Feature Article

Fast Reading of C-Reactive Protein in Whole Blood in Hematology Analyzers

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A new homogeneous immunoassay of whole blood C-reactive protein based on the use of magnetic field-assisted agglutination of polyclonal anti-CRP coated superparamagnetic particles is described. These particles self-organize into linear chains under an appropriate magnetic field which considerably increase the colliding frequency and thus, the probability to form links between ligand and receptor. Therefore, reaction rates are highly accelerated so that results are obtained well before the system has reached equilibrium. This immunoassay can be adapted to perform high sensitivity as well as high speed assays depending on analyte concentration. The high capabilities of magnetic agglutination immunoassay allow the integration of a whole blood CRP immunoassay to an existing high speed hematology analyzer. The feasibility of such a concept is reported with a prototype that combines the sampling process of a hematology analyzer with an immunodetection module. As a result, a complete whole blood CRP analysis cycle is achieved in less than one minute. The successful application of these investigations should reinforce the competitiveness and leading position of HORIBA medical in whole blood immunoassay.

Introduction

C-Reactive Protein (CRP) is a well-established biomarker in clinical laboratories for the diagnosis of infection or inflammation. It is an acute-phase protein whose concentration increases rapidly in response to various stimuli including bacterial infection, inflammation, trauma, surgery. Many commercially available CRP immunoassays performed on plasma or serum samples are mainly based on particle-enhanced turbidimetry or nephelometry using latex microbeads.[1] This technology has been further adapted to whole blood samples by HORIBA Medical. Actually, C-reactive protein assay in whole blood where blood cells are present (leucocytes, erythrocytes, thrombocytes) has led to specific developments in HORIBA Medical.[2] For example, an analyzer such as ABX MicrosCRP200 provides biologists with efficient equipment which allows performing in less than 5 minutes a Complete Blood Count (CBC) including the determination of 18 parameters together with a CRP immunoassay in the range 2-200 mg/L. The determination of all these parameters on a unique blood sample which avoids separation of plasma or serum from blood cells, constitutes an important procedure simplification and reduction of analysis time. This was made possible by use of an infrared light source for which cell debris and hemoglobin diffusion/absorption is negligible with respect to the specific signal due to particle aggregates formation in response to the presence of CRP. With latex beads, formation of aggregates results roughly from two events, capture of the antigen by the beads and formation of links between beads. However, the agglutination rate which obviously depends on antigen concentration and antibody surface density, is also limited by particles diffusion. The technique proposed in this article, first described by Baudry et al.[3] and further developed by HORIBA Medical for blood proteins, allows a considerable reduction of reaction time between particles due to the use of superparamagnetic particles whose aggregation is accelerated by a magnetic field.

This article describes the adaptation of a magnetic agglutination assay to whole blood CRP which combines high analytical sensitivity and fast reading. This technique fast enough to be integrated to a high speed hematology analyzer (60-120 tests/h), will offer new opportunities for HORIBA Medical within routine high throughput hematology analyzers.

Magnetic Agglutination Assay:

Magnetic agglutination assay is a homogeneous assay like usual Latex Agglutination Assay (LAA) used in many diagnostic assays.[4] In LAA, particles grafted with a specific antibody self-aggregate in the presence of the antigen. However aggregation rate is limited by diffusion time. In the case of magnetic particles assay, aggregates formation is accelerated by submitting the particle suspension to a homogeneous magnetic field reducing the diffusion time. Actually, a colloidal suspension of superparamagnetic particles can form linear chains under an appropriate magnetic field. This chaining process brings the particles together, considerably increasing colliding frequencies between adjacent particles, which leads to a substantial increase of antibody-antigen bond formation rate. Upon release of the magnetic field, unreacted particles and aggregates rapidly diffuse in the solution (Figure 1). Aggregates are then detected by light scattering, the signal is expressed as the difference in optical density at 650 nm (OD650 nm) measured before and after application of the magnetic field. Whereas analytical sensitivity is in the nanomolar range for LAA, it drops to the picomolar range for magnetic agglutination assay which makes it comparable to heterogeneous approach such as ELISA (enzyme-linked immunosorbent assay) in terms of sensitivity, but much faster.

The accelerating effect of magnetic field is illustrated on Figure 2. Progress curves obtained for magnetic particles grafted with an anti-CRP antibody in the presence of 14 picomoles/L of C-reactive protein (CRP) with and without several pulses of a magnetic field of 20 mTesla (mT), were compared. In the absence of magnetic field, no significant change in the optical density was observed indicating that nearly no reaction occurs between particles. On the contrary, under a field, particles aggregation took place, causing a progressive increase of optical density which demonstrates the extent of the accelerating effect induced by the magnetic field. Typical aggregation kinetics is shown on Figure 3. Repeated pulses of magnetic field corresponding to alternating particles chaining and relaxation phases lead to a progressive increase of suspension turbidity. Particles chaining starts when magnetic field is on. In the first 100 msec, a drop of OD is observed which corresponds to the orientation of already formed aggregates in the direction of the field, and then the chaining process itself occurs, resulting in a slow decrease of OD. When magnetic field is off, relaxation of the system takes place. Disruption of chains leads to an almost instantaneous increase of turbidity. This rapid phase is followed by a slow decrease of OD which corresponds to the breakdown of non-specific aggregates,

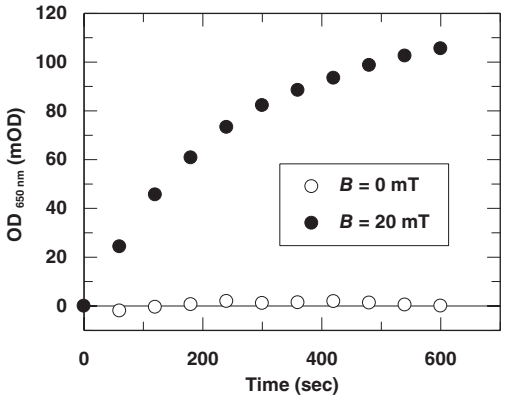

Figure 2 Progress curves obtained with magnetic particles (200 nm) grafted with an anti-CRP antibody in the presence of 14 pM of CRP, with or without pulses of a magnetic field of 20 mT.

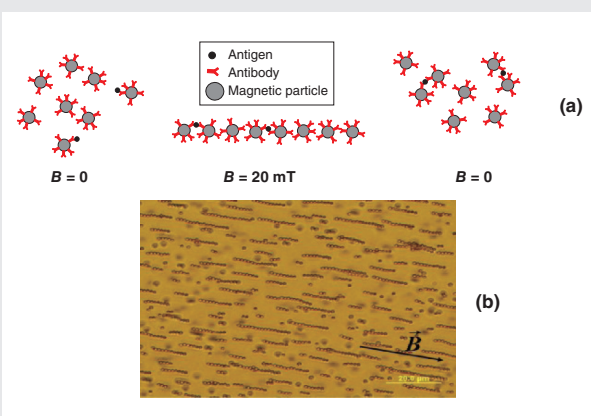

Figure 1 Principle of magnetic agglutination assay.
(a) Chaining process; (b) Linear chains of magnetic particles (1 µm, Dynal) under a magnetic field (*B*). The arrow indicates field direction.

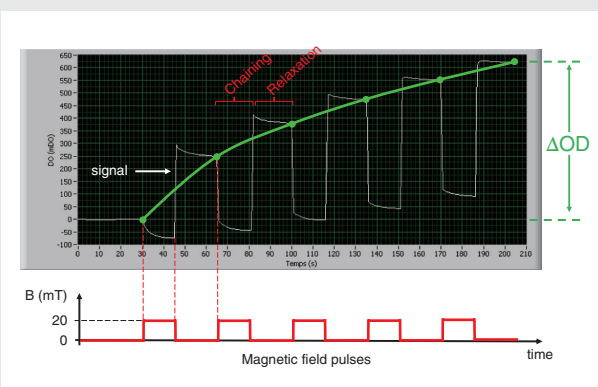

Figure 3 Optical density variation during field-induced aggregation of magnetic particles (200 nm) grafted with an anti-CRP antibody in the presence of CRP. Magnetic field was 20 mT.

and finally a plateau is reached whose level is proportional to the number of stable bonds formed between particles.

C-Reactive Protein Assay in Whole Blood

First results were obtained with an in-house built magneto-optical device described in Figure 4(a). This device included a disposable spectrophotometric cuvette surrounded by an electromagnet able to deliver a roughly homogeneous field, a LED emitting at 650 nm as a light source and a photodiode. Sample illumination and light collection were performed by optical fibers. Carboxyl-modified superparamagnetic particle of 200 nm diameter (Ademtech, Pessac France) were covalently coated with a goat polyclonal antibody directed against human CRP (Meridian life sciences, Memphis, USA) using a carbodiimide coupling chemistry. Before mixing with particles, blood sample was first diluted in a buffer containing saponin in order to disrupt blood cells. Then the diluted sample was mixed with particles in the measuring cell, and 3 pulses of 30 sec of magnetization at 20 mT followed by 30 sec of relaxation, were applied. Aggregation kinetics was monitored at 650 nm, ΔOD was plotted against CRP concentration (Figure 4(b)). For CRP concentrations less than 20 mg/L, ΔOD is directly proportional to the amount of CRP. This linear relationship corresponds mainly to the formation of only doublets. Then the slope of the curve decreases progressively and for CRP concentrations higher than 200 mg/L, ΔOD finally decreases slightly. In LAA, this is usually explained by a progressive saturation of grafted antibodies by excess antigen or "hook effect" which is characterized by a bell-shaped curve. Rather, the curve profile obtained in the conditions described above for the magnetic agglutination assay could mainly result from an optical effect due to formation of large aggregates when CRP exceeds 20 mg/L[5]. High dilution of blood sample together with detection at a wavelength where hemoglobin absorption is minimal allowed a drastic reduction of interferences due to cell debris and hemoglobin. Dynamic range and analytical sensitivity can be modulated through modification of either dilution factor or total magnetization time, or both. This is illustrated in Figure 5. When blood sample was diluted 150 times and magnetization time was 105 sec, dynamic range was approximately from 0.1 to 10 mg/L. With a 1:1500 dilution and magnetization time of 10 sec, dynamic range was approximately from 2 to 200 mg/L. Thus, with simple modifications of sample treatment and conditions of magnetization, a dynamic range of about three orders of magnitude could be explored in whole blood.

Fast Reading Automated Assay

Our objective is to combine an immunoassay module to an existing high speed hematology unit such as Pentra80 or Pentra120 in order to perform simultaneous biochemical and hematology analyses without impairing hematology analyses rate. Fast CRP immunoassay described above should fulfill the conditions to reach this goal. The flow diagram of such a machine is reported in Figure 6. A transfer module including a sampling valve

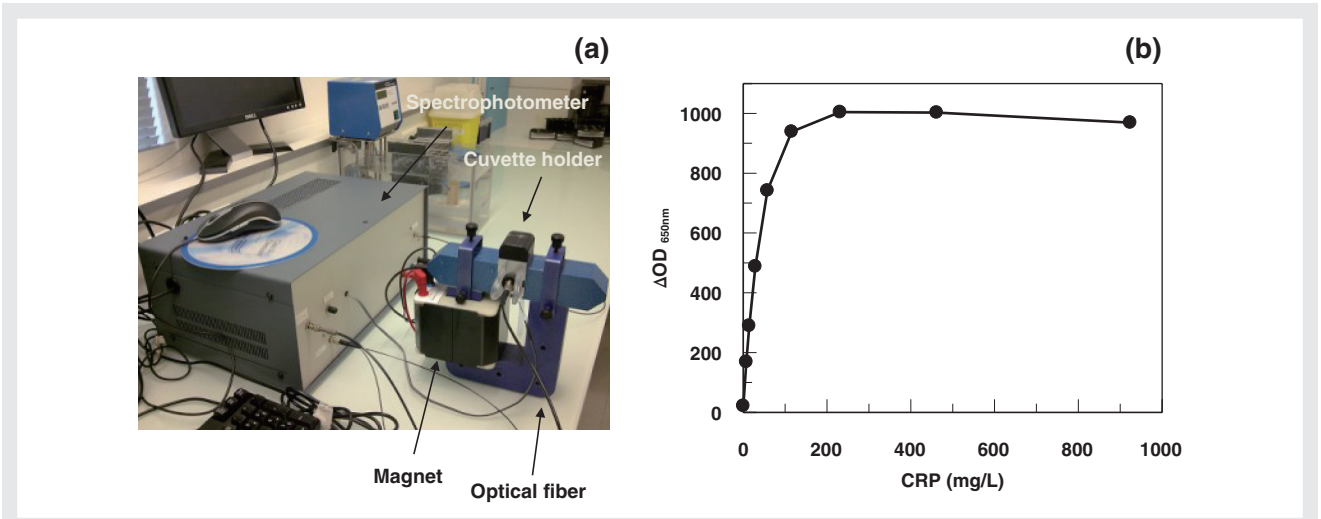

Figure 4 Whole blood CRP immunoassay.
(a) Magneto-optical device.
(b) Dose-response curve obtained using magnetic particles (200 nm) grafted with a polyclonal anti- human CRP with 3 × 30 sec of a field of 20 mT. Final sample dilution is 1:1500.

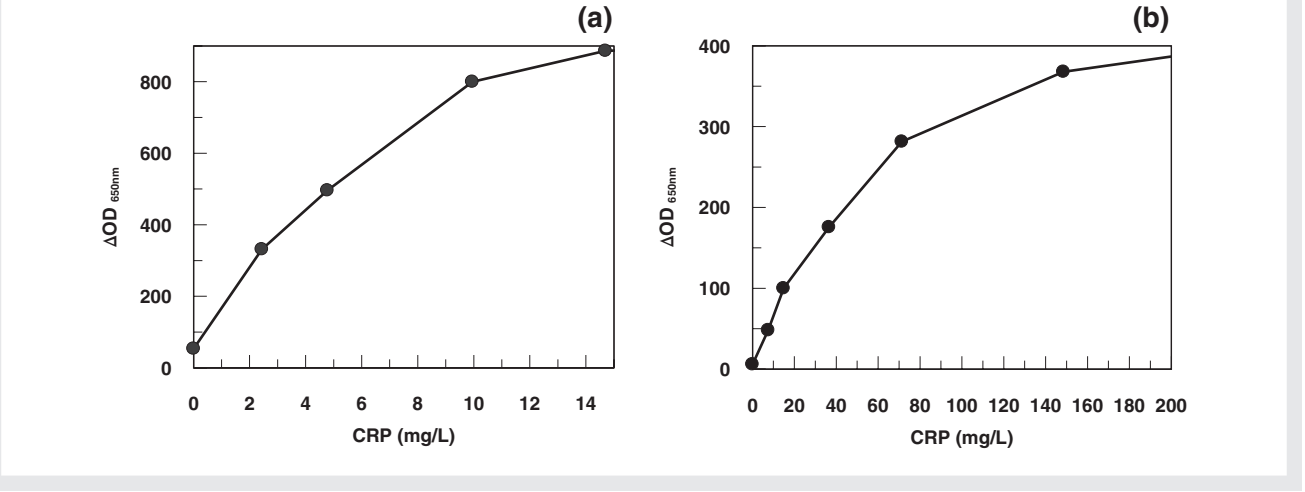

Figure 5 Dose-response curves of whole blood CRP in different conditions.
(a) Dilution factor 150, total magnetization time 105 sec, (b) Dilution factor 1500, magnetization time 10 sec

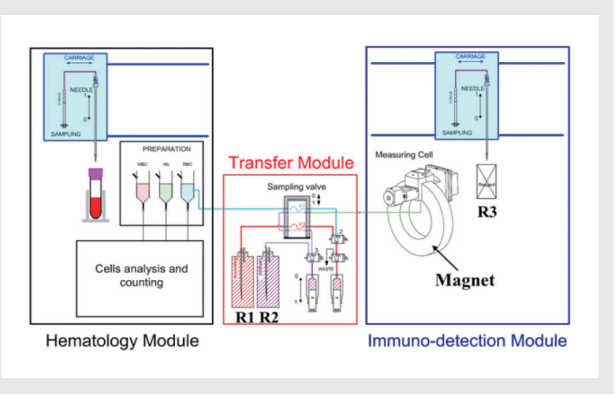

Figure 6

and two syringes for R1 (Saponin solution) and R2 (Buffer solution) will constitute an interface between hematology and immunodetection modules. The hematology module will typically comprise a sampling needle, preparation tanks for hemoglobin determination, and blood cell analyses, and a counting device. The immunodetection module will include measuring cell, magnet, optical unit and sampling needle for R3 (magnetic beads). In this approach, there will not be a specific pre-dilution tank for CRP analysis; the immunodetection module will use a pre-diluted blood sample from one of the preparation tanks of the hematology module. This configuration will allow simultaneous cell counting and CRP immunoassay at high speed (patent pending).

With this goal in mind, preliminary results were obtained with a prototype built in a Pentra60 frame which simulated such a configuration, i.e. only the sampling and dilution sections of a hematology module associated with the transfer and immunodetection modules.

The sequence of events was as follows

First step: a blood sample was taken and subsequently prediluted (1:40) in Eosinofix which is the reagent used in hematology analyzers such as Pentra80 for white blood cell analysis. In the meantime, the sampling valve of the transfer module was loaded with R1 and R2

Second step: diluted sample was loaded in the sampling valve while R3 was taken by the needle of the immunodetection module.

Third step: the diluted sample, R1 and R2 were transferred from the transfer module to the immunodetection module, added to the measuring cell together with R3 and rapidly mixed by air bubbling. Magnetization cycle then began including 7 sec of magnetization, and 3 sec of relaxation after which OD was recorded. Total reaction volume was 500 µL. Final sample dilution was 1:1500.

The complete cycle was achieved in 45 sec before next sample analysis. These conditions should be fully compatible with analysis rate of a P80, i.e. 80 samples analyzed per hour.

The imprecision profile of the assay obtained with various amounts of serum CRP indicated that CVs were less than 5% from 1 to 250 mg/L, and less than 3% from 2.5 to 100 mg/L. Detection limit determined on blood samples was less than 1 mg/L(*) and no “hook effect” was observed for blood CRP up to 1 g/L.

A preliminary correlation study was performed against the ABX MicrosCRP200 with 25 samples constituted of a pool of human EDTA-blood spiked with various amounts of purified human CRP (Euromedex, France) (Figure 7). Prototype calibration was performed with ABX Pentra400 serum CRP calibrators (ABX Pentra CRP Cal, HORIBA Medical). Measured blood CRP concentrations were corrected with hematocrit value (HCT) determined on ABX Micros CRP200.

Although very preliminary, these results were quite

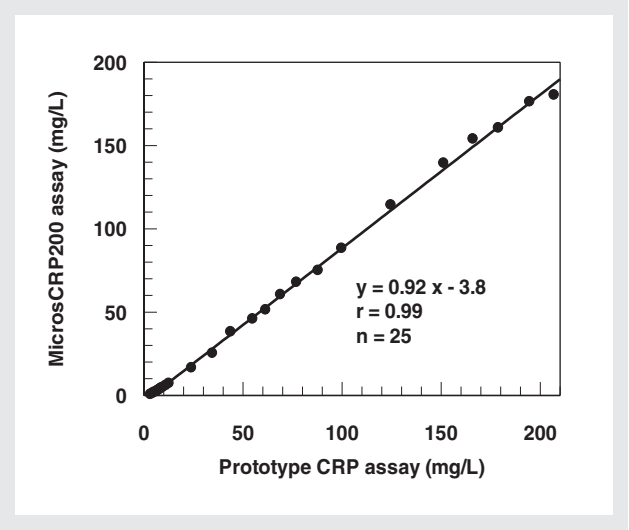

Figure 7 Correlation between prototype and HORIBA MicrosCRP200 using EDTA-blood samples spiked with purified human CRP. Concentrations were corrected with HCT.

satisfactory and promising. The high y-intercept value of the correlation curve was essentially due to not corrected differences in optical density observed between blood CRP and the serum CRP calibrators used for the prototype. These differences are related to a "matrix effect". Actually, we found that the presence in normal blood (or serum) of high molecular weight proteins caused some non-specific interactions between magnetic beads. Surprisingly, these interfering proteins are absent from the CRP calibrators used, probably eliminated by a chemical or physical treatment of serum during the production process. This matrix effect is considerably reduced by addition of an appropriate concentration of saponin or other related detergents to the reaction mixture. However, in the example reported here, it is possible that the prototype did not deliver the right quantity of saponin. Efforts are underway to improve this particular point and the overall analytical performances of the prototype. We are also focusing on the reduction of reaction volume to 300 µL in order to limit reagent consumption and waste.

Conclusion

Magnetic agglutination assay is a very sensitive and flexible technique. One can perform high sensitivity assays in few minutes or fast medium sensitivity assays in few seconds. This allows determination of low abundant blood proteins (~ 10-100 µg/L) as well as high throughput assays of more abundant proteins (>1 mg/L) such as C-reactive protein. Work is underway to adapt this technology to Pentra hematology analyzers. Throughout these investigations and results, the uniqueness and competitiveness of HORIBA Medical in the field should be maintained over years, taking into account these last achievements.

However, despite its sensitivity, the method is limited to the detection of few picomoles/L of an antigen. There are different causes for this limitation; one comes from the optical detection system itself, i.e. turbidity measurement for which the limit of resolution is higher than a 1% intensity variation (3). We are currently working on a different optical approach that should lower the detection limits down to a few hundreds of femtomoles/L.

(*) Detection limit was determined empirically and represents the lowest CRP concentration at which the mean value minus2 sigma does not overlap with blank mean value plus 2 sigma.

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