



Biosensing of D-dimer, making the transition from the central hospital laboratory to bedside determination

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ABSTRACT

Since the disclosure of the fibrinogen degradation mechanism, around half a century ago, a significant number of papers have been published related to the clinical relevance of D-dimer, a molecule immune to additional enzymatic decomposition by plasmin. Due to the obliquity of regulating blood coagulation in pathological events, the number of diseases and conditions associated with abnormal levels of D-dimer includes deep vein thrombosis, pulmonary embolism, sepsis, myocardial infarction, disseminated intravascular coagulation, among many others. D-dimer not only is an important player in medical diagnosis but also its role as a prognosis biomarker is being revealed. However, the number of analytical alternative methods has not accompanied this trend, even though novel simple point-of-care devices would certainly boost the relevance of D-dimer in emergency medicine. Some reasons for that could be related to the fact that D-dimer is a challenging analyte present in complex samples like blood. In this manuscript, subsequent to a fibrinogen degradation process introduction, it is provided a historical overview of the early D-dimer assays, followed by an extended focus on innovative solutions, with a spotlight on the electrochemical bioanalytical devices. The discussion is accompanied with a critical analysis and concluding thoughts concerning future perspectives.

1. D-dimer and its clinical relevance

When a hemorrhage occurs, caused by external and/or internal injuries, the process of hemostasis is initiated in order to prevent the loss of blood from the torn vessels. Blood vessels constrict in an attempt to stop the hemorrhagic process, and, in the very proximity of the affected site, platelets bind with exposed collagen fibers to produce a sort of plug, which eventually turns into a stabilized cross-linked fibrin clot that stops the bleeding. Clot formation is accompanied by a series of platelets modifications and reactions related to the coagulation factors. In the course of the mentioned events, the enzyme thrombin initiates degradation of the coagulation factor I (fibrinogen) forming self-adhesive fibrin monomers consisting of fibrinopeptides, and then converts them into soluble polymers. However, thrombin also enables the coagulation factor XIIIa to form insoluble fibrin polymers by crosslinking between the outer D domains of adjacent fibrin monomers and the central E domain of a third fibrin monomer (D-E-D), as presented in Fig. 1. These polymers are prone to further degradation by the means of plasmin, leading to the formation of fragments with different molecular weights, ranging from 190 to 530 kDa [1]. The smallest fragment among them, resistant to further plasmin activated degradation and

consisting of one E fragment and two crosslinked D domains (D-DE, molecular weight 240 kDa), is called D-dimer [2,3]. Alternatively, higher molecular weight fragments are also collectively referred to as D-dimer [3]. The issue of terminology for macromolecular derivatives of crosslinked fibrin, including D-dimer containing fragments was addressed by Mosesson, with suggestions for denotation of fragments and their assumed molecular weights [1]. Pioneer publications related to D-dimer, mainly focused on unveiling its complex formation mechanism and detection methods [4]. More recently, taking into account the growing clinical relevance of D-dimer, research focus has switched to development and optimization of methods for its quantification, pointing at high selectivity procedures along with fast and unambiguous results.

Classically, D-dimer is acknowledged as a biomarker for the activation of hemostasis and fibrinolysis, introduced in the 1970s [5]. However, the clinical pertinence of D-dimer comes from initial reports showing a strong correlation between its elevated levels in blood/serum and the occurrence of deep vein thrombosis (DVT), recurrent venous thromboembolism, disseminated intravascular coagulation (DIC) and thrombosis in cancer [6,7]. Nevertheless, pulmonary embolism (PE) is perhaps the pathology in which there is larger solid clinical evidence

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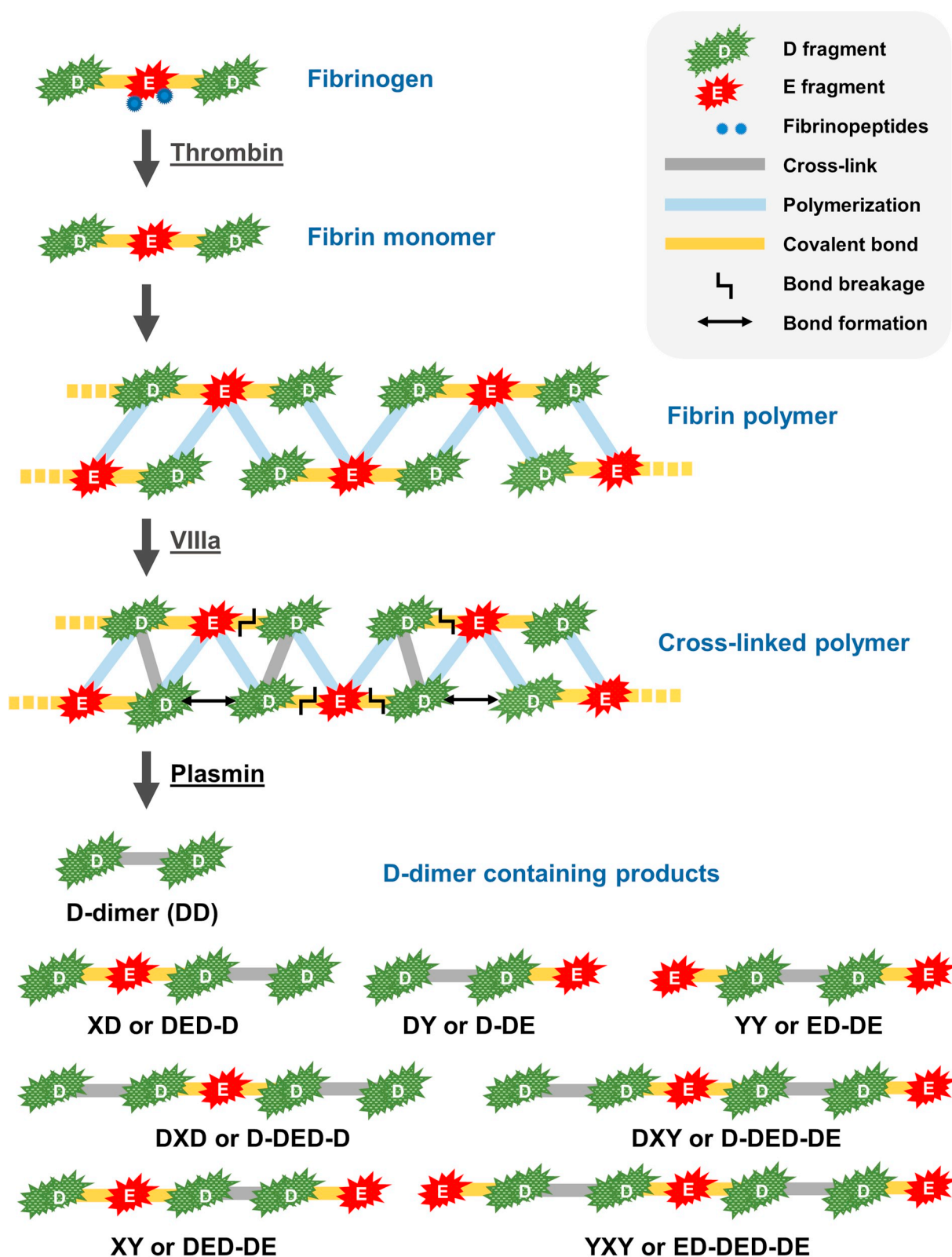


Fig. 1. – Schematics of the DD formation. This process involves 3 enzymes: plasmin, activated factor XIII (factor XIIIa) and thrombin.

that D-dimer measurement can make a difference [8]. PE occurs when a thrombus travels along the veins and ends up clogging the lungs' arteries causing difficulties in breathing that, not rarely, can ultimately cause a death. PE is indeed an acute life-threatening but, thankfully, potentially reversible with a suitable and opportune diagnosis. PE is suspected by the presence of common and non-specific symptoms like

difficult breathing, chest pain or palpitations, however a D-dimer value lower than $500 \mu\text{g L}^{-1}$ can rule out PE [9]. Elevated levels of D-dimer were also correlated with meager overall survivability and increased mortality risk in patients with cancer, such as lung [10,11], breast [12], lower gastrointestinal tract [13,14], pancreas, stomach, kidney, prostate and brain [6]. An elevated level of D-dimer has been associated

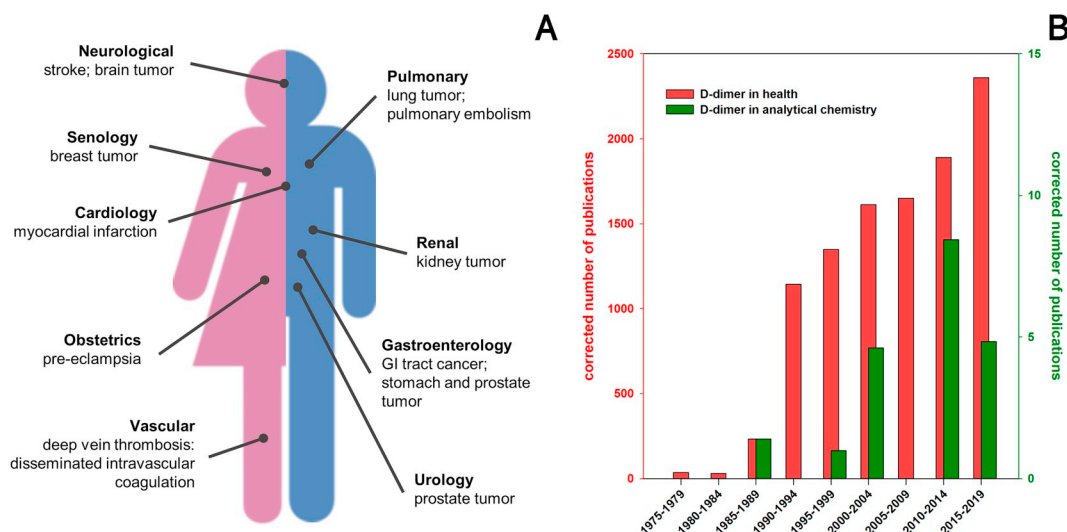


Fig. 2. – A) A few pathologies in which D-dimer can be useful for either diagnosis or prognosis. B) Corrected number of publications about D-dimer in hematology (all papers in D-dimers were divided by the number of all papers in hematology in the same periods, and then multiplied by 100,000) and corrected number of publications about analytical methodologies for D-dimer in analytical chemistry (all papers in D-dimers were divided by the number of all papers in analytical chemistry and electrochemistry in the same periods, and then multiplied by 100,000), source: Web of Science.

with patients suffering from sepsis and myocardial infarction [15] and even pre-eclampsia [16]. D-dimer level might be a prognostic indicator for patients with acute upper gastrointestinal hemorrhage [17] or for outcome prediction and mortality after acute ischemic stroke [18]. And these are just a few examples of pathologies in which D-dimer can be of great relevance, while still a lot of seminal research is currently being performed (Fig. 2 - A). May the reader please bear in mind that the majority of these pathologies are increasing in frequency since most people live longer, dying proportionally less of preventable causes like accidents, famine or infections [19].

Despite all the above mentioned, the clinical use of D-dimer is surprisingly mediocre. The average D-dimer concentration could be, for example, an extra factor in the thromboembolic risk score (CHA2DS2-VASc) [20–22]. In the authors' opinion, the lack of reliable, simple, low-cost, and, notably, bedside assays for D-dimer quantification may be the main reason for such fact. Perhaps the attention towards D-dimer would significantly increase if some of the proposed quantification techniques could match the simplicity of the contemporary devices for rapid quantification of glucose. As can be observed in Fig. 2 - B, where are shown the number of publications about D-dimer and the number of publications concerning analytical ways for its determination during the last decade (both numbers were corrected by the number of publications in their scientific field) one can assume there should have been more analytical research. The emerging interest in the analysis of D-dimer and efforts to incorporate it into the diagnostics routine has yet to result in many engaging solutions. A quick explanation is that, from the standpoint of a classical analytical chemist, precise quantification of D-dimer is somewhat of a nightmare since D-dimer does not have a clear molecular weight and is heterogeneously constituted of the fragments produced throughout the process of fibrinogen degradation, originating both from soluble fibrin polymer before it has been incorporated into a cross-linked fibrin gel, and from high molecular weight complexes released from an insoluble clot [23]. Some studies have shown that D-dimer results are not comparable among different assays, even among those using similar formats and significant efforts have been invested into their harmonization [2,24,25]. This is primarily due to the dissimilar operating mechanisms of the proposed assays and, secondly, due to the usage of monoclonal antibodies with divergent specificities towards various D-dimer epitopes. Moreover, the complexity of blood as a sample matrix certainly does not help.

Taking into consideration the above mentioned, D-dimer biosensing seems to be of utmost importance. This review aims to provide an overview of the published literature and the current “state of the art” in the field of detection and quantification of D-dimer, based on a critical discussion of these methods and its limitations, having a more focused look at electrochemical analytical methods.

2. Immunoassays, chromatographic and other approaches

Upon the disclosure of fibrinogen degradation process, its related degradation products and their clinical relevance towards DIC and thromboembolism (TE), the succeeding analytical techniques were nonspecific and insensitive towards D-dimer, such as agglutination inhibition tests with tanned red blood cells, staphylococcal clumping tests, immunoelectrophoresis or immunodiffusion [2,26–29]. However, the introduction of monoclonal antibodies specific towards different D-dimer epitopes [30–32] has led to the creation of many viable D-dimer immunoassays. The most prominent were latex-agglutination assays comprising of the monoclonal antibodies in conjunction with latex beads [32–34], in which the quantification of the specific analyte is proportional to the degree of clumping. The obvious shortcoming of this technique was that it required visual reading of the agglutination/clumping magnitude, which compromised its accuracy. In contemporary assays, the agglutination level is registered by turbidimetry using the calibration standards that include products from both partially and fully digested fibrin clots, meaning that they possess distinct sensitivity to a variety of D-dimer included compounds [23]. Interference caused by hemoglobin, bilirubin and triglycerides can be diminished by choosing the appropriate turbidimetric wavelength [35]. The main disadvantage of this technique is that it must be performed in laboratory conditions, and typically on centrifuged citrated venous blood [36]. There are reports on the usage of undiluted plasma in this category of assays, with only 140 s necessary for the result expressed in fibrinogen equivalent units (FEU) [37]. So far, at least 13 assays based on this technique have been commercially introduced, with certified manufacturers cut-off values in the range of 200–500 $\mu\text{g L}^{-1}$ [2].

Whole-blood agglutination assays were introduced as a successful attempt to decrease the time required for immunoassay (i.e. 1–2 min to obtain positive results) [38]. These semiquantitative assays utilized a double specific antibody which bonds both to the D-dimer and to the

red blood cell membrane antigen so that the red blood cells agglutinate when D-dimer levels are above the threshold. The level of detection for this assay was $60 \mu\text{g L}^{-1}$. The most obvious advantage of these bed-side assays is that they are performed on whole blood, without the requirement to centrifuge or process plasma [39]. The extensive usage of these assays in comparative clinical tests have revealed that its sensitivity is largely affected by the experience of health care workers that perform the assays [40].

Enzyme-linked immunosorbent assays (ELISA) derive on the usage of D-dimer binding antibodies and “tagging” antibodies, which subsequently quantify captured antigen, via spectrophotometry or fluorimetry. An initial assay of this type (known under the commercial name of VIDAS D-dimer) was presented in mid 1990s and consisted of pipetting device coated with anti-D-dimer mouse monoclonal antibody and reagents-sensitized strip onto which plasma sample was pipetted. The measuring procedure was performed on an automated analyzer provided by the assay manufacturer, while the results could be obtained after 35 min with the cut off value of $500 \mu\text{g L}^{-1}$ [41,42]. Similarly, some commercial assays (Asserachrom DDi from Stago) used murine antihuman D-dimer monoclonal antibodies fixed on the surface of test microtitration plates, onto which the D-dimer is bound during incubation. Polyclonal rabbit anti-D-dimer coupled with peroxidase was used as “tagging” factor, and the amount of D-dimer fixed to the wells was quantified by adding a substrate that is converted to a colored substance using peroxidase with the cut off value of $500 \mu\text{g L}^{-1}$ [43]. Contemporary ELISA assays incorporate the magnetic particles conjugated with the anti-D-dimer antibodies, for better separation of the analyte, followed by a chemiluminescent reaction as a detection tool [44].

Immunofiltration assays are another type of clinical tests for fast quantification of D-dimer. They consist of a thin, porous membrane sensitized with a monoclonal antibody specific towards D-dimer. The device's configuration is such that, upon the suction of citrated plasma sample through the membrane, D-dimer antigens are bonded to the monoclonal antibodies. Then, an antibody conjugate coupled with gold colloids (4 nm) is added to react with the D-dimer antigens captured on the membrane. The gold colloids provide the intensive stain, and the amount of D-dimer-configured antigens is proportional to the amount of red color visible on the membrane. However, the biggest disadvantage of this assay is its low cut off value of $500 \mu\text{g L}^{-1}$, which limits its practical usage despite good reactivity towards all fibrin degradation products containing D-dimer [45–47].

Chromatographic methods were recently introduced and are established on more sophisticated operating mechanisms, resulting in increased specificity and sensitivity towards D-dimer. Wang et al. indirectly quantified D-dimer by aiming to the cross-link at the interface of the two fibrin D domains, originating from XIIIa-catalyzed dimerization of fibrin γ chains. This so-called “true D-dimer signature” of ca. 3.995 kDa was enriched using an antipeptide antibody and quantified by liquid chromatography with tandem mass spectrometry (LC-MS), obtaining a limit of quantification (LOQ) of $170 \mu\text{g L}^{-1}$ [7]. This was a clever way to “overcome the ambiguity of the D-dimer identity” as nicely put by the authors. Similarly, Toulon et al. tested qualitative immuno-chromatographic assay in more than 400 patients with low or intermediate pretest probability in aiding the diagnosis of pulmonary embolism with a sensitivity of 100% and a specificity of 48.8%. The proposed assay comprised of murine monoclonal antibody (specific for D-dimer – gold conjugate), D-dimer specific antibody and a sheep anti-murine IgG antibody. Whole blood samples were used for D-dimer semi quantification (cut-off value of $8 \times 10^{-5} \mu\text{g L}^{-1}$), which could be finalized after 10 min of incubation followed by the visual readings on the display [48].

Besides the above described techniques and devices for detection of D-dimer in whole-blood and/or plasma samples, there are several other proposed that due to their distinctive operating mechanisms cannot clearly be classified as immunochromatographic. For instance, Ruivo

et al. have fabricated a semi-quantitative lateral flow microfluidic paper-based immunoassay for colorimetric detection of D-dimer (Fig. 3). The operational principle is based on the colorimetric changes proportional to the analyte content that occur as the D-dimer from the running fluid reacts with the capturing antibody-gold nanoparticles conjugates dispersed on the sensing paper. One of the shortcomings of this method is that the gold particles are not incorporated into the sensing paper, but need to be added prior to the analysis. Furthermore, the running buffer cannot be added at once but throughout the measuring procedure. The detection limit of the presented immunoassay was $1.5 \times 10^{-5} \mu\text{g L}^{-1}$, while the test duration was in the range of 10–12 min. The tests could be tailored to yield positive results in compliance with the clinically relevant threshold [49]. Gao et al. have proposed a rapid method for determination of D-dimer in human plasma by using giant magnetoresistance sensor integrated with microfluidic technique and superparamagnetic particles. The function of this sensor is based on the variations of the magnetoresistance signal of the magnetic particles which bond to the D-dimer-antibody complex during the flow of the plasma through the sensing area. The positive correlation between the amount of the particles attached on the sensing chip and D-dimer was observed, thus the magnetoresistance signal changes as the magnetic particles react with the D-dimer-antibody complex [50]. Koukouvinos et al. fabricated a dual-analyte assay for simultaneous determination of C-reactive protein (CRP) and D-dimer in human blood plasma based on a white light reflectance spectroscopy (WLRS) sensing platform [51], previously introduced for the determination of other biomarkers [52,53]. Real-time measurements were performed by scanning the sensing surface on which distinct antibody areas have been created with a reflection probe that was used for surface illumination and collection of the reflected data. The proposed assay exhibited a LOD of $0.25 \mu\text{g L}^{-1}$ for D-dimer, dynamic range up to $1000 \mu\text{g L}^{-1}$ and an assay duration of 45 min.

Most of the methodologies mentioned in this section have to be applied in a controlled environment, such as the central hospital laboratory, making them a bit impractical to be used in emergency medicine either in the hospital services or in an ambulance.

3. Bioelectroanalysis

Researchers in the electrochemistry field have been trying to replicate the successful example of the glucose biosensor with many other analytes [54,55]. Although developing an electrochemical sensor has many challenges it is indeed viable if it holds its typical advantages like simplicity, low-price, speed of analysis, and the possibility of becoming a ‘point-of-care’ or ‘point-of-need’ miniaturized device [56]. However, it may indeed require some kind of sample preparation step [57] and/or a recognition element since electroanalytical techniques are susceptible to matrix effects; in the case of D-dimer, all literature is based on the use of a selective antibody (Table 1).

Publications in the field of electrochemistry include the use of carbon nanotubes, modified with a nitrile-triacetic-acid/copper complex, with measurements by cyclic voltammetry (CV) based on the decreasing of the electrochemical redox signal of the complex when there was an increasing D-dimer-antibody interaction [58] (Fig. 4 - d). Another copper complex, this time *N*- α -bis(carboxymethyl)-L-lysine, had also been applied, with a lower LOD, on top of a polypyrrole layer; the electrochemical detection was performed with differential pulse voltammetry (DPV) [59] (Fig. 4 - e). Gamella et al. published a work with amperometric approaches based on two different electrode configurations (indirect competition using horseradish peroxidase and a sandwich format) using magnetic beads functionalized with a carboxylic acid on a commercial screen-printed carbon electrode (SPCE) (Fig. 4 - a) [60]. Ibupoto et al. detected D-dimer using an electrode with zinc oxide (ZnO) nanotubes containing electrodeposited silver nanoparticles, while the antibody-antigen interaction on the surface of the working electrode was measured potentiometrically [61]. Other

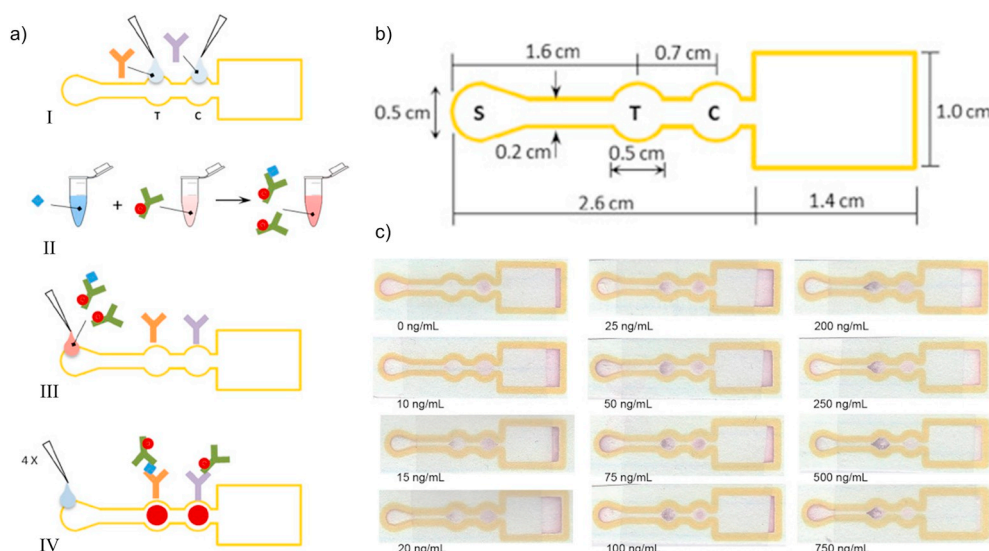


Fig. 3. – a) Schematics of the analytical protocol used for the detection of D-dimer in paper-based analytical devices: I) adsorption and control (mouse and goat anti D-dimer, in orange and purple, respectively) antibodies on the test (T) and control (C) areas; II) test solutions by mixing the anti D-dimer (green)-conjugated gold nanoparticles (red) with D-dimer (blue) samples; III) sample (S) dispensing; and IV) washing. b) Design and dimensions of the paper-based analytical device. c) Concentration of D-dimer samples on immunodetection. All images were adapted from the source [49] with permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Electroanalytical methodologies for D-dimer. How the working electrodes were modified is simplified, more information can be found in the corresponding reference.

Technique	WE	Sample	LOD/ $\mu\text{g L}^{-1}$	Reference
CV	Au/MWCNT/Cu/Ab	buffer	0.1	[58]
DPV	Au/ppy/Cu/Ab	buffer	0.01	[59]
amperometry	SPCE/MB/Ab	blood serum	20	[60]
potentiometry	Au/ZnO/Ab	buffer	0.01	[62]
potentiometry	Cu/graphene/Ab	buffer blood serum	3×10^{-4}	[63]
potentiometry	Au/ZnO/AgNP/Ab	buffer	0.001	[61]
EIS	Au/SAM/Ab	buffer	0.5	[65]
EIS	Au/SWCNT/Ab	buffer	1×10^{-4}	[66]
DPV and EIS	Au/ppy/Cu/Ab	buffer	0.1	[67]
EIS	Au/SAM/Ab	buffer	0.01	[69]
		blood serum	1.4	
EIS	SPPtE/AuNP/Chi	buffer	0.9	[70]

WE – working electrode, CV – cyclic voltammetry, Au – gold electrode, MWCNT – multiwalled carbon nanotubes, Cu – copper complex, Ab – anti-D-dimer antibody, DPV – differential pulse voltammetry, ppy – polypyrrole, SPCE – screen-printed carbon electrode, MB – magnetic bead, ZnO – zinc oxide, AgNP – silver nanoparticles, EIS – electrochemical impedance spectroscopy, SAM – self-assembled monolayer, SWCNT – single walled carbon nanotubes, SPPtE – screen-printed platinum electrode, AuNP – gold nanoparticles, Chi – chitosan.

potentiometric methods can also be found in literature, either by using ZnO nanorods grown on a gold coated glass (Fig. 4 - c) [62] or by using graphene nanosheets (Fig. 4 - g) [63]. In both cases, the D-dimer antibody was immobilized on stabilized lipid films. In these last three cases, a simple 2-electrode cell configuration was used, and the obtained potential of the working electrode was observed against the silver-silver chloride (Ag|AgCl) reference electrode.

A few impedimetric approaches have also been published in recent years, and some important advantages over amperometric biosensors could be obtained. In the latter case, biomolecules are immobilized on the surface of the conductive electrodes and typically, some mediators may be required to avoid that the electron transfer rates drop exponentially with the distance. Hence, one of the advantages of the impedimetric quantification in biosensing is that the biomolecule or antibody-antigen binding could be directly detected without the use of any mediators [64]. Notwithstanding, impedimetric sensors need to be perfectly designed in order to avoid any other non-specific adsorption. Hafaid et al. used two different electrode configurations, both had a

glutaraldehyde-aminothiols on the bottom connected to the gold electrode surface, and both had an antibody on top. However, in between one of the configurations functionalized magnetic iron particles with streptavidin were used while the other used neutravidin (Fig. 4 - b) [65]. Bourigua and co-workers used gold microelectrodes modified with functionalized single wall carbon nanotubes (SWCNT) linked with an 11-carbon thiol self-assembled monolayer (SAM). The thiol made the connection to the gold surface, and the antibody was immobilized by covalent binding to the SWCNT. The microelectrodes were produced in two photolithographic steps [66]. Chebil and co-workers immobilized the antibody on a polypyrrole layer functionalized with a copper coordination complex, while the antibody was connected to the copper centre [67]. This work was recently adapted to a portable microfluidic cartridge (Fig. 4 - f) [68]. Marques et al. developed a label-free approach with an electroactive SAM mounted on a commercial gold electrode. The SAM had two different thiols, one was used to directly connect with the antibody while the other had a ferrocene attached. The electronic transfer of this ferrocene varied according to the amount of D-dimer that had connected with the antibody (Fig. 4 - h) [69]. Also, rather recently, a work was published using a layer-by-layer film containing alternate immobilized antibodies and chitosan/gold nanoparticles [70].

Though, to the best of the authors' knowledge, for reasons that have not been fully described or discussed, none of these methodologies managed to become commercially available.

4. Final thoughts and the future perspectives of DD analysis

D-dimer is a robust molecule, derived from the heterogeneous cross-linked polymeric structures that can exist either independently (190 kDa) or as constituent of 7 different fibrinogen degradation products with molecular weights ranging from 240 to 530 kDa. This variety of molecular composition presents an obstacle in meeting the standards for speed, selectivity, and specificity, found in the devices for detection and quantification of common analytes such as the commercial glucose sensor, and consequently, it has been inhibiting a more pronounced clinical use. Still, although these drawbacks are just slowing down the process, it is quite likely just a matter of time until D-dimer assessment becomes more prevalent and more generalized in medicine.

A promising path to speed up the creation of devices which are cheaper and with longer shelf life is by developing alternatives to antibodies; antibodies not only are expensive but also require specific conditions of temperature and pH to remain fully functional. Substitutes may pass through developing molecular imprinted polymers

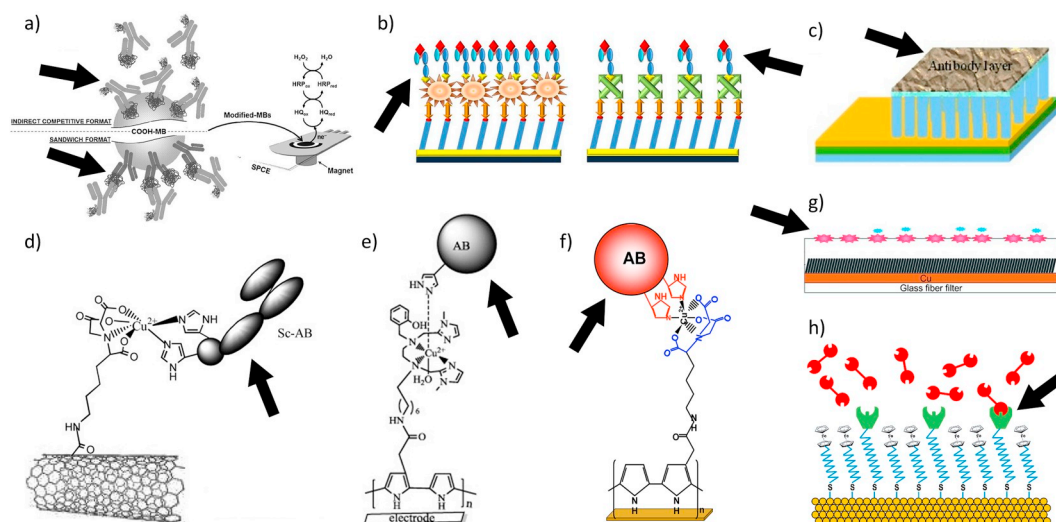


Fig. 4. – Schematic representation of a few electroanalytical methodologies, the black arrows point to the antibody element. All images were adapted from their sources: a) [60], c) [62], d) [58] and g) [63] with permission from Wiley; b) [65] with permission from Hindawi; e) [59], f) [68] and h) [69] with permission from Elsevier.

(MIPs) [71,72], or by synthesizing aptamers [73,74]. Although D-dimer is a too large molecule to be considered as an ideal analyte for MIP development, there have been many advances in this field. It is encouraging that MIPs for large compounds like proteins (e.g. albumin or cytochrome C) have already been successfully developed [75,76].

Authors would like to leave one last encouraging word to the reader, particularly scientists working in this research field: good analytical work with D-dimers is difficult and laborious but one should not give up since it can make a difference. It may eventually save many lives in an emergency room, hopefully in a not so far away future.

Conflicts of interest

All authors declare no conflicts of interest.

Note

The graphical abstract is a D-dimer complexed with Gly-His-Arg-Pro-Tyr-amide [77], drawn using VMD software [78], within a schematized blood vessel.

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