Immunoassays for diagnosis of coagulation disorders

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Summary
Immunoassays play a pivotal role in the clinical laboratory. In the coagulation section of the laboratory, they are used as an aid for diagnosis of deep vein thrombosis or pulmonary embolism, thrombophilia screening, or detection of coagulation factor deficiencies, respectively. Enzyme-linked immunosorbent assay (ELISA) and latex agglutination immunoassay technologies are currently most widely used, while Luminescent Oxygen Channeling Immunoassay (LOCI®) and other chemiluminescence-based immunoassays are emerging technologies for the coagulation laboratory. However, not all immunoassay technologies employed are compatible with the workflow requirements of the coagulation laboratory, and, not all technologies are suitable for detection or quantification of every marker.

This review focuses on technical and performance aspects of those immunoassay technologies that are most widely used in the coagulation laboratory, and provides a description of markers that are typically tested by immunoassays.

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In contrast to other sections of the clinical laboratory, the coagulation section has special requirements for immunoassay technologies to integrate them into the workflow. This makes solutions which may be practical in other areas of the clinical laboratory, impractical for the coagulation section. This review will
• provide an overview over the markers typically tested with immunoassays in the coagulation laboratory, and
• present the immunoassay technologies that are commonly employed in the testing of those markers.

Coagulation markers

The protein coagulation markers that are mainly tested by the haemostaseoologist include
• coagulation activation and/or reactive fibrinolysis markers such as
  – D-dimer,
  – prothrombin fragment 1+2 (F1+2),
  – thrombin-antithrombin (TAT) complex,
• anticoagulant factors such as free or total protein S,
• coagulation factors such as factor XIII.

Moreover, emerging markers such as the FSAP (factor VII-activating protease) are also tested. Besides these plasma protein markers, serological markers such as antibodies associated with heparin-induced thrombocytopenia or antibodies associated with antiphospholipid syndrome exist.

D-dimer

The D-dimer is the most frequently used indicator for in vivo coagulation activation. Assays for the quantitative determination of D-dimer are widely used in the diagnosis

Although the diagnosis of coagulation disorders commonly relies on methods that determine the activity of single coagulation factors or of parts of the coagulation cascade (1), highly sensitive immunoassays are today widely used for specific diagnostic questions addressed by the haemostaseoologist. In particular, immunoassays are used as an aid for e. g.
• diagnosis of deep vein thrombosis (DVT) or pulmonary embolism (PE),
• thrombophilia screening, or
• detection of coagulation factor deficiencies.
of deep vein thrombosis or pulmonary embolism, as D-dimer concentrations below a cutoff specifically defined for each assay have a high negative predictive value (NPV) for exclusion of these thromboembolic events (2). D-dimers are degradation products of cross linked fibrin generated during fibrinolysis. D-dimer, however, is not a single entity in plasma but rather a heterogeneous mixture of fibrin degradation products. Different assays measure different types of those degradation products.

As a consequence, it is difficult to standardize D-dimer tests with different design.

Respective attempts for standardization or harmonization of test results from different assays are still under debate (3, 4).

F1+2

Clotting activation always leads to the generation of thrombin. F1+2 is a 35 kDa protein generated during the conversion of prothrombin into thrombin in an 1:1 molar ratio, and is a direct marker for thrombin formation. Under physiological conditions, this process occurs in the presence of factor Xa, factor Va, calcium ions, and platelets. During this reaction factor Xa cleaves two peptide bonds in the prothrombin molecule. Cleavage at Arg 273-Thr 274 results in liberation of F1+2 (residues 1–273) and prothrombin 2 (residues 274–581) (5). Monoclonal antibodies specific for the C-terminal neoepitope, generated during the cleavage, in combination with prothrombin specific antibodies allow the quantification of F1+2 in plasma samples (6) in the presence of a surplus of prothrombin. F1+2 plasma concentrations increase in many clinical conditions associated with hypercoagulability, and decrease in patients during e.g. oral anticoagulation treatment (5, 7). Elevated F1+2 levels can be found e.g. in patients with thrombosis, pulmonary embolism (5, 8), disseminated intravascular coagulation (DIC) (9), polytrauma (10), sepsis (11).

Increased F1+2 concentrations can be found e.g. in patients under oral anticoagulation or heparin therapy (1, 7).

TAT

As F1+2, TAT is an indicator of active thrombin generation. Free thrombin is not detectable in plasma samples, as thrombin that leaves a site of clot formation is immediately inactivated by antithrombin III at the vessel wall to generate the enzymatically inactive TAT-complex. The 90 kDa large TAT-complex can be quantified by immunoassays, thereby allowing the specific detection of a latent activation of the clotting pathway (12). Elevated TAT-concentrations can be found in patients predisposed to thrombosis, DIC patients (9, 13, 14) and during heparin or fibrinolysis therapy (15, 16).

Determination of TAT is also of interest in patients with multiple trauma, liver dysfunction, septicemia (17) and preeclampsia (18).

Protein S

Protein S is a vitamin K-dependent single chain plasma glycoprotein of approximately 75 kDa that is present in human plasma at a concentration of 20–25 mg/l (19). About 60% of plasma protein S is present in a high affinity 1:1 stoichiometric complex with C4b-binding protein (C4b-BP), a component of the complement cascade. Only the free, but not the complexed form, acts as cofactor of protein C in the proteolytic inactivation of coagulation factors Va and VIIIa (19). It is by this means that the coagulation process is tightly regulated. As a consequence of its pivotal role in the haemostatic process, deficiency in protein S is a risk factor for venous thrombosis (20).

Protein S deficiency can be inherited or acquired (21) during
- hormonal contraceptive treatment,
- pregnancy,
- inflammatory diseases or cancer.

The diagnosis of protein S deficiency is part of the evaluation of thrombosis patients and high-risk individuals.

It is performed in the clinical laboratory using either functional tests (22) or immunoassays (23–25) for quantification of protein S plasma levels. Measurements of free protein S have been shown to be superior to total protein S (i.e. free protein S plus C4b-BP complexed protein S) measurements in diagnosing protein S deficiency due to higher sensitivity and specificity for the underlying genetic defects (26).

FXIII

Coagulation factor XIII (FXIII) is a member of the transglutaminase family of enzymes (27). The enzymatically active factor XIIIa can crosslink adjacent fibrin molecules, thereby strengthening the blood clot. Other substrates of FXIIIa are fibronectin or vitronectin, which link the blood clot to the surrounding matrix, or α-2 antiplasmin, which inhibits plasminogen-mediated clot lysis when linked by FXIIIa to the clot (28). Acquired or congenital deficiency in FXIII is associated with a risk for spontaneous bleeding and impaired wound healing (29–31).

Substitution therapy of patients with low plasma levels of FXIII requires diagnostic quantification of the factor before and during therapy (31). FXIII substitution therapy is recommended in particular for patients with FXIII activity plasma levels below 5%, but abnormal bleedings can also occur in patients with FXIII activity plasma levels at 30% (32). Several methods for quantification of either FXIII plasma activity or antigen levels have been developed in the past (33) and are well established in the coagulation laboratory.
APLs

Antiphospholipid antibodies (APLs) such as antibodies to cardiolipin (aCL) or β2-glycoprotein 1 (β2GPI) are strongly associated with the antiphospholipid syndrome (APS), an autoimmune disorder characterized by arterial and venous thrombosis, and recurrent fetal loss, respectively (34, 35).

According to the current classification criteria for APS, identification of APLs in moderate to high serum titers is a significant finding when it persists for more than 12 weeks and is closely associated with clinical features (36).

Although aCL or β2GPI antibodies are equally listed in the classification criteria, their individual role in the association with APS and hence their diagnostic and predictive values are still under debate (37, 38). In the coagulation laboratory, APLs are either detected by

- immunoassays or
- Lupus anticoagulant (LA) testing (38), a clot-based assays that can be performed on coagulation analyzers.

HIT

As with APS, the diagnosis of heparin-induced thrombocytopenia (HIT) requires the combination of a laboratory test result with the clinical picture (39). HIT is clinically defined as a decrease in platelet count shortly after starting heparin therapy, which resolves after stopping heparin and has no other apparent cause.

The severe thrombocytopenia observed in HIT, usually occurring between four and 14 days after starting with heparin therapy, is associated with both arterial and venous thrombosis. As a consequence, HIT should always be considered in any patient who develops a 50% reduction of platelet count while on heparin treatment (40). HIT is strongly associated with antibodies to complexes of platelet factor 4 (PF4) bound to heparin (41, 42), which can be detected by platelet activation tests or immunoassays (43).

Imмуnoassay technologies

Since their first description by Yalow and Berson (44), immunoassays rely on the specific interaction of the analyte that shall be quantified in the test, and analyte-specific antibodies. The discovery of monoclonal antibodies (45) further boosted the development of immunoassays for detection of a broad range of analytes. Originally, the antibodies employed in immunoassays were labeled with radioactive substances (46, 47). Although the resulting Radioimmunoassays (RIA) were very suitable for automated applications, the rather sophisticated and expensive equipment as well as strict regulatory controls on the use of isotopes have excluded RIA from many laboratories. Nowadays, antibodies are usually labeled with organic substances or enzymes, or are coated to specific surfaces.

ELISA

Enzyme-linked immunosorbent assays (ELISA) (48) are routinely used for the quantitative determination of markers for coagulation disorders (5, 12, 49–54), such as

- D-dimer,
- F1+2,
- FSAP,
- free or total protein S,
- factor XIII,
- F1+2,
- TAT,
- APLs,
- heparin/PF4 complexes.

ELISA tests rely on the so-called antibody sandwich technique (Fig. 1a): A solid phase of a microtitre plate is coated with specific antibody to the analyte to be quantified. This reacts with the test sample containing the analyte. Subsequently, a second specific antibody to the analyte is added, which is linked directly to an enzyme, or indirectly by an antibody conjugated to the enzyme. The analyte in the sample is thereby captured and immobilized on the solid phase via the first antibody, where it can itself then fix the enzyme-linked antibody directly or via a second antibody.

The steps described are usually separated by one or more washing steps. After completion of these steps, the enzyme substrate is added to the microtitre plate. It is converted by the enzyme linked to the antibody into a water-soluble, coloured product. This conversion depends on the amount of enzyme-labeled antibody and thereby on the amount of antigen in the sample. The intensity of the colour is proportional to the concentration of the analyte present in the sample.

A typical enzyme used in ELISA is horse-radish peroxidase (55), which can convert the colourless substrate TMB (tetramethylbenzidindihydrochloride) to a soluble, blue dye. Other enzymes commonly used in ELISA assays include β-D-galactosidase and alkaline phosphatase (48, 56).

Problems

Although ELISA assays can be fully automated today, many laboratories still perform the pipetting or even washing steps manually. This makes ELISA assays quite time consuming.

Another weakness of ELISA assays is that they are most often used in a batch mode, meaning that samples have to be collected – sometimes over days – until an ELISA run is performed. This makes it often difficult to integrate ELISA assays into the workflow of a coagulation laboratory. The batch mode procedure is due to the fact that the ELISA has to be calibrated at every run, and that only entire strips (usually 8 tests) can be used as a minimum for one assay run. This makes ELISAs cost-effective only when multiple samples are analyzed at the same time.

Another issue with ELISA assays is that not every coagulation section of the clinical laboratory has immediate access to ELISA equipment. This makes the parallel analysis of samples on the coagulation analyzer and on ELISA instruments often impractical.

Latex agglutination

In contrast to ELISA, immunoassays based on the latex agglutination technique are highly compatible with the workflow of a coagulation laboratory, because they
Fig. 1  Schematic representations

a) ELISA assays: A first analyte-specific antibody (capture antibody) is coated to the surface of a microtitre plate and reacts with the analyte present in the sample. Subsequently, a second analyte-specific antibody (detection antibody) is added. The detection antibody is either directly linked to an enzyme such as horseradish peroxidase (HRP), or indirectly linked by an antibody conjugated to the enzyme. Finally, the enzyme substrate (tetramethylbenzindihydrochloride or TMB in this case) added, and is converted by the enzyme to a colored product. All steps are usually separated by one or more washing steps.

b) Latex agglutination assays: Latex particles (blue spheres) coated with specific antibodies agglutinate in the presence of analyte (red squares). The agglutinates cause scattering of a light beam, which can be measured by nephelometry or turbidimetry.

c) LOCI assays: LOCI reagents include two latex bead reagents and a biotinylated analyte receptor. One bead reagent (sensibead) is coated with streptavidin and contains a photosensitive dye. A second bead reagent (chemibead) is coated with an analyte-specific binding partner and contains a chemiluminescent dye. During an assay the three reactants combine with analyte to form a bead-aggregated immunocomplex. Illumination of the complex by light at 680 nm generates singlet oxygen from sensibeads, which channels into chemibeads to trigger a chemiluminescent reaction that is measured at 612 nm.
can be adapted to modern coagulation analyzers,
● have short time to results,
● allow random access test of samples.

The integration of latex agglutination tests to coagulation analyzers allows the parallel analysis of a sample with coagulation and immunoassays. The capability for random access testing, i.e. a single sample can be analyzed using a stored calibration curve, makes latex agglutination tests also attractive under cost aspects.

Latex agglutination assays are based on latex particles coated with antibodies specific for the analyte that shall be quantified in a sample (57–61). Upon addition of the latex reagent to the sample, the antibodies react with their cognate analyte (Fig. 1b). The reaction between the antigen and the particle-bound antibody leads to aggregate formation through bridging of the antibody between antigen molecules. A light beam that passes through the reaction vessel is scattered depending on the number and size of aggregates formed. The amount of light scattering is proportional to the amount of aggregates generated, and thereby proportional to the concentration of the analyte in the sample. The light scattering can be quantified by
● turbidimetry, which measures the change in absorption during the reaction, or
● nephelometry, which measures the scattered light directly.

In contrast to heterogeneous immunoassays such as ELISA, latex agglutination assays are homogenous, meaning they do not involve any washing steps to separate free from bound analytes or antibodies. This significantly reduces assay duration and complexity of automation, making homogenous assays convenient methods for the clinical laboratory.

Markers of coagulation disorders that can be quantified by latex agglutination immunoassays on automated coagulation analyzers (24, 25, 62–67) include
● D-dimer,
● von Willebrand factor (VWF) antigen or activity,
● free and total protein S.

Latex agglutination assays based on low-angle light scattering would have the theoretical ability to provide exceptionally high sensitivity by measuring particle pairs instead of larger agglutinates as in conventional assays (68). However, the measurements would require extremely low particle concentrations in the sample, which is not practical with the typical samples used in the clinical laboratory. This typically limits latex agglutination tests to coagulation markers with plasma concentrations in the nanomolar range or higher. However, exact quantification of low levels of coagulation markers as well as establishment of new markers with low plasma concentrations that result from proteomics studies (69) will require more sensitive immunoassay technologies.

LOCI

The Luminescent Oxygen Channeling Immunoassay (LOCI®) technology combines
● the advantages of latex agglutination assays (short time to result due to homogenous assay format, high precision and ease of automation) with
● an extremely sensitive detection technology.

This allows the development of immunoassays that can quantify plasma analytes present in pico- or even femtomolar concentrations.

The technology (Fig. 1c) is based on the analyte-mediated association or dissociation of two different types of latex nanobeads (70, 71). One nano-bead type called sensibeads is coated with streptavidin and contains a photosensitive dye such as phthalocyanine. A second type of nano-bead called chemibead contains a chemiluminescent dye such as thioxene, and is coated with an analyte-specific antibody. A third reagent involved in LOCI assays is a biotinylated receptor antibody to the analyte that binds in most cases to a different epitope than the antibody coated to the chemibead.

During a LOCI assay, the biotinylated antibody and the antibody coated to the chemibead both react with the analyte, thereby forming an immunocomplex. The complex formation is completed by binding of the streptavidin-coated sensibead to the biotinylated antibody through biotin-streptavidin interaction. This brings chemibead and sensibead in close proximity. Illumination by light at 680 nm generates singlet oxygen from sensibeads, which can only diffuse a few nanometers into the medium before it converts to triplet oxygen. This is due to the short half-life of singlet oxygen of about 4 μs in aqueous solutions. Singlet oxygen can trigger a chemiluminescent reaction with the chemiluminescent reagent of the chemibeads, which can be measured at 612 nm. This reaction does only occur, when sensibeads and chemibeads are in close proximity, as in the absence of an association of sensibeads and chemibeads, singlet oxygen produced by a sensibeads decays in the reaction mixture without signal generation. Thus, no separation step is necessary, as unassociated beads do not generate a signal.

Besides the high sensitivity and precision, short time to result and ease of automation, LOCI assays provide an extremely broad dynamic range and require only small sample volumes.

For example, a D-dimer LOCI assay prototype with excellent sensitivity, precision, turnaround time, and dynamic range suitable to measure reliably D-dimer in a broad range without any sample dilution was developed (72). The LOCI D-dimer assay prototype has a time to the first result of 10 minutes. Preliminary data have shown within-run precision of <1.2 % CV and total precision <3.2 % CV (Tab. 1). No interference (>10 % bias) was observed in the presence of fibrinogen or fibrinogen degradation products (Tab. 2). Comparison of results from 60 patient samples processed by the new method (Y) and the D-dimer method on the Stratus® CS (SCS) STAT fluorometric analyzer (X) showed the assay prototype has the potential for very good agreement by linear regression analysis: Y = 0.999 X – 37 ng/ml, r = 0.999, range 26–3.094 ng/ml (Fig. 2).
Chemiluminescence assays

Other chemiluminescence-based immunoassay technologies are well established in the clinical laboratory, but have so far not penetrated the coagulation section of the laboratory, mainly due to workflow constraints. Chemiluminescence assays are typically based on magnetic beads coated with an antibody that captures the analyte from a sample. After a washing step, a secondary antibody to the analyte that is conjugated to a tracer molecule such as acridinium esters or isoluminol is added. After a second washing step that removes unbound tracer conjugate, reagents that trigger the luminescent reaction are added, and the emitted light is measured as relative light units. The light energy in these reactions is generated as a result of the decomposition of a weak bond, which produces excited-state intermediates that decay to a ground state with the emission of light (73).

As with ELISA assays, the heterogeneous test format of chemiluminescence-based assays that employs washing steps increases assay duration and makes automation more complex than with homogenous assay formats. So far, chemiluminescence-based assays for quantification of D-dimer or antiphospholipid antibodies have been specifically developed for the coagulation laboratory (74, 75).

It is noteworthy to mention that not all immunoassays for coagulation markers are performed in the coagulation section of the clinical laboratory, even if respective equipment is available. In particular, a significant fraction of D-dimer test is performed on immunoassay analyzers of the central laboratory (76, 77) or even at the point of care (78), whereas tests for antiphospholipid antibodies are sometimes performed on specialty immunoassay analyzers for autoimmune diseases (79).

Conclusion

There are several technical options for performing immunoassays in the coagulation laboratory. However, not all immunoassay technologies employed are compatible with the workflow requirements of the coagulation laboratory, and, not all technologies are suitable for detection or quantification of every marker.

It can be anticipated that there is a continuous trend for further integration of immunoassays into the workflow of the coagulation laboratory, which will eventually provide the haemostaseologist with access-on-demand to perform highly sensitive immunoassays.

### Tab. 1 Imprecision of a LOCI-based D-dimer prototype assay

<table>
<thead>
<tr>
<th>D-dimer (ng/ml)</th>
<th>445</th>
<th>1353</th>
<th>4391</th>
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<td>CV (%)</td>
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<td>1.0</td>
<td>0.9</td>
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<td>2.7</td>
<td>3.0</td>
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<tr>
<td>run-to-run</td>
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<td>2.9</td>
<td>3.1</td>
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<tr>
<td>total</td>
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<td>2.9</td>
<td>3.1</td>
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### Tab. 2 Cross-reactivity of a LOCI-based D-dimer prototype assay

<table>
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<tr>
<th>interference substance</th>
<th>cross-reactivity (%)</th>
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</tr>
<tr>
<td>fragment D (20 μg/ml)</td>
<td>0.74</td>
</tr>
<tr>
<td>fragment E (20 μg/ml)</td>
<td>0.01226</td>
</tr>
</tbody>
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Conflict of interest
Andreas Kappel and Matthias Ehm are employed by Siemens Healthcare Diagnostic Products GmbH.

Literature