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Interfering lipoproteins in magnetic field-assisted agglutination of superparamagnetic particles immunoassay

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ABSTRACT

Objective: The technology of magnetic field-assisted immuno-agglutination of superparamagnetic particles allows sensitive detection of biomarkers in whole blood. However, we observed non-specific agglutination (NSA), due to interfering plasma proteins, that negatively affects C-reactive protein immunoassay. The objective of the study was to identify the plasma proteins involved and to eliminate these interferences.
Design and methods: Plasma was fractionated by size exclusion HPLC and each fraction was tested for non-specific agglutination. In addition, plasma proteins bound to magnetic particles were analyzed by SDS-gel electrophoresis and identified by mass spectrometry.
Results: We found that NSA was due to the binding of some lipoproteins to the particles. NSA was observed in the presence of purified LDL and VLDL but not HDL. NSA was mediated by the binding of ApoB100 to magnetic particles through its heparin binding sites. These interferences could be eliminated by addition of heparin or other polyanions like dextran sulfate to the assay buffer.
Conclusion: NSA results from the binding of some plasma lipoproteins to magnetic particles. The use of a polyanion to eliminate these interferences allows the formulation of a stable reagent.

1. Introduction

Magnetic field-assisted agglutination of superparamagnetic particles is a powerful immunoassay method for routine analyses of biomarkers such as C-reactive protein (CRP) directly in whole blood. The acceleration of particle aggregate formation induced by the magnetic field make this technology much more sensitive and faster than the widely used latex agglutination technique. Furthermore, this technology has been successfully integrated in a hematology analyzer combining simultaneously fast CRP assay and a complete blood count on the same blood sample (patent US2014377771). However, like other immunological methods, it is subject to non-specific interactions, especially with samples containing high concentrations of proteins such as blood and blood derivatives. This represents a serious drawback that can result in significant and unacceptable performance degradation of the assay.

Non-specific interactions are of different types: interaction of some blood proteins with antibodies used in the assay as

Abbreviations: NSA, non-specific agglutination; LDL, VLDL, HDL, low density, very low density and high density lipoprotein, respectively; CRP, C-reactive protein; SMP, superparamagnetic particles; SEC-HPLC, size exclusion chromatography-high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; DXS, dextran sulfate; ApoA1, ApoB100, apolipoprotein A1 and B100, respectively.

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observed with rheumatoid factor or heterophile antibodies [11], or direct binding of proteins on particle surfaces. It has been shown that various blood proteins spontaneously bind to polymeric particles forming a corona [8]. The protein composition of this corona depends largely on particle surface material, the plasma concentration as well as on/off rate constants for each protein and hydrophobic/hydrophilic and/or electrostatic interactions [3].

In this article, we describe the identification of plasma proteins responsible for non-specific agglutination in our immunoassay and propose a solution to completely eliminate these interactions.

2. Materials and methods

2.1. Chemicals

Porcine heparin, purified apolipoproteins, and Lipid Removal Adsorbent were from Sigma (Sigma-Aldrich Chimie, Lyon, France). Purified lipoproteins were from Interchim (Montlucon, France). CRP-free plasma was from BBI solutions (Le Perray en Yvelines, France).

2.2. Magnetic agglutination

Carboxyl-modified superparamagnetic particles of 200 nm diameter (Carboxyl Adembeads, Ademtech, Pessac, France) comprising a magnetic core surrounded by a layer of polymer, were covalently coated with a goat polyclonal antibody directed against human C-reactive protein (Meridian Life Sciences, Memphis, TN, USA) using a carbodiimide coupling chemistry. Magnetic agglutination assay was performed as previously described [4]. Briefly, a homogeneous magnetic field generated by an electromagnet is applied to the reaction medium for 7 s at 20 mT. Then the magnetic field is turned off and the optical density is measured at 650 nm after 3 s of relaxation. The signal is expressed as the difference in optical density before and after application of the field. For routine CRP assay, blood samples were first diluted in Horiba lysis buffer (ABX Eosinofix, Horiba Medical, Montpellier, France). An aliquot was then taken and mixed with the assay buffer (glycine 50 mM pH8.5) and magnetic particles (0.04%). The final sample dilution factor was 1500.

2.3. SDS-PAGE analysis of proteins bound to magnetic particles

Magnetic particles were incubated either with 100 μL of CRP-free human plasma or purified lipoproteins for 30 min at 25 °C, washed, re-suspended in non-reducing SDS buffer, and heated at 95 °C for 5 min. SDS-PAGE analysis of the supernatant was performed in preformed SDS-polyacrylamide gels (Biorad, Hercules, CA, USA). Proteins were stained with Coomassie Blue G250.

2.4. In-gel trypsin digestion and mass spectrometry

In-gel trypsin digestion was carried out essentially as described previously [9]. Briefly, following SDS-PAGE, stained protein bands were cut out from the gel and digested with sequence-grade modified bovine trypsin (Promega Corporation, Madison, WI, USA). The peptides generated were subsequently extracted and submitted to mass spectrometry on a MALDI-TOF instrument (Ultraflex, Bruker Corporation, Billerica, MA, USA). Monoisotopic peptide masses obtained were used for protein identification by database searching.

2.5. Size exclusion HPLC

Human plasma (100 μL) was fractionated by SEC-HPLC on a Yarra 3 μ SEC-3000 column (300 × 7.8 mm, Phenomenex, Macclesfield, UK) at 1 ml/min in 50 mM phosphate, NaCl 300 mM, pH 6.8. UV absorption was monitored at 230 nm. The column was calibrated using molecular weight standards (Biorad).

2.6. Cholesterol assay

The presence of cholesterol bound to magnetic particles after incubation with human plasma was determined by an enzymatic assay using ABX Cholesterol CP (Horiba Medical). Superparamagnetic particles (SMP) (0.4%) were incubated with a pool of human plasma or 0.9% saline solution for 15 min at room temperature, and then washed with a glycine buffer (50 mM, pH 8.5). Washed particles were re-suspended directly in cholesterol assay reagent and incubated at 37 °C for 7 min. Absorbance at 505 nm was measured on the supernatant. In some experiments, dextran sulfate (DXS; 1 g/L) was added to the plasma sample.

2.7. Other assays

Plasma cholesterol, ApoA1, ApoB100 and CRP were determined on a ABX Pentra 400 automatic analyzer (HORIBA Medical, Montpellier, France) using dedicated reagents.

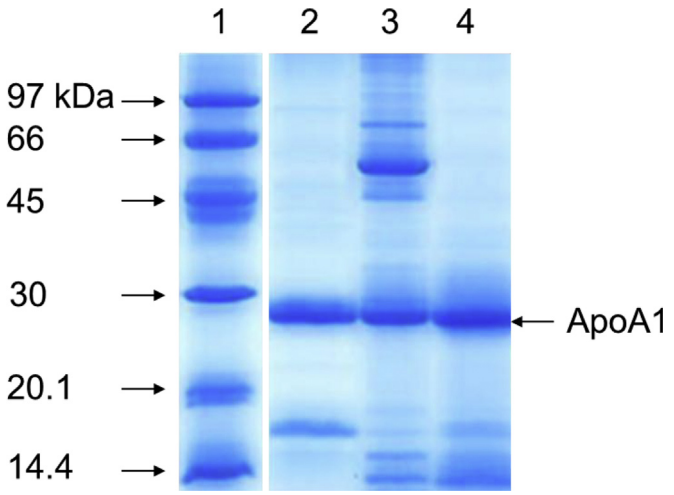

Fig. 1. SDS-PAGE analysis (12%), under non-reducing conditions, of proteins bound to magnetic particles: (1) molecular weight standards (reduced); (2) purified HDL; (3) proteins bound to SMP after incubation with human CRP-depleted plasma. The protein band at about 50 kDa co-migrated with human serum albumin; (4) proteins bound to SMP after incubation with purified HDL.

3. Results and discussion

3.1. Lipoproteins are involved in non-specific agglutination of magnetic particles

Magnetic field-assisted agglutination of superparamagnetic particles (SMP) is the basis of a new homogeneous immunoassay with high sensitivity and reduced analysis time [1]. During the development of a C-reactive protein immunoassay in whole blood based on this technology, we observed significant non-specific agglutination (NSA) of SMP when submitted to a magnetic field despite an important sample dilution.

NSA was observed independently of the animal origin of the antibody immobilized on SMP. It was also observed with SMP not grafted with antibodies. This ruled out possible involvement of heterophile antibodies or rheumatoid factor, but seemed directly related to the SMP surface itself. It has been shown that numerous blood proteins were able to bind to the surface of the nanoparticles forming a corona and that the protein composition of this corona depended on plasma dilution [2]. Thus, it was hypothesized that some of these particle binders could have been involved in NSA.

It is well known that many plasma proteins such as albumin and immunoglobulins bind to polymer particles. However, we found that none of these proteins were capable of inducing NSA. In order to know which proteins could bind to SMP surface, grafted SMP were pre-incubated with human CRP-depleted plasma. After washing, particles were re-dispersed in a denaturing but non-reducing sample buffer in order not to release immobilized antibody fragments. The supernatant was then analyzed by SDS-PAGE (Fig. 1). As well as human serum albumin, a prominent protein band at about 28 kDa was found associated with the particles. This band was excised from the gel, and digested with trypsin. Trypsin-generated peptides were eluted from the gel and submitted to MALDI-TOF mass spectrometry. The peptide mass profile obtained was unambiguously attributed to ApoA1 (data not shown). The same protein band was found when particles were incubated with

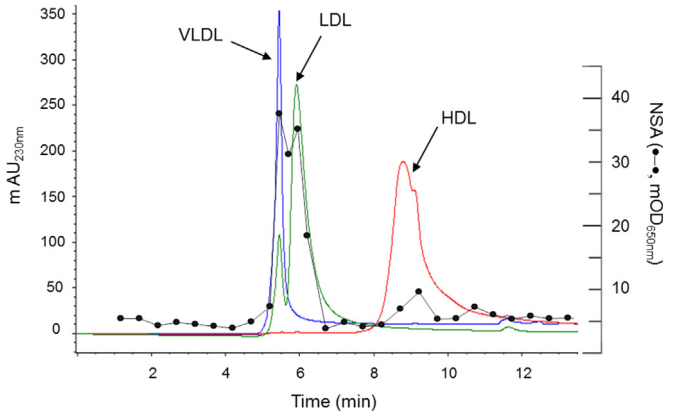

Fig. 2. Fractionation of human plasma by SEC-HPLC. Human plasma (100 μL) was loaded on the column and fraction (0.25 mL) were tested for NSA (●). Purified lipoproteins were analyzed under the same conditions (lines marked VLDL, LDL and HDL).

purified HDL (Fig. 1, lane 4). In order to know whether binding of ApoA1 was correlated with NSA, SMP were submitted to a magnetic field in the presence of different amounts of purified ApoA1. However, NSA was not observed which demonstrated that ApoA1 was not responsible for NSA.

However, we observed that a pool of plasma previously delipidated after treatment with LRA (lipid removal adsorbent) was no longer capable of inducing any agglutination of SMP under a field. This result strongly suggested the involvement of lipoproteins in non-specific agglutination of SMP. ApoA1 is the major protein constituent of HDL and we theorized that HDL instead of isolated ApoA1 was responsible for NSA. However, purified HDL was not capable of inducing any agglutination of SMP when submitted to a magnetic field. This result indicated that other lipoproteins were likely to be involved in NSA.

3.2. Lipoproteins other than HDL are responsible for NSA

In order to further confirm this hypothesis, a pool of CRP-depleted human plasma was submitted to size exclusion chromatography (SEC-HPLC) (Fig. 2). Fractions (0.25 ml) were collected and tested for NSA on grafted SMP under a magnetic field. We found that fractions inducing NSA were eluted as a double peak corresponding to high molecular compounds of more than 700 kDa. Thus if lipoproteins are involved in NSA, this points to high molecular weight structures such as LDL or VLDL. In order to identify which type of lipoproteins was responsible for NSA, purified VLDL, LDL and HDL were successively loaded on the SEC-HPLC column. We found that the double peak inducing NSA was eluted with retention times corresponding to those of VLDL and LDL. The small peak present at RT = 9 min, was due to residual amounts of pentameric human CRP still present in the CRP-depleted plasma used.

The role of LDL in NSA was further confirmed by using purified human LDL as the sample in a magnetic agglutination assay. The results clearly showed that LDL induced NSA under a field in a dose-dependent manner (Fig. 3). The curve reached a maximum and then slowly decreased. This behavior resembled a hook effect and suggested direct binding of LDL to the particles resulting in the formation of aggregates. As the LDL concentration increased, binding sites were progressively saturated which resulted in a reduction of aggregate formation. Indeed, it has already been shown that complete lipoproteins could bind to polymer particles [5]. Thus, if whole LDL particles could bind to SMP, we should find the main components of LDL (i.e. ApoB100 and such as cholesterol) associated with the particles.

To address this question, grafted magnetic particles were incubated with purified LDL in buffer, and subsequently washed several times to remove any loosely-bound material. A fraction of these particles was incubated with a non-reducing SDS denaturing buffer. The supernatant was analyzed by SDS-PAGE. The protein profile obtained showed a high molecular weight band which co-migrated with purified human ApoB100 (Fig. 4).

In order to determine whether complete LDL was bound to SMP, the remainder of the washed particles were assayed for cholesterol using a routine enzymatic assay. The absorbance obtained was significantly higher when SMP were incubated with LDL than with physiological saline, which indicated that cholesterol was bound to SMP (Table 1). These results confirmed that not only apoB100 but intact LDL was able to bind to magnetic particles. The binding of LDL probably induced agglutination of SMP by forming bridges between particles when submitted to a magnetic field.

3.3. Heparin eliminates NSA

Non-specific agglutination of SMP could be significantly reduced by increasing the ionic strength of the assay. This result

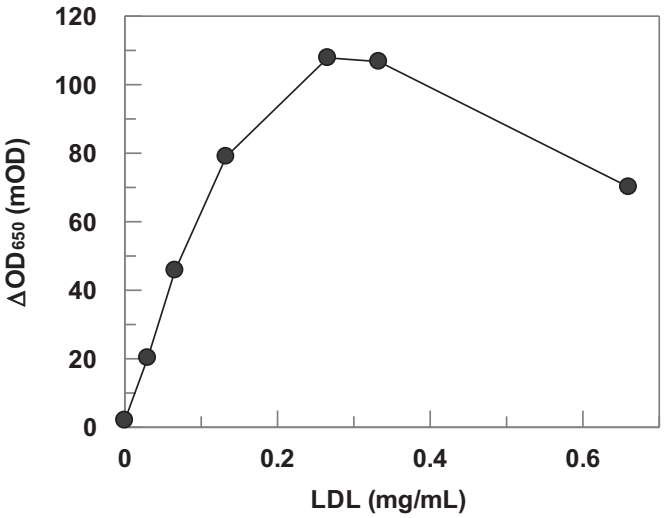

Fig. 3. Dose–response curve as a function of purified LDL concentration in our magnetic agglutination assay. SMP (0.04%) were mixed with various concentrations of purified human LDL and submitted to a magnetic field under the conditions described in Section 2.

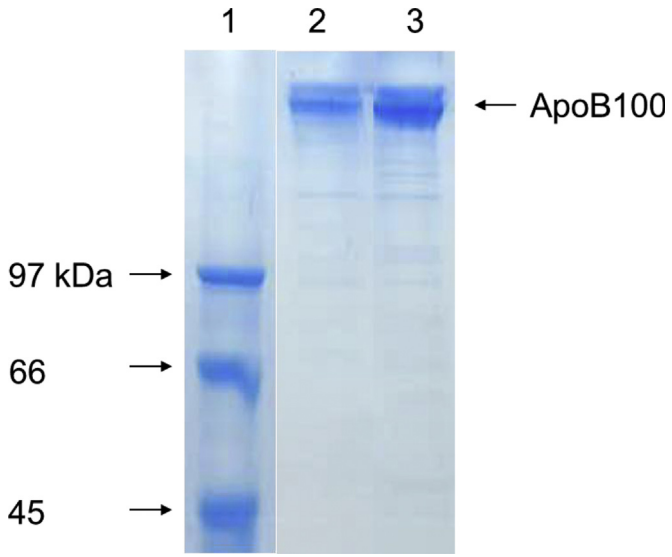

Fig. 4. SDS-PAGE analysis under non-reducing conditions of proteins bound to magnetic particles: (1) molecular weight standards (reduced); (2) purified ApoB100; (3) proteins bound to magnetic particles incubated with purified LDL.

Table 1
Cholesterol binding to SMP. SMP (0.4%) were incubated either with a pool of human plasma (with or without 1 g/L of dextran sulfate, DXS) or 0.9% NaCl. Cholesterol was assayed enzymatically directly on washed particles. Optical density change is shown ($\Delta OD_{505\ nm}$).

	$\Delta OD_{505\ nm}$
SMP+0.9% NaCl	0.126
SMP+Human plasma	0.203
SMP+human plasma+DXS	0.131

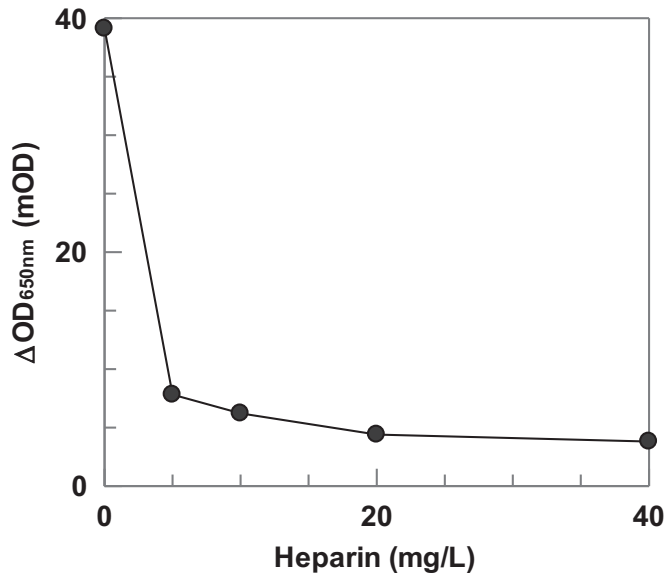

Fig. 5. Effect of heparin on NSA. Non-grafted SMP (0.04%) were mixed with a pool of human plasma diluted 1500 times in buffer in the presence of increasing amounts of porcine heparin and submitted to a magnetic field.

suggested that binding between SMP and LDL was due to ionic interactions although at pH 8.5 (the pH of the immunoassay) the net charge of both SMP and LDL is negative [6]. This normally should have prevented any electrostatic interactions between SMP and LDL. However, it has been already noted that effective charge is not the major driving force regulating particle–proteins interaction [12]. On the other hand, it has been shown that apoB100, which is the major protein

Table 2
Effect of heparin (0.5 g/L) on CRP measured with SMP immunoassay in two human plasma samples with different level of lipoproteins as determined by cholesterol and apolipoproteins concentrations.

	Plasma 1	Plasma 2
Cholesterol (mM)[a]	7.03	3.74
ApoA1 (g/L)[a]	1.62	1.25
ApoB100 (g/L)[a]	1.55	0.73
CRP (mg/L)[a]	6.16	0.27
CRP (mg/L) SMP without heparin	17.76	6.03
CRP (mg/L) SMP with heparin	5.73	0.22

[a] Measured on a ABX Pentra 400 automatic analyzer.

constituent of LDL, possesses several distinct clusters of basic residues. These highly positively charged regions have been recognized as heparin binding sites [13]. Thus, the possibility that binding between SMP and LDL could have occurred through these heparin binding sites was investigated. If so, heparin should inhibit interactions between SMP and LDL. Indeed, we found that addition of heparin significantly reduced non-specific agglutination of SMP in a pool of human plasma (Fig. 5). This result strongly suggested the involvement of ApoB100 heparin binding sites in non-specific agglutination of SMP under a field in the presence of plasma. This inhibitory effect of heparin on NSA could also be obtained when heparin was replaced by other polyanions like dextran sulfate. This is consistent with the finding that dextran sulfate prevented cholesterol from binding to the particles (Table 1).

The effect of heparin on NSA was further confirmed with native plasma samples with different level of lipoproteins (as reflected by cholesterol and apolipoproteins concentrations). We compared CRP concentrations obtained in the presence or absence of heparin in our SMP immunoassay with CRP concentrations measured on a ABX Pentra 400 automatic analyzer (Table 2). We found that, without heparin, CRP concentrations were substantially overestimated whereas in the presence of heparin, CRP concentrations obtained were very close to those measured by the independent method. We obtained comparable results when heparin was replaced by dextran sulfate.

Thus both heparin and dextran sulfate are capable of eliminating interference due to lipoproteins independently of their plasma level.

3.4. ApoB100 is involved in NSA

To further confirm the role of basic clusters of ApoB100 in NSA, lysine residues were indirectly modified by oxidation of LDL phospholipids. It has been shown that lipoprotein phospholipids can undergo oxidation, and that oxidation products are capable of modifying lysine residues, resulting in loss of the positive charge [10]. These modifications were found to interfere, for example, with the ability of LDL to bind to negatively-charged glucosaminoglycan chains of proteoglycan [7]. This oxidation process, which is quite slow, could be accelerated by using divalent metals like Cu^{2+}. Accordingly, LDL was incubated with 5 μM copper chloride at room temperature. Aliquots were taken at various time intervals and tested for NSA. Copper-induced oxidation of LDL resulted in a progressive reduction of NSA (not shown). This result confirmed that binding of LDL to SMP was due to ionic interactions between heparin binding sites on ApoB100 and negatively charged SMP.

Although it was demonstrated that LDL was involved in NSA, VLDL contribution to NSA cannot be excluded since (1) part of NSA co-eluted with VLDL on SEC-HPLC and (2) ApoB100 is also present on this lipoprotein.

4. Conclusion

The incidence of NSA on SMP in our immunoassay was eliminated by introducing a polyanion such as heparin in the assay buffer. Other polyanions (dextran) were equally efficient in reducing NSA. Although these non-specific interactions could also be eliminated by increasing the buffer ionic strength, we found that too high a salt concentration induced a progressive self-agglutination of magnetic particles upon storage which required separation of the assay buffer and the particle suspension. In contrast, addition of heparin or dextran sulfate to the assay buffer allowed the formulation of a stable single reagent consisting of magnetic particles suspended in the assay buffer. The use of a single reagent is particularly important for assay automation in terms of improved analysis time and reduced cost per test.

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