NaCl and Tween[R] 20.

- ● = covalent coupling according to the complete procedure
- + = binding without the reduction step with sodium cyanoborohydrid
- o = adsorptive binding

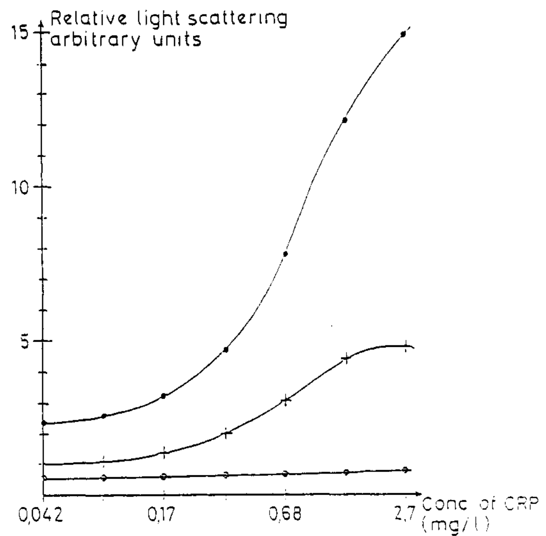

Fig. 4 Agglutination reaction for the 20 mg antibody/g latex preparation (Fig. 3). Reaction of latex reagent with CRP. A series of geometrical dilutions (1:10 - 1:640) of the CRP-standard was performed in N-Diluens, and the reaction with the reagent was measured on the analyzer.

- ● = covalent coupling according to the complete procedure
- + = binding without the reduction step with sodium cyanoborohydrid
- o = adsorptive binding

RESULTS

According to this technique we have developed reagents for the detection of C-reactive protein (ref. 9) (CRP), immunoglobulin E (ref. 10) (IgE) and myoglobin (ref. 11). The particle diameter for the CRP test is 180 nm because the sensitivity requirement for this parameter is not so high. The sample predilution is 1 : 400 which is performed automatically by the instrument. Because of this high predilution of the sample interfering factors from the serum are removed. The tests for IgE and myoglobin use 250 nm particles because of the higher sensitivity demand. Sample predilution is in both assays 1 : 20 for the basic measuring range. This means a range between 30 and 1 000 IU/ml for the IgE test and a range between 25 and 400 ng/ml for the myoglobin test (Fig. 5). Samples with higher concentrations of both proteins will be remeasured from the instrument automatically using a higher sample predilution. No false negative results at antigen excess are found for CRP-concentrations up to 1 600 mg/l, IgE-concentrations up to 24 000 IU/ml and myoglobin-concentrations up to 200000 ng/ml. Samples with a lower concentration of both parameters may be remeasured from a 1 : 5

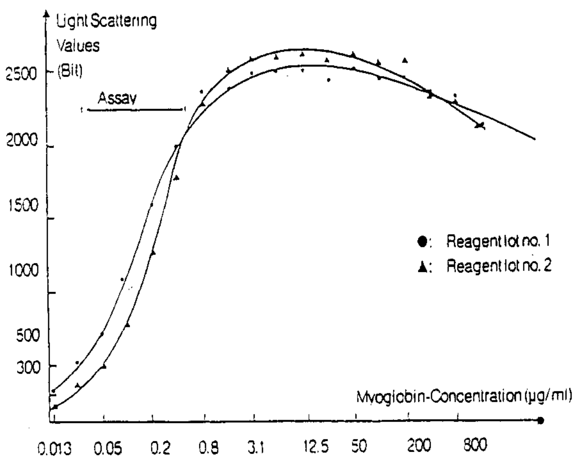

Fig. 5 N-Myoglobin Test. Assay range and antigen excess.

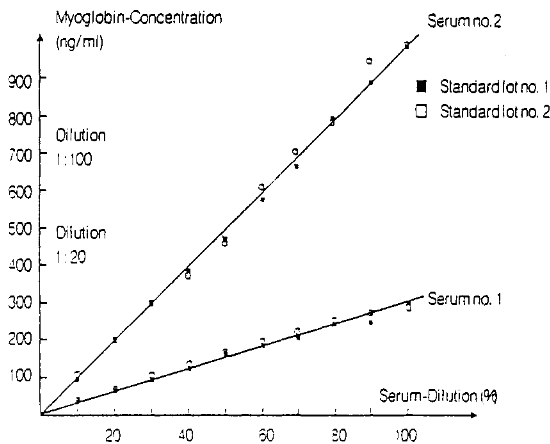

Fig. 6 N-Myoglobin Test. Linearity studies. Measurement of 2 sera.

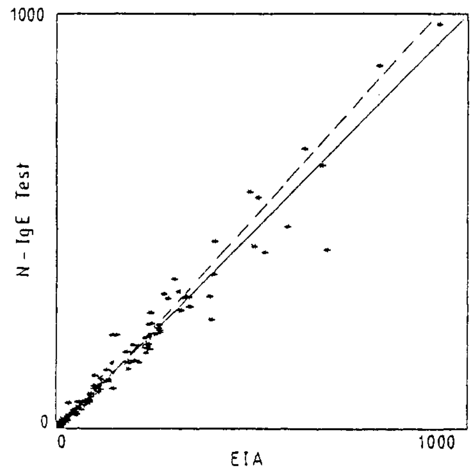

Fig. 7 N-IgE Test. Correlation study. Comparison with EIA.

Linear regression:

N-IgE (y) = 1.085 EIA (x) - 10.06 IU/ml
Correlation coefficient: r = 0.985
Number of samples: n = 144

sample predilution to increase sensitivity. Because of the lower sample predilution of the IgE and the myoglobin test special precautions have to be undertaken to prevent unspecific reactions from interfering factors in the patient sera. For this reason special supplement reagents were developed which contain detergents and special salts. To remove interference from rheumatoid factors the supplement reagents contain high concentrations of rabbit immunoglobulins as well.

The test precision for the three latex-enhanced assays is good. The intra assay variance studies gave values below 5 %. For the inter assay variance (between independent assays) coefficients of variation are below 8 %. The linearity of all three tests is given throughout the measuring ranges (Fig. 6). For these tests no interference could be observed when high levels of rheumatoid factors are present in serum. Bilirubin and hemoglobin at increased concentrations do not interfere.

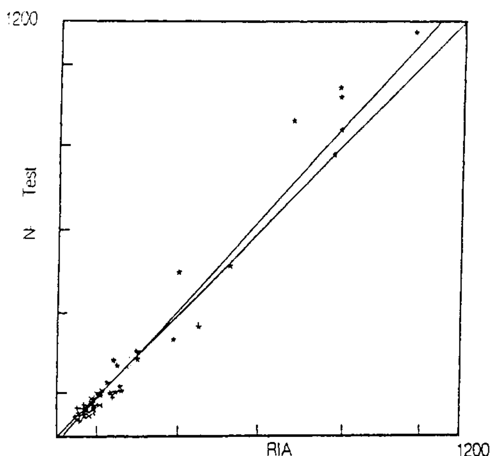

Fig. 8 N-Myoglobin Test. Correlation study. Comparison with RIA.

Linear regression analysis:

N-Myo (y) = 1.08 RIA (x) - 18.89 ng/ml
Correlation coefficient: r = 0.999
Number of samples: n = 41

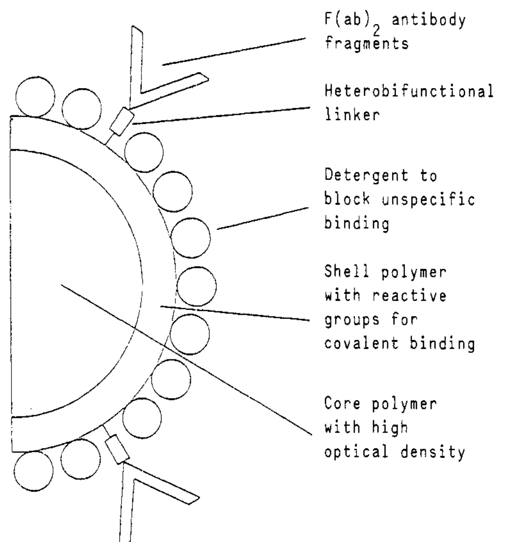

Fig. 9 "Ideal" Reagent Particles

Lipemic samples which exhibit a high turbidity might have to be clearified by a centrifugation step prior to measurement. Correlation studies were performed with classical tests for the three proteins. The CRP test showed a good agreement with the radial immuno diffusion. The IgE test was correlated with an enzyme immunoassay and gave excellent results (Fig. 7). The myoglobin test was compared with a radio immuno assay, the correlation showed very good results as well (Fig. 8). For all three tests regression analysis indicated a good conformity between the corresponding assays. The nephelometric particle-enhanced tests utilize a simple test protocol. They are fully compatible to all other tests on the analyzer. So profiles with other tests can be run for patient-by-patient operation. The reaction time is short.

SUMMARY

An ideal latex reagent could be designed according to the principles of latex technology which were examplified in this report (Fig. 9). Such a reagent should have a core/shell structure with a high optical density of the core and a thin shell featuring special anchor groups, charged groups for stabilization and a hydrophylic polymer surface. The binding procedure should be simple to perform at a physiological pH and without interfering side reactions. Bifunctional spacers may have advantages for positioning antibodies in such a way that their recognition sites are freely movable and well enough away from an interfering polymer surface. Coupling of $F(ab)_2$ fragments can be advantageous for preventing interference with rheumatoid factors which may derive from the Fc portion. But some antibodies lose activity during this fragmentation step. An important point is to bind the antibody in such a way that the antibody recognition sites show away from the polymer surface. This may be achieved by utilizing a SH-group in the Fc portion for binding. To achieve a maximum sensitivity of the test the particles should have a diameter between 0.1 and 0.4 µm. The particle size has to be adjusted to the wavelength of the instrument which is used for the test. Instruments using a low wavelength work better with smaller particles. Instruments with a high wavelength as for instance in the infra-red are better suited for larger particles. As a perfect particle system is rather complex it may only be required for a very sensitive test.

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