Review

Bioanalytical methods for circulating extracellular matrix related proteins: new opportunities in cancer diagnosis

Ramón Lorenzo-Gómez, Rebeca Miranda-Castro, Noemí de-los-Santos-Álvarez, María Jesús Lobo-Castañón*.

Departamento de Química Física y Analítica. Universidad de Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain.

Instituto de Investigación Sanitaria del Principado de Asturias, Avenida de Roma, 33011 Oviedo, Spain.

* Corresponding author:

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Abstract

The role of the extracellular matrix (ECM) remodeling in tumorigenesis and metastasis is becoming increasingly clear. Cancer development requires that tumor cells recruit a tumor microenvironment permissive for further tumor growth. This is a dynamic process that takes place by a cross-talk between tumor cells and ECM. As a consequence, molecules derived from the ECM changes associated to cancer are released into the bloodstream, representing potential biomarkers of tumor development. This article highlights the importance of developing and improving bioanalytical methods for the detection of ECM remodeling-derived components, as a step forward to translate the basic knowledge about cancer progression into the clinical practice.

Keywords: circulating cancer biomarkers; extracellular matrix**;** immunoassays; aptamer-based assays

1. Introduction

Early cancer diagnosis is widely recognized as a key to tackling the disease and achieving its cure. However, most cancers are currently diagnosed by molecular pathology tests performed in the biopsied tumor tissue, when they become symptomatic. In this way, the tumor is identified, providing information to guide the most appropriate therapeutic strategy. An alternative approach, with potential to allow the diagnosis of cancer in asymptomatic individuals, is the so-called liquid biopsy that consists of detecting tumor-related components in a biological fluid sample. Tumor cells are characterized by a rapid turnover. This implies a continuous release into the bloodstream of not only circulating tumor cells (CTCs) but also tumor-derived nucleic acids (circulating-free tumor DNA, (ctDNA) and RNA such as microRNAs), as well as proteins or exosomes characteristic of tumor cells. All of them have proven useful in the early diagnosis of cancer, its therapeutic guidance, and recurrence monitoring [1].

Besides the tumor cells themselves, there are other components of the tumor that may act as a source of circulating biomarkers. Tumor cell progress depends on the establishment of a favorable niche or microenvironment, which is constituted by blood vessels, several types of cells such as mesenchymal (fibroblasts, adipocytes), immune or neuronal cells, and the surrounding extracellular matrix (ECM) (Figure 1). The latter is an essential component of the tumor stroma that is continuously remodeled by a bidirectional communication with the cells [2]. The interactions between the tumor cells and ECM are critical to promote tumorigenesis and metastasis, and influence all the cancer hallmarks [3]. The dynamic changes that occur in the composition of the ECM during tumor progression involve the release of soluble ECM proteins to the patients' peripheral circulation. The potential for these components to act as cancer biomarkers is rather unexplored, although increasing evidences suggest that we, as analytical chemists, should broaden our focus to include their detection in the design of new early cancer diagnostic tests [4]. In this article, ECM-associated proteins, their detection technologies, as well as their potential use in the diagnosis of cancer are described, identifying the challenges we have to face to translate the extensive knowledge about the ECM-tumor cell cross-talk into information that could be used for clinical decisions.

<Figure 1>

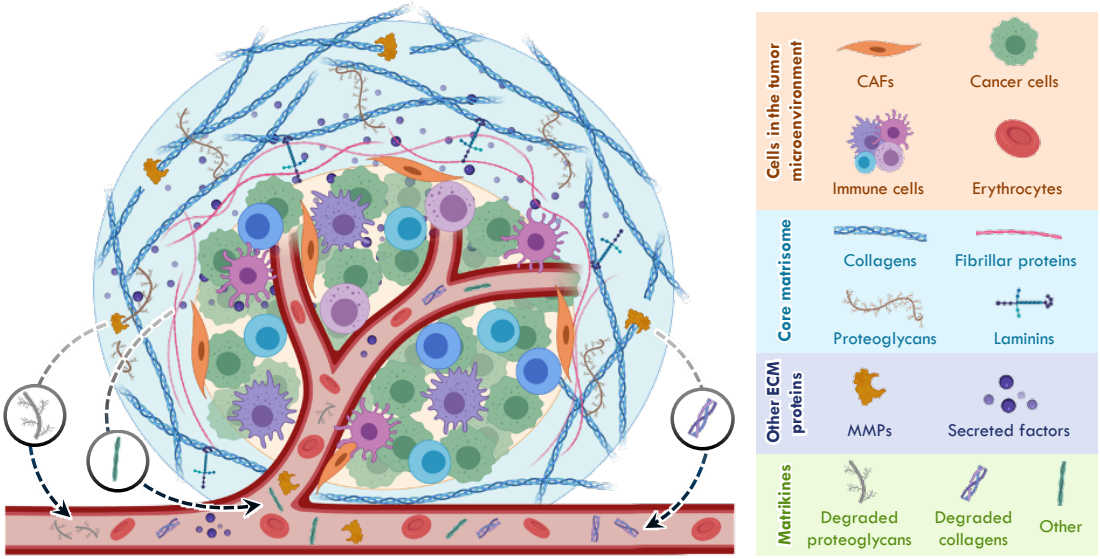

Figure 1 Components of the tumor microenvironment, highlighting the main constituents of the extracellular matrix (ECM). CAFs: cancer-associated fibroblasts; MMPs: matrix metalloproteinases. Created with BioRender.com

2. ECM as a source of circulating biomarkers

Tissue cells are surrounded by a complex meshwork of proteins called ECM. This three-dimensional scaffold not only provides physical support for cells but also facilitates cellular communication, actively participating in signaling to control cell behavior [5]. ECM has two main forms: the basement membrane on which cells sit in close apposition and the interstitial matrix that interconnects the cells and modulates processes such as cell differentiation and migration, guaranteeing the structural integrity of tissues. It is accepted that tumor development usually leads to an activation of ECM-producing cells, resulting in an accumulation of ECM proteins known as tumor desmoplasia, a sign widely used by pathologists to identify tumors with a poor prognosis [6]. The characterization of ECM composition or 'matrisome' is a challenging task because its core structure is largely made of insoluble proteins, although the use of proteomic techniques is providing its thorough description, and a constantly expanding knowledge about its role and influence on cancer progression and metastasis [7].

Matrisome can be categorized into core matrisome, encoded by around 300 genes, and matrisome-associated proteins, a list of more than 770 molecules (Figure 1) [7, 8].

The core matrisome comprises a framework of collagens, the most abundant class of ECM proteins, together with proteoglycans and glycoproteins. The superfamily of collagens is integrated by 28 types, which are divided into fibrillar collagens (types I, II, III, V, XI and XXIV) and non-fibrillar ones. The last group includes among others network-forming collagens (types IV, VIII and X), collagens associated with fibrils (types IX, XII, XIV, XVI, XIX, XX, XXI, XXII) and collagens with a transmembrane domain (types XIII, XVII, XXIII and XXV) [9]. The structure of these collagens is markedly altered during the remodeling of ECM associated to tumorigenesis. Proteoglycans, which expression is often tissue-specific, consist of a core protein onto which one or more glycosaminoglycans (GAG) chains are covalently attached. Some proteoglycans, such as versican, are accumulated in the tumor stroma and are associated to tumorigenesis [10]. Finally, the largest group of core matrisome proteins is the group of glycoproteins, encoded by around 200 genes, including laminins, fibronectins, osteopontin, tenascin C and different elastin-associated proteins such as fibrillins, microfibril-associated glycoproteins (MAGPs) and fibulins [9]. The core matrisome assembles into a complex and mostly insoluble structure, but it is undergoing a wide range of continuous changes, which include its breakdown into smaller fragments during degradation.

In addition to these core components, the ECM acts as a reservoir for secreted factors (growth factors and cytokines), affiliated proteins that share features with ECM proteins or can associated to them (mucins, galectins, etc), and ECM remodeling enzymes that work extracellularly to modify and degrade the ECM proteins. The excessive deposition of structural components of ECM in the tumor microenvironment is accompanied by their reorientation and cross-linking, catalyzed by enzymes as lysyl oxidase (LOX) and transglutaminase families. In consequence, an increased activity of these enzymes is strongly associated with cancer progression [11]. Tumor and stromal cells also produce extracellular proteases such as matrix metalloproteinases (MMPs) and cathepsins, which are deregulated in cancer. These enzymes can cleave most ECM proteins to overcome ECM barriers during metastasis. MMPs constitute the largest family of ECM proteases, and high levels of several MMPs have been correlated with poor prognosis of certain types of cancer [12], such as breast, lung or urological cancers (Table 1), although overexpression of other MMPs has also been related to a reduced metastatic potential in cancer [13].

During the ECM remodeling processes associated to cancer, proteases release into circulation soluble peptides, called matrikines or matricryptins. The serum levels of these fragments can be correlated with tumor activity and invasiveness and may be useful for cancer diagnosis and prognosis [14]. In this context, MMP-degradation products of collagen I (C1M), collagen III (C3M) and collagen IV (C4M and C4M12) or matrikines derived from different collagens such as tumstatin (from type IV collagen) and endostatin (from type XVIII collagen) have been described as potential cancer biomarkers. Proteoglycans are another source of matrikines. Table 1 summarizes the most studied circulating ECM-related components identified so far and the type of cancer they are related.

Table 1 Representative examples of released ECM-components and the type of cancer they are related

Biomarker	**Type of cancer**	**Reference**
MMPs		[4, 12, 15]
MMP-1	Breast	
MMP-2	Lung, prostate, urological	
MMP-7	Breast, gastric, esophageal, brain, oral, renal, pancreatic, urological	
MMP-9	Colorectal, breast, lung, prostate, urological	
MMP-11	Breast	
Collagen family		[4, 14]
From COL1		
C1M	Breast, lung, colorectal, pancreatic	
From COL3		
C3M	Breast, colorectal, pancreatic	
Pro-C3	Colorectal	
From COL 4		
Tumstatin	Lung	
Tetrastatin	Lung	
C4M	Breast, colorectal, pancreatic	
From COL18		
Endostatin	Lung, colorectal, pancreatic	
From COL19		
NC1 XIX	Melanoma	
Glycoproteins		[4, 14]
Elastin	Lung, melanoma	
Fibronectin	Breast, urinary, pancreatic	
Tenascin C	Pancreatic	
Osteopontin	Lung, pancreatic	
Proteoglycans		[10]
Decorin	Breast, esophageal, pancreatic	
Versican	Breast, ovarian, gastric, hepatocellular	
Lumican	Lung	

The presence of the released ECM components in serum and biofluids can be followed over the course of the disease, helping in the early detection, diagnosis and progression monitoring of cancer. The main limitations to adapt these potential biomarkers to the clinical practice are their low concentration levels and the lack of suitable reagents and assays for their high selective and sensitive detection. In the following sections, representative examples of these ECM-derived biomarkers are presented from an analytical point-of-view, comparing the analytical performance of existing and emerging assays and how they fulfill the specific requirements for the application in clinical laboratories.

3. Bioanalytical tools for detecting components released from ECM

Aberrant ECM remodeling in tumor tissues leads to release into the bloodstream of abnormal amounts of certain components, for example the ECM remodeling enzymes themself or soluble matrikines generated from the ECM breakdown. They may be used to indicate the presence of malignancy at an early stage or to provide information about the patient outcome (the likeliness of tumor progression or its response to a certain therapy). These potential biomarkers are identified by first establishing the ECM gene signature associated to the tumor tissue and then applying proteomic approaches to identify circulating proteins or peptides that may be originated from the tumor microenvironment. To translate these findings into effective diagnostic tools, it is essential to develop new analytical tools for the accurate and rapid detection of these targets, mainly in blood where they would enter by diffusing into the leaky vasculature of the tumor tissue, although other biofluids such as saliva or urine can also be explored. The ECM derived biomarkers are classified as: i) MMPs; ii) collagen family; iii) glycoproteins; and iv) proteoglycans. For each group, different analytical assays are presented, which can facilitate and improve the feasibility of using these new biomarkers into the clinical laboratories.

3.1. Matrix metalloproteinases

Human matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, which are named as MMP-1 to MMP-28 and share a similar structure. They are synthesized as inactive pro-peptides (zymogens), being enzymatically activated in the ECM. In addition, their activity is reversibly inhibited by a group of

structurally related endogenous inhibitors known as tissue inhibitors of metalloproteases (TIMPs). MMPs proteolytic activity contributes to the breakdown of the ECM, and their destructive function is associated to different inflammatory disorders [12]. They are also upregulated in different types of cancer, thus serving as potential biomarkers for early detection, disease progression and metastasis [15].

Assays for the determination of MMPs are classified into two groups: enzymatic methods detecting the active forms of MMPs on the bases of their proteolytic activity and affinity methods, which use antibodies or aptamers for their specific recognition [16]. The immunoassays cannot distinguish between the active and zymogen forms of the proteins. MMPs are typically measured in serum, although their measurement in tissue samples results also of interest. There is a lack of validated methods or standards and different assays provide significant differences in the reported values, which makes interpretation difficult from a clinical point of view.

3.1.1. Enzymatic Methods

Among the methods that take advantage of the MMPs proteolytic activity, zymography analysis is the gold standard. This method involves the electrophoretic separation of the enzymes through polyacrylamide gels containing a natural protease substrate (typically gelatin or collagen), under denaturing conditions. Upon separation, the cleavage of the substrate takes place after renaturation of the enzymes by changing the detergent from sodium dodecylsulphate to Triton X-100. In this way, the staining of the resulting bands provides qualitative and semi-quantitative information on the enzymes, with typical detection limits in the picograms range and relatively long digestion periods (18- 43 h) [17].

Short synthetic peptides are most often used to measure the proteolytic activity of MMPs. Linear custom-made peptides, which act as specific substrates for a given MMP, are either labeled or bound to a surface to design homogeneous and heterogeneous assays, respectively. The transduction step is usually performed by fluorescence resonance energy transfer (FRET)-based detection or electrochemical techniques.

3.1.1.1. FRET-based assays

Regarding homogeneous assays, research focuses mainly on the use of FRET measurements (Figure 2), which use as a substrate a short peptide linked at one end to a

donor fluorescence molecule and at the other to a fluorescence acceptor. While both species are in close proximity (1–10 nm), the emission of the fluorescence donor is quenched by the acceptor, but when the MMP breaks the peptide, donor and acceptor move away, resulting in an increase in the donor fluorescence. Traditionally, donor and acceptor are organic molecules, such as the couple fluorescein isothiocyanate (FITC) and 4-(4-dimethylaminophenylazo) benzoic acid (Dab). A short peptide specific for MMP-2 labeled with these two molecules (Dab-GPLGVRGY-FITC) allowed the sensitive and specific detection of MMP-2 overexpressed in gastric cancers (Figure 2a). This method has a limit of detection (LOD) of 42 ng/mL for MMP-2 in buffer samples, with the ability to specifically recognize gastric cancer cells, both through *in vitro* and *in vivo* measurements [18]. To overcome the long-term stability and photo-bleaching issues related to the use of organic fluorescent labels, semiconductor quantum dots (QDs) have been proposed as FRET donors, allowing the efficient and reliable analysis of MMPs [19]. QDs may also act as energy acceptors for a bioluminescence emission process, in a methodology that is called bioluminescence resonance energy transfer (BRET). Thus, when the bioluminescent protein luciferase is fused to a 15 amino acid peptide containing an MMP-2 substrate (GGPLGVRGGHHHHHH), and then conjugated to QDs with carboxylic groups, the radiation released in the presence of the luciferase substrate is transferred to the QDs. As a consequence of this BRET process, the light emission from QDs is observed. In the presence of MMP-2, the cleavage of the peptide separates the luciferase from the QDs and there is a decrease in the QD emitted radiation (signal-off, Figure 2b), improving the detectability of MMP-2 to 2 ng/mL (30 pM) [20]. Nanomaterials are also used as efficient acceptors. For example, gold nanoparticles (AuNPs) have been exploited in the design of a signal-on BRET assay for detecting MMP-2 activity [21]. Luciferase was conjugated through a substrate peptide (IPVSLRSG) to AuNPs, which efficiently quenched the bioluminescence emission from the protein. The cleavage of the peptide in the presence of active MMP-2 led to an increase in the bioluminescent signal that is related with the MMP-2 concentration in the range between 50 ng/mL and 1 µg/mL. In most of these studies, only the proof-of-concept is shown, without evaluation in patient samples in order to establish their feasibility for diagnosis or prognosis purposes. For practical applications, it would be necessary to evaluate a panel of different MMPs. However, homogenous assays are characterized by a limited multiplexed detection capability because only a reduced number of probes providing different signals can be simultaneously used. This

limitation can be overcome by designing heterogeneous assays, where different substrates can be immobilized at different locations on a solid surface for multiplex detection.

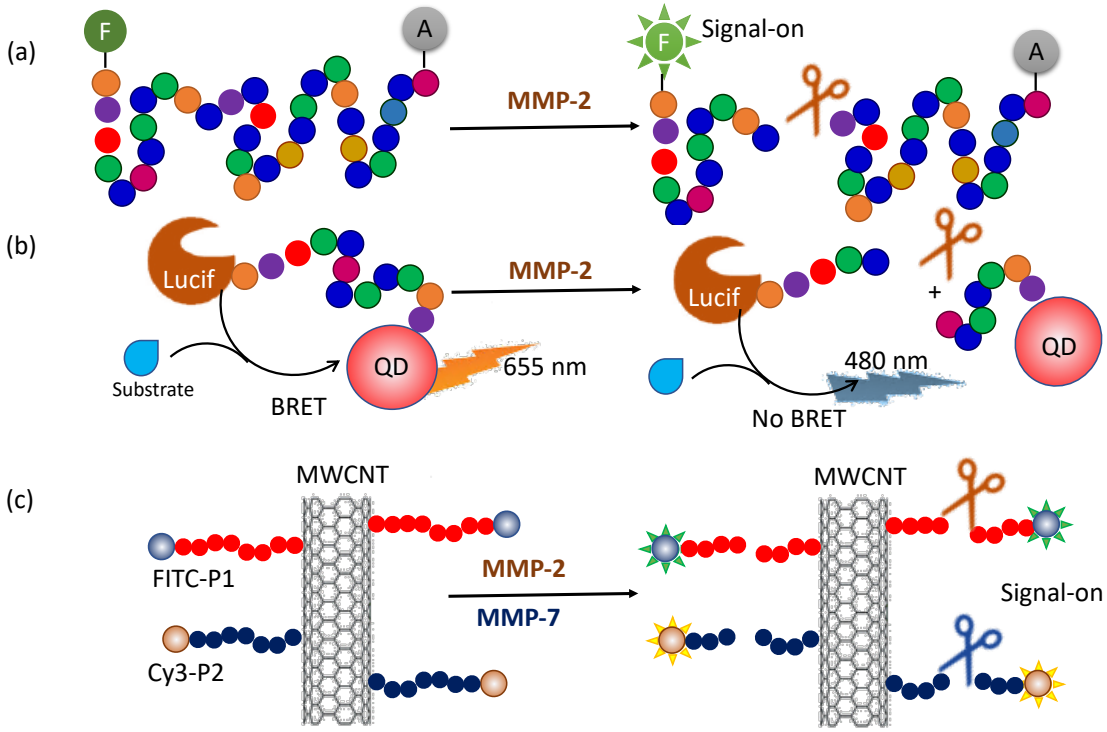

Figure 2 FRET-based assays for measuring MMP activity. a) Homogeneous signal-on assay using a peptide substrate labeled with a fluorescence molecule (F) and an acceptor (A). b) Bioluminescence resonance energy transfer from luciferase (lucif) substrate to a quantum dot (QD). The cleavage of the peptide by MMP causes a decrease in the luminescence of QD. c) Multiplex detection of MMPs using two specific peptides labeled with different fluorophores immobilized on MWCNT

Thus, FRET-based sensors are prepared by conjugating suitable peptides labeled with a fluorescence donor on graphene oxide (GO) or carbon nanotubes (CNTs). These carbon nanomaterials act not only as a solid support for immobilization but also as a fluorescence quencher. This scheme has been applied to the detection of MMP-2 activity by covalently immobilizing the peptide CALNNGPLGVRGRAK-FITC onto graphene oxide [22]. The signal-on assay achieved a detection limit of 2.5 ng/mL and was applied to the analysis of serum samples. In a similar approach, peptides labeled with different fluorophores (GVPLSLTMGK-FITC for MMP-7 and GCGPLGVRGK-Cy3 for MMP-2) were immobilized onto multiwalled carbon nanotubes (MWCNT) for

the multiplex detection of MMP-2 and MMP-7 (Figure 2c), in both buffer solution and human serum samples, with a LOD of 5 pg/mL and 0.5 pg/mL, respectively [23].

3.1.1.2. Electrochemical sensors

Electrochemical transduction offers the possibility of designing label-free sensors, easy to operate and miniaturize, which can be integrated into sensor arrays (Figure 3). In this context, a field-effect transistor (FET)-based biosensor has been described for the detection of MMP-9 [24]. In this example, molybdenum disulfide (MoS_2) was deposited on the gate of a FET, between the drain and the source. This film was further modified by adsorbing a peptide (amyloid-β_{1-42}, DAEFRHDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVVIA), which acts as substrate of MMP-9 and also as n-type dopant amplifying the electrical signal from the transistor. This amplification led to a significant improvement in the detection of MMP-9 with a dynamic detection range from 1 pM to 10 nM (67 pg/mL to 670 ng/mL, assuming a MW of 67 kDa). The cleavage of specific peptides immobilized on electrodes leads to a change in the electrode interfacial properties such as impedance or capacitance, which can be used for transduction purposes. Interdigitated array microelectrodes are very useful for this kind of measurements. Thus, the immobilization of an MMP-9 specific peptide (GPLGMTSRC) by chemisorption of the terminal cysteine onto gold surfaces (Figure 3a) induced a better electric isolation of the electrodes, and consequently a decrease in their capacitance. The digestion of the peptide by cell-secreted MMP-9 led to a recovery on capacitance in a relatively short detection time (30 min), showing a wide detection range from 10 pM to 10 nM [25]. This system was integrated into a microfluidic device for relating the amount of cell-secreted protease with cellular morphology changes, observing a significant increase of MMP-9 release from activated cells (1.19 pg/cell h) when compared with normal cells (0.04 pg/cell h). Alterations in the charge transfer resistance can also be monitored by faradaic impedance spectroscopy (FIS) in the presence of an electroactive probe, typically ferro/ferricyanide. This transduction principle has been exploited for developing another MMP-9 biosensor on interdigitated gold electrodes [26]. In this approach, a specific peptide (LGRMGLPGK) was cross-linked to dextran on the electrode surface. The degradation of this hydrogel by the action of MMP-9 led to a decrease in the electron transfer resistance, measured by FIS, which allowed the detection of the enzyme in the range between 50 to 400 ng/mL in only 5 min. Differential pulse voltammetry (DPV) in the presence of the electroactive

probe may also provide information about the changes in the electron transfer resistance of the electrode surface. This is the approach exploited in an electrochemical biosensor for MMP-7, based on the immobilization of a specific peptide substrate: NAADLEKAIEALEKHLEAKGPCDAAQLEKQLEQAFEAFERAG onto a gold electrode modified with a nanocomposite of gold nanoparticles and carbon nanotubes [27]. The nanomaterials improved the sensitivity of the assay, with a LOD of 6 pg/mL. The sensors have been applied to the detection of MMP-7 in diluted serum and undiluted synthetic urine samples.

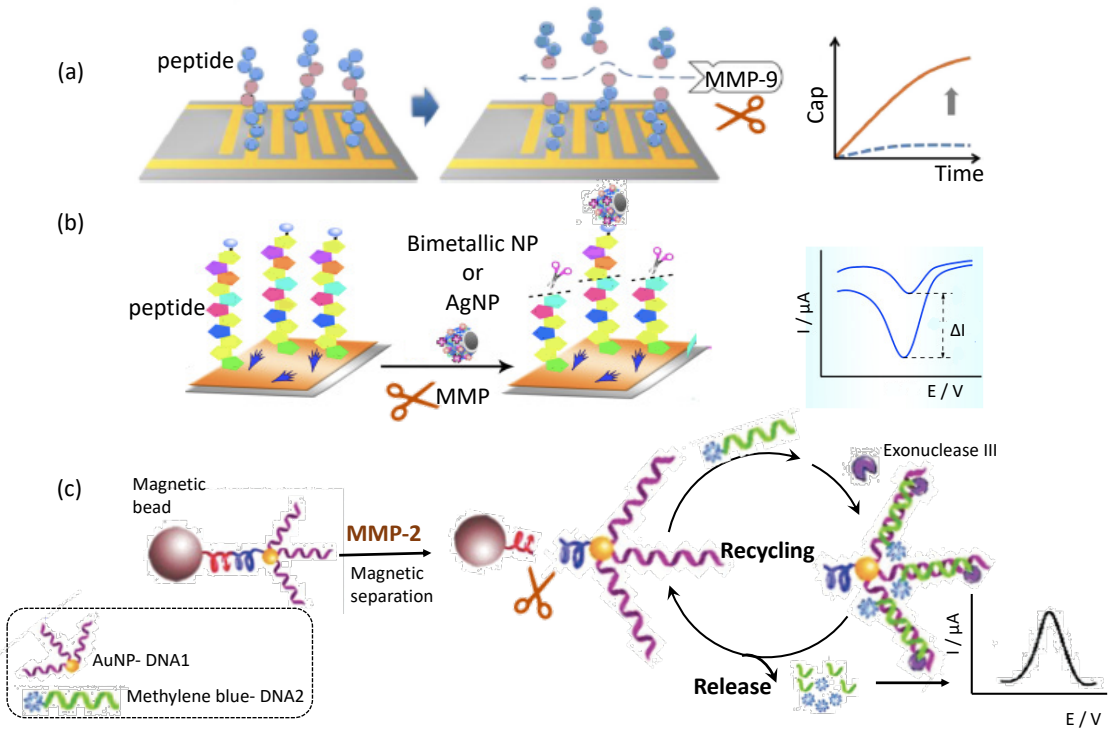

Figure 3 Electrochemical sensors for measuring MMP activity. a) Capacitive sensor based on the immobilization of a specific peptide on interdigitated electrodes (adapted with permission from [25]. b) The specific peptide is labeled with a nanoparticle. The cleavage of the peptide causes a decrease in the voltammetric signal associated to the nanoparticle (adapted with permission from [31]. c) The specific peptide is immobilized onto magnetic beads, combined with AuNP-DNA1. MMP cleavage leads to the release of DNA, which is detected using an exonuclease-III recycling scheme (adapted with permission from [33]

Electrochemical sensors may also rely on the immobilization of the peptides labeled with a redox reporter, which is responsible for the analytical signal. The cleavage of the peptide catalyzed by the enzyme causes the release of the redox reporter with the consequent decrease of the signal. This was the approach adopted to detect MMP-9, using methylene blue (MB) chemically bound to the N-terminus of the peptide GPLGMTSRC as redox reporter [28, 29]. This approach led to a LOD higher than the previous label-free designs, in the range of 0.5-5 ng/mL. The poor detectability may be attributed to a signal-off format. To increase the signal, different amplification mechanisms have been proposed involving nanomaterials [30, 31] or coupling the recognition event to an oligonucleotide sequence, which is then measured by amplification methods [32, 33]. The proof-of-concept of these strategies, summarized in Figure 3, has been stablished for MMP-2 measurements. Peptide-templated silver nanoparticles (AgNPs) [30] and a complex composite of hollow ceria nanospheres with bimetallic Pt/Pd nanoparticles, which incorporated streptavidin for their bioconjugation and thionine as electroactive probe [31], have been coupled to a peptide specific for MMP-2, GPLGVRGKGGC (Figure 3b). The cleavage of this peptide causes a release of the nanomaterial, which is responsible for the electrochemical signal, with a consequent signal-off response. Despite the complexity of the design, the increased costs associated with the use of nanomaterials is not justified by the slightly improvement in sensitivity, with a LOD about 0.1 pg/mL. Platinum nanoparticles can be coupled not only to the specific biotinylated peptide but also to a ssDNA sequence, in such a way that after their directed immobilization on the electrode surface through streptavidin-biotin interactions, ssDNA molecules trigger a hybridization chain reaction (HCR) [32]. The resulting dsDNA interacts electrostatically and through intercalation with thionine to generate the electrochemical signal. Again, a signal-off assay is obtained, because in the presence of MMP-2, the peptide is hydrolyzed with the subsequent release of the ssDNA-PtNP. Although this sensor combines the multivalence of the PtNP with an isothermal DNA chain reaction for signal amplification, the sensitivity is not improved. The first concentration of MMP-2 that is distinguished from the blank is 1 pg/mL and a detection limit of 0.32 pg/mL is claimed, but the criteria to obtain it is incorrect (it is estimated as 3 s_B/m, where s_B is the standard deviation of the blank and m is the slope of the corresponding calibration curve, but the signal is linearly correlated with the logarithm of the concentration). The possibility of using this sensor in biological samples has only been demonstrated in 10-fold-diluted serum sample. It

should be noted that this is not an aptasensor, as mentioned in the work, because the molecular recognition of the MMP takes place through the peptide and ssDNA is used for signal generation and amplification purposes. A similar approach used exonuclease III-assisted recycling for the amplification of the signal related to ssDNA attached to the peptide substrate [33] (Figure 3c). However, in this approach molecular recognition and transduction are not integrated. The molecular recognition takes place on the surface of magnetic microparticles and after the hydrolysis of the peptide substrate in the presence of MMP-2, ssDNA-AuNPs are released from the microparticle and hybridized with MB-labeled DNA. The introduction of exonuclease III led to an amplification by recycling, in which each DNA-AuNP triggered the digestion of many MB-DNA with the subsequent release of MB, responsible for the electrochemical signal onto a suitable electrode surface. The advantage of this scheme is that it leads to a signal-on assay although the sensitivity improvement is only moderate (LOD 0.15 pg/mL). These strategies, where the detection of MMP is subrogated to a DNA sequence bound to a substrate peptide, can suffer from interferences related to the presence of circulating DNA in the samples, which is not usually explored. Recovery assays on diluted samples are the only validation tests to demonstrate the feasibility of these approaches on clinical samples.

3.1.2. Affinity methods

The specific recognition of MMPs by suitable antibodies is the most commonly used molecular recognition reaction for the development of affinity assays. Various MMPs- antibodies and enzyme-linked immunosorbent assays (ELISAs) with colorimetric detection are commercially available. Sandwich ELISAs are the most widely used assays in the clinical studies reporting increased levels of several MMPs in body fluids from cancer patients, although it is uncertain if the recognition antibodies discriminate between free- and inhibitor-bound forms of the MMPs [34, 35]. The same sandwich-assay format is used on multiplexed microsphere-based immunoassays commercially available for the measurement of different panels of human MMPs [36]. These assays are based on the use of fluorescent-coded magnetic beads coated with MMPs -specific antibodies. A secondary biotinylated-antibody is used for signaling through streptavidin-phycoerythrin conjugates. After magnetic separation, a Luminex® analyzer, incorporating two lasers, is used for reading; one laser is microparticle-specific and identifies the MMP that is being detected and the other laser excites the

phycoerythrin-fluorescent signal, which is correlated with the amount of each MMP. The minimum detectable concentration depends on the MMP, it is 2 pg/mL for MMP-9 and 200 pg/mL for MMP-2, probably because the different affinities of the corresponding antibodies. Research in this field is focused on the search for new labels for improving the sensitivity of the commercial tests. For example, the use of silver nanoparticles-labeled secondary antibodies in combination with CdTe quantum dots (QDs) allowed the detection of MMP-7 in a paper-based visual fluorescence immunoassay with a LOD of 7 pg/mL [37].

Electrochemical immunosensors are also explored for MMPs detection [38-43]. The preferred design is the sandwich format, which is directly performed onto the electrode transducer, focusing the attention on new labels for the signaling antibodies to improve the analytical performance. The activity of the enzyme classically used in ELISAs, horseradish peroxidase (HRP), can be electrochemically monitored. Multiple units of this enzyme were incorporated to the secondary antibody through polystyrene beads [38] or polydopamine-functionalized graphene sheets [39] leading to LODs of 4 pg/mL and 0.11 pg/mL for the detection of MMP-3 and MMP-2, respectively. Multiple HRP have also been incorporated to the immunoassay using a DNA-labeled signaling antibody. This sequence triggered a hybridization chain reaction using two additional ssDNA, misnamed as aptamers, one labeled with HRP and the other with AuNP and thionine [40]. This scheme was used for the amperometric detection of MMP-2 at -0.4 V in the presence of H_2O_2, which was catalytically reduced by AuNPs and HRP, and thionine acted as an electron transfer mediator for HRP, being electrochemically regenerated. The LOD obtained was 35 fg/mL. The labelling of the DNA sequences was performed by cross-linking with glutaraldehyde, which may lead to poor reproducibility. The selectivity of the sensor was only examined for certain proteins, although it was tested in a reduced number of serum samples with recoveries between 98 and 102%. Several nanomaterials, which are detected by different electrochemical techniques, have also been proposed as labels [41-43]. The loading-capacity and the detection technique seem decisive to establish the analytical performance. Thus, the use of copper nanoclusters incorporated to the signal antibody through a DNA sequence by a hybridization chain reaction involving copper nanocluster-modified hairpin probes, allowed the potentiometric detection of MMP-7 with a LOD of 5.3 pg/mL [41]. Although only a portable potentiometer is required to make the measurement, a

cumbersome protocol is needed, with a final solubilization of the nanoclusters before the transduction. Sensitivity is improved by using silver nanoparticles as a label in combination with its measurement by anodic stripping analysis. This is the approach followed to analyze MMP-9, with the ability to detect 5 fg/mL [42]. Similar sensitivity was reported for a photochemical sandwich immunoassay based on the use of a composite of TiO_2 nanotubes and CdS:Mn/ CdTe quantum dots as matrix for the immobilization of an MMP-2 antibody [43]. This structure enhanced the photocurrent used as an analytical signal due to its high light-absorption capability, promoting a fast electron transfer and inhibiting the charge recombination. The signaling antibody was modified with SiO_2 nanoparticles, which reduced the photocurrent intensity because of a steric hindrance effect. Though a signal-off assay, it exhibits a detection limit of 3.6 fg/mL for MMP-2 detection.

A much simpler design is a direct immunoassay, where the immunological recognition of the antigen leads to the analytical signal without the need for labels. This was scarcely explored for MMPs analysis, in combination with surface plasmon resonance (SPR) detection. This approach allowed the rapid detection of MMP-9 in saliva samples, with a LOD of 8 pg/mL [44]. Multiplexing is challenging in SPR measurements, and to overcome this limitation a nanoplasmonic immunosensor array with a high spatial and temporal resolution was developed [45]. This sensor was used for real-time monitoring of MMP-9 secretion from human cells.

The selection of nucleic acid aptamers to MMPs could provide new functional ligands for their specific recognition with high affinity. In addition, aptamers can be selected so that they distinguish zymogen and active enzyme forms of the enzymes. So far, few aptamers have been selected using MMPs as targets [46-49]. There are various aptamers targeting MMP-9, both RNA aptamers (F3B: 5′-UGCCCUGCCCUCACCCGUUAGCCUGAGCGCCCCGCA-3′, K_d = 20 nM [46], and DNA aptamers (Apt-9: 5′- TACGGCCGCACGAAAAGGTGCCCCATAACT CAATGCCGTA-3′ [47] and 8F14A: 5′- TCGTATGGCACGGGGTTGGTGTTG GGTTGG-3′ [48]), and only one aptamer for the recognition of MMP-2 (Apt-2: 5′-TCGCCGTGTAGGATTAGGCCAGGTATGGGAACCCGGTAAC-3′; K_d = 5.6 nM [49]). The selectivity of these receptors made it possible to design very simple MMP-9 measuring devices, in which aptamers were immobilized onto a conductive surface to entrap the target with the subsequent transduction of the recognition event without the

need for additional labels [48, 50]. A capacitive biosensor has been developed, using the aptamers 8F14A immobilized onto MWCNT-modified interdigitated electrodes for capturing MMP-9, with a LOD of about 165 pg/mL [50]. Similarly, the RNA aptamer F3B was immobilized onto the gold surface of a quartz crystal microbalance (QCM), which in combination with the DNA aptamer 8F14A for signal improvement through the formation of a sandwich complex with MMP-9, led to a LOD of 560 pg/mL in untreated serum [48]. The same RNA aptamer was immobilized on a microchip for the fluorescence detection of MMP-9 [51]. This is a much complex design, where a second ssDNA sequence binds to Zn^{2+} metal site of MMPs (in a non-specific chelating interaction) after capturing. A third assistant molecular beacon sequence is subsequently incorporated for fluorescence measurement after elongation of the second probe by rolling circle amplification (Figure 4a). Although rapid (30 min), the improvement in sensitivity that is achieved with such a complex combination of sequences is not clear. FRET has been exploited for the simultaneous detection of MMP-9 and MMP-2, using the aptamers Apt-9-labeled with FAM and Apt-2-labeled with Texas Red for their specific recognition [52]. These aptamers were initially bound to polydopamine nanospheres, which quenched the fluorescence from the labels. In the presence of MMP-2 and MMP-9, the aptamers were released from the nanospheres by forming the corresponding aptamer/target complexes, with the subsequent recovery in the fluorescence. DNase-I allowed target recycling and signal amplification (Figure 4b). This strategy was useful for the detection of MMPs in urine samples with a detection limit of 9.6 and 25.6 pg/mL for MMP-9 and MMP-2, respectively.

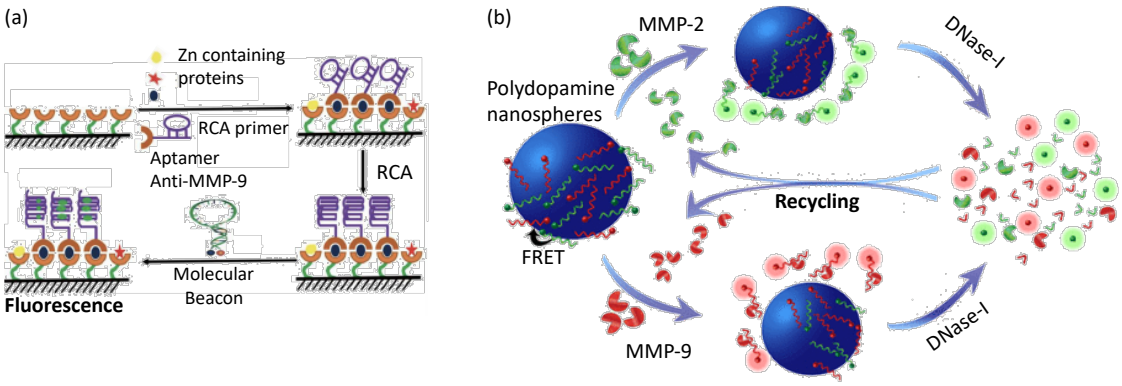

Figure 4 Aptamer-based assays for MMP detection. a) Sandwich between a DNA sequence recognizing Zn-containing proteins and an anti-MMP aptamer, which is elongated by RCA. A molecular beacon is finally used for fluorescence detection (adapted with permission from [51]). b) Multiplex fluorescence assay with two different

aptamers immobilized onto polydopamine nanospheres that quench the signal from the labels. The formation of the aptamer-MMP complex releases the aptamer from the nanosphere recovering the fluorescence. A DNAse-I recycling amplifies the signal (adapted with permission from [52])

The assays reported above have in general potential for obtaining information about the increased levels of several MMPs in body fluids from cancer patients. However, taking into account that the same MMPs are upregulated in many different types of cancer as well as in many inflammatory diseases, such assays should be used for screening purposes.

3.2. Collagen family

All collagens share a core structure consisting of three polypeptide chains (denoted as α) equal (homotrimer) or not (heterotrimer). They are named as COLxAy, in uppercase letters for human collagens, where x is the collagen type and y the number of the α chain [53]. They contain repetitive proline and hydroxyproline rich-peptides with a glycine every three amino acids (Gly-X-Y). The three chains form a right-handed triple helix around a central axis with glycine in the inner central region while bulky side chains from other amino acids are oriented to the molecular surface [54]. This collagenous domain is the major part of fibrillar collagens. Other collagens present several non-collagen domains (NC) interrupting the pure triple helix domain. This NC regions are more diverse in sequence and structure and more amenable to proteolysis. NC domains at N and C-terminal ends govern the triple helix assembling in all collagens [55]. Once fully folded, the fibrillar collagens are secreted into the ECM through vesicles and then the terminal domains (N and/or C propeptides) are enzymatically cleaved to yield the mature collagen acquiring the ability to assemble into fibrils. Other collagen types are also processed forms [53].

Constant rebuilding of ECM implies not only neosynthesis and rearrangements but also degradation of ECM components including collagens. Proteolytic cleavage generates fragments biologically active (matrikines), whose function is different from the parent molecule or peptides that regulate cell activity. Collagen fragments can have opposite functions in tumorigenesis. The most studied group of matrikines are those derived from basement associated collagens, mainly type IV, XV, XVIII and XIX. They play antiangiogenic and antitumoral roles [56]. Since their expression is regulated

during tumor invasion they are being studied as potential drugs. Endostar, the N-terminal fraction of endostatin (derived from COL18) has been approved in China for the treatment of non-small cell lung cancer (NSCLC) [55].

3.2.1. Enzyme linked immunosorbent assays

Several collagen fragments of basement membrane and fibrillar collagens have been found in serum stimulating their study as tumor biomarkers. Other minor collagens, which might also generate biomarkers of clinical utility, are extremely challenging to detect in biological fluids due to minute concentrations. Current bioassays rely mostly on antibodies. Until recently most monoclonal (mAb) or polyclonal (pAb) antibodies available recognize the repetitive collagenous region or the NC domain but not the denatured or proteolyzed collagen. The detection of epitopes unveiled upon degradation has been achieved upon generation of suitable Ab by subtractive immunization [57]. HU177 epitope from COL4 was detected in serum of melanoma patients. The specific mAb was used as the capture receptor and a biotinylated anticollagen IV Ab as the secondary one. An antibiotin mAb conjugated to HRP was used to generate the colored product. The sandwich format indicates that the assay is aimed at detecting the cryptic site within the entire denatured collagen, which was used as a standard for calibration from 5 to 100 ng/mL.

Relatively large matrikines such as endostatin (20 kDa) from COL18A1 and tumstatin (28 kDa) from COL4A3 are also detected in sandwich formats. Both compounds have a protective role in tumor growth thanks to its antiangiogenic activity. The former is usually detected with commercial assays. Tumstatin was detected with two novel mAbs obtained by immunization with the recombinant matrikine expressed in *E. coli*. The most sensitive Ab was employed as a capture receptor and the detection one was biotinylated to label it with streptavidin-HRP. The analytical range, 1.4-500 ng/mL, is suitable for detection of tumstatin in serum from healthy people and lung cancer patients with or without metastasis. Cross-reactivity with structurally or functionally similar ECM components was negligible [58]. More recently, a short peptide from human tumstatin, PGLKGKRGDS, highly similar to the mouse tumstatin, was the immunizing peptide to obtain mAb finally used in a competitive assay. The biotinylated peptide anchored to streptavidin-coated plates was incubated in one-step with a mixture of the sample and the HRP-mAb. Addition of HRP substrate tetramethylbenzidine (TMB) allowed the detection of peptide concentrations between 0.26 and 9.92 ng/mL.

Serum levels in NSCLC patients were found significantly elevated in comparison with healthy individuals but differentiation among disease stages was not possible [59].

The matrikine derived from NC1 domain of COL4A4, tetrastatin (29 kDa), is another compound that can be found in serum. In this case, a competitive assay using only one receptor was developed. The pAb was obtained after immunization with a small peptide TLKESQAQRQKISRC. The microtiter plate was covered by adsorption with recombinant human tetrastatin and challenged with a previously incubated mixture of the biotinylated pAb and increasing concentrations of the standards or samples. As usual, a secondary HRP-conjugated antiIgG antibody and TMB addition allowed the colorimetric detection. The linear range, from 2 to 2500 ng/mL, was wide enough to detect the until then unknown values of tetrastatin in serum, bronchial aspiration, bronchoalveolar lavage fluids and tumor tissues with a reproducibility below 10% [60]. An identical approach was also developed for the NC1 (XIX) peptide, NPEDCLYPVSHAHQRTGGN. The analytical range from 0.019 to 40 µg/mL allows the detection of the full-length peptide in serum samples [61].

The Danish company Nordic Bioscience is strongly engaged in developing novel biomarkers including neoepitopes from ECM. They always design competitive assays based on biotinylated peptides bound to streptavidin-coated plates. They obtain their own mAbs for each neoepitope, that includes fragments from mature COL1 generated by MMP-9, -2 and -13 cleavage (C1M), from COL3 and COL4 generated by MMP-9 (C3M and C4M, respectively) and from COL3 propeptide cleaved by a N-protease (PRO-C3). The selection of the neoepitope relies in enzymatic cleavage of the collagen by several proteases, identification of fragments through LC-MS and selection based on sequence specificity. C1M, with sequence GSPGKDGVRG, was selected to contain a cleavage site for cathepsin K, the main protease in bone, ensuring the non-bone origin of the neoepitope. The competitive ELISA with a LOD of 0.83 ng/mL does not detect intact type I collagen nor cross-react with highly homolog sequences from types V and VII collagens [62]. Similarly, the competitive assay to detect C3M, whose sequence is KNGETGPQGP, presents a LOD of 0.5 ng/mL and it does not recognize intact type III and type II collagens. MMP-9 degraded fragments from the latter do not interfere its detection [63]. The protease degradation of type IV collagen released several unique peptides but C4M (GTPSVDHGFL) was chosen after confirming that the corresponding mAb was able to distinguish between the full length and cleaved collagen [64]. The

analytically useful concentration range varied from 1.5625 to 100 ng/mL with a LOD of 0.61 ng/mL. Analogous collagen fragments from type I and VI do not interfere the detection [64]. The C-terminal neopeptide from N-terminal propeptide of COL3A1 (PRO-C3), whose sequence is CPTGPQNYSP, was also determined in a competitive assay with a dynamic range from 0.867 to 60.1 ng/mL and a LOD of 0.606 ng/mL [65]. All these neoepitopes are being studied as a fingerprint of overall survival in pancreatic ductal adenocarcinoma (PDAC) [66] and metastatic breast cancer [67] or stratification of colorectal cancer patients [68]. The PRO-C3 antibody recognizes both the cross-linked and monomeric propeptide. Since tumor growth and metastasis is associated to ECM stiffness, a sandwich assay only detecting the cross-linked propeptide might be a biomarker in hepatocellular carcinoma (HCC). The detection range from 0.4 to 17.3 ng/mL was enough to study the plasma values of HCC or other liver patients and healthy people [69].

3.2.2. Aptamer-based assays

To the best of our knowledge only one aptamer has been derived from collagen fragments. The 26 amino acid C-telopeptide of COL1A1 (CTX) (SAGFDFSFLPQPPQEKAHDGGRYYRA), associated to bone metastases, was the target for the selection by SELEX of a molecular beacon aptamer [70]. Of the three stem-loops of the two winning aptamer sequences, only one near the 3'-end was found to exhibit FRET behavior when labeled with TYE-665 fluorophore and Iowa Black RQ quencher at each end. By molecular dynamics this binding site was confirmed. The final truncated aptamer contains only 15 nt of which 13 belongs to the primer region, a rather unusual finding. However, all 15 nt are needed to exhibit FRET in the presence of increasing concentrations of CTX. This is one of the few examples of primer-containing aptamers. The application in urine samples is not straightforward due to the principle of detection. Urea and creatinine amines disrupt the aptamer secondary structure yielding large background signals and lack of recognition. Removal of these components through a size-exclusion column is compulsory prior to analysis though the recovery of the target is modest (78.6%). The aptamer does not recognize the epitope of some commercial antibodies opening the door to hybrid antibody-aptamer sandwich assays.

The above discussion reveals that antibody development against collagen-derived matrikines is an active research area and competitive assays are the most extended. In contrast, aptamer selection, that is animal-free, is mostly unexplored for this class of

ECM components. The insoluble nature of collagen might discourage its obtention but the increasing knowledge of matrikine sequences and the affordable cost of chemical synthesis of short peptides might fuel the selection of anti-collagen aptamers. Their availability will open a wide range of analytical possibilities.

3.3. ECM glycoproteins

Glycoproteins present in ECM are a heterogeneous set of proteins with diversity of functions. Elastin, fibronectin and laminins form large assemblies and have structural roles. In contrast, matricellular proteins comprise an increasing group of modular proteins associated to structural ECM components, though are not part of them, involved in cell to cell or cell-ECM interaction and cell migration [9].

3.3.1. Structural glycoproteins

The degradation fragments of elastin, the main component of elastic fibers is the result of the increase secretion of proteinases during tumor invasion and metastasis. A panel of unveiled matrikines, ELM12 (GVAPGIGPGG), ELM7 (IKAPKLPGGY), EL-NE (GGPGFGPGVV), EL-CG (LGGVAARPGF) and ELP-3 (LPGGYGLPYT), have been recently studied in lung cancer patients using competitive assays as those described for collagen cryptic peptides. Assays present limits of detection between 0.2 and 3 ng/mL and extended detection range up to 17.5 -197 ng/mL. Their clinical utility as biomarkers needs further investigation [71].

Fibronectin (FN) is a large adhesive glycoprotein. It exists as an inactive (non adhesive) soluble dimeric molecule in plasma and other fluids, but as an insoluble active polymeric form in ECM. Its expression is dysregulated in several pathologies including cancer [9]. Urinary fragments have been found elevated in several cancers by sandwich ELISA [72]. Though fragments such as anastellin were identified as implicated in tumor processes, the target of most analytical methods is the full-length protein. According to its large size, commercial sandwich assays are widely available. Direct assays have the advantage of requiring only one Ab. These assays were designed using Ab-modified AuNPs [73] or Ab-modified SPR chips built on a SAM of cysteamine [74]. The former relied on the unique distance-dependent plasmon resonance properties of AuNPs that allows naked-eye but only qualitative detection because there is no correlation between the number of cells and the ECM amount containing the FN [73]. The SPR method with a detection range from 5 to 400 ng/mL

required 1000-5000-fold dilution of the human plasma because of the high concentration of soluble FN (µg/mL level) [74].

In addition to antibodies, an anti-FN aptamer was developed against a short peptide (RGDSPASSKP) containing the tripeptide RGD, critical for cell-adhesion. This aptamer (5'-CATGCGCCTTCCCCCTGTGGTTGGTGTCAGTCGGCCTGTG-3'), recognizes the peptide within the full protein [75]. It was used in a target recycling scheme coupled to a AuNPs colorimetric assay (Figure 5a). The assay consists of two hairpin sequences that only can hybridize each other through a short sequence (blue line in Figure 5a) when the target is present. Indeed, FN opens the aptamer-containing hairpin 1 leaving the complementary sequence as a "sticky end" (ssDNA) for hybridization. The double helix formation is thermodynamically favorable and implies the release of the target that can bind another hairpin 1, initiating a cycle only limited by the available amount of the hairpin 1. The hairpin 2 contains a short fragment that becomes a hanging ssDNA upon duplex formation (green line). This way this short ssDNA can only bind its complementary sequence, the reporter probe adsorbed on AuNPs, when there is FN in the sample. Addition of salt induces AuNPs aggregation and the corresponding red shift in the absorption spectra (blue color) when the reporter probe is released from the AuNPs due to hybridization. When the target is not present the negative charges of the wrapping reporter probe keep the AuNPs apart (red color). The design relies in a very delicate equilibrium between the length and the concentration of the reporter probe to observe significant analytical signal changes (ratio between absorbances at 650 and 520 nm). The assay is homogeneous but needs 75-fold dilution of the sample, which compromises the real applicability [76].

Laminin is a heterotrimeric glycoprotein that exists in 18 isoforms depending on the five heavy α chains, four light β chains and three light γ chains present. It has a characteristic cross shape with three short arms (Figure 1). Laminin-5, now known as laminin 332 indicating the type of each chain, contains a γ2 chain. The lack of simultaneous expression of α3 and β3 chains in a variety of invasive carcinomas from epithelial origin leaves the γ2 chain as a monomeric form. Since this monomer is not found in normal cells γ2 chain is considered as a specific tumor biomarker of invasiveness and metastatic cancers. The γ2 chain is proteolytically degraded by MMP into smaller soluble fragments that are released into the circulation. N-terminal 85 kDa and 50 kDa fragments were detected in human sera. Their low concentration required a

sandwich assay with electrochemiluminescence detection because with conventional peroxidase-based ELISA they were undetectable. The Abs were developed against the trimeric form but only those with specificity to γ2 chain were selected. One of the mAb was labeled with ruthenium (II) tris-bipyridine. The capture mAb was immobilized onto magnetic beads so that all labeled material can be collected on the working electrode [77]. This assay cannot distinguish between the complete laminin 332 and the monomeric specific biomarker. To solve this ambiguity, an antibody (2H2) specific of a region of the monomeric form hidden in the full protein was developed and used as a capture element of a chemiluminescent immunoassay on carboxylated magnetic beads. The K_d of this mAb estimated by SPR is not very low, 76.1 nM, but enough to obtain a LOD of 10 pg/mL, 20-fold lower than a conventional ELISA (using another capture mAb). The detection pAb was labeled with acridinium and the assay was performed in 30 min automatically in urine samples of patients with benign or tumoral bladder pathologies [78].

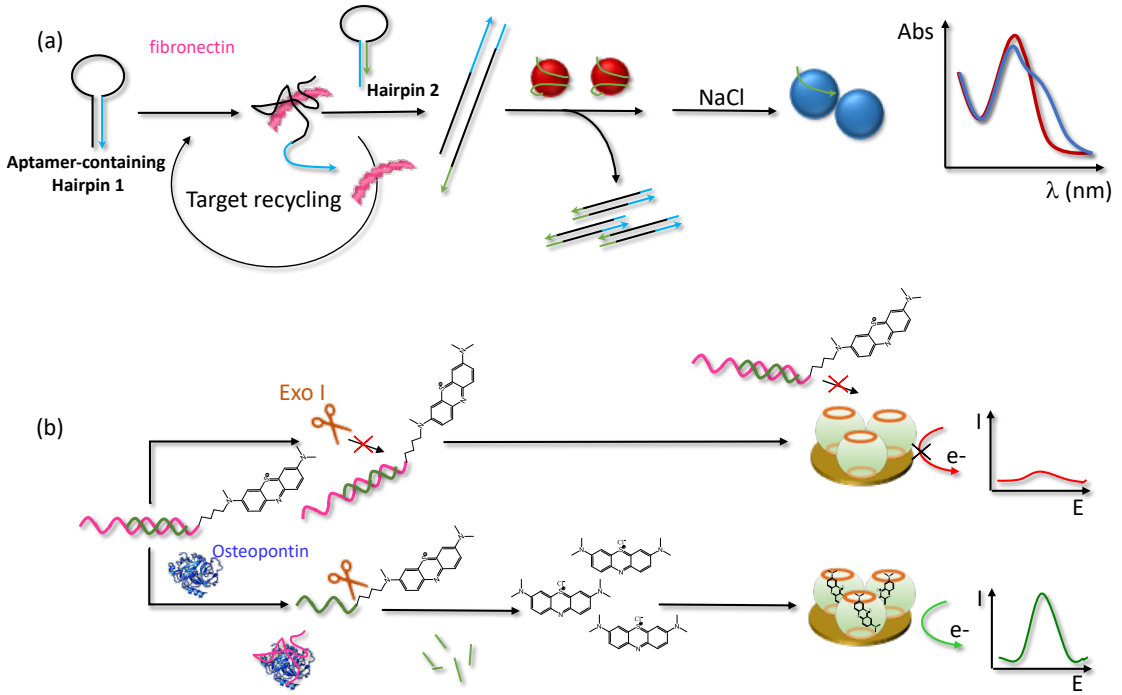

Figure 5 Aptamer-based assays for glycoprotein detection. a) Colorimetric assay where the recognition of FN initiates a catalytic cycle due to the opening of hairpin 1 that can then hybridize with hairpin 2 releasing the target. The reporter probe adsorbed on the AuNPs keep them separated until the duplex formed releases it from AuNPs by hybridization. As a result, aggregation is visible upon salt addition. b) Voltammetric assay where the aptamer is partially hybridized with a MB-labeled probe. Addition of exonuclease I breaks the labeled probe only when it is released upon OPN recognition.

The free-MB size is suitable for accumulation onto a host-modified electrode. Its electroactivity is measured by square wave voltammetry.

Direct label-free techniques such as SPR are also useful for the sensitive detection of laminin 5. A pAb was linked to the Au chip through a cysteamine SAM. The reflectivity at a fixed angle of incidence was measured before and after protein addition and its difference plotted as the analytical signal against the concentration of the target. The LOD, 4 pg/mL, is well below the range detected with a commercial ELISA (15.6-1000 ng/mL). Plasma samples from bladder cancer patients were 10^3-10^4 fold diluted to fit in the detection range (14 to 100 pg/mL) [79].

3.3.2. Matricellular proteins

Many matricellular proteins have been monitored in body fluids to follow disease progression including cancer. As examples of the potential of these molecules we will focus the discussion on the three original members; secreted protein, acidic and rich in cysteine (SPARC also known as BM40 or osteonectin), thrombospondin (TSP-1) and tenascin (TN), and two additional members; osteopontin (OPN) and periostin (POSTN).

3.3.2.1. Enzyme linked immunosorbent assays with optical detection

There are commercial ELISAs for all of them. However, they are not validated or certified and there are no international standards for calibration. Besides the result of OPN depends on the ELISA test used [80]. The evolution experienced in sandwich assays is valuable to mention. In 1993 two sandwich ELISA to detect TN were developed using Fab mAb on polysterene balls and another Fab mAb labeled with β-D-galactosidase. The fluorescent assay used 4-methylumbelliferyl β-D-galactoside as an enzyme substrate and obtained a 200-fold lower LOD than the UV absorption assay that used chlorophenolred-β-D-galactopyranoside as the substrate [81, 82]. The use of microtiter plates with peroxidase-labeled secondary antibody made easier the analysis of numerous samples in several fluids including urine, cerebrospinal, amniotic or pleural fluids [83]. The advent of magnetic particles (MP) facilitated the performance in automated instruments [84]. The immunological recognition of TN was carried out in solution between the sample and two antibodies labeled with fluorescein and alkaline phosphatase, respectively. Then, the complex was entrapped on MPs modified with anti-fluorescein Ab and the spectrophotometric detection was achieved after addition of

the substrate p-nitrophenylphosphate. The introduction of nanomaterials might also improve the performance. In an OPN assay, Ab-covered Au nanorods lowered the LOD though the detection range was identical and the slope very similar, which indicates that the improvement is related to the precision of the linear regression. This method was applied to saliva samples [80]. Another strategy to boost sensitivity is the use of polymeric HRP. The in-house sandwich assay developed for TSP was eight times cheaper than the commercial assays but presented a marked hook effect at relatively high target concentrations. This obliged to make appropriate dilutions which hampers the applicability for routine analysis [85].

A single biomarker has limited clinical utility so multiplexing is a clear trend in analytical chemistry to allow the detection of a panel of biomarkers. A commercial device has been designed to detect TSP and cathepsin D that automatically combines the concentrations of common prostate cancer biomarkers (fPSA and tPSA) with those of these novel biomarkers to predict the risk and help in the diagnosis of high-grade prostate cancer. This sandwich assay did not exhibit remarkable hook effect [86]. The wide availability of fluorophores makes fluorescent measurements very appealing for this purpose. Bio-Plex system from Bio-Rad uses Luminex carboxylated MPs containing different ratio of fluorophores that conferred unique fluorescent properties to each particle type. Each type is conjugated to a specific Ab. After incubation with the sample and a biotinylated secondary Ab the complex is labeled with streptavidin-phycoerytherin. As explained above, the particles are sorted by fluorescence measured in two channels; one to identify the target through the detection of the particle type and the second one for quantification by measuring the intensity of the phycoerytherin emission [87]. Point of care devices for cancer diagnosis are desirable though not very common. Very recently an integrated miniaturized system was described. It combines a microfluidic cartridge for sample pretreatment (conversion of whole blood into plasma or serum samples) with a photonic sensor based on interferometric measurements. The immobilization of the Ab is achieved through hydrofluoric acid treatment of Si_3N_4 and glutaraldehyde followed by laser-induced Ab deposition. Direct detection of POSTN was possible in spiked and breast cancer blood samples [88]. Measurements in certain body fluids such as interstitial fluid is challenging. An attempt to solve this issue is the recent work on microneedles patches containing antiPOSTN-Ab on their surface. They are applied *in vivo* for several minutes, removed and then quantified by fluorescence

imaging. The sandwich assay is revealed using an ultrabright fluorescent label consisting of a gold nanorod (AuNR) coated with biotin and 800CW fluorophore [89].

The current pandemics has demonstrated the importance of having rapid and reliable methods of analysis. Lateral flow devices (LFD) are those tools that can be also developed for cancer monitoring. As an example, a mixed antibody-aptamer LFD for OPN was recently described. The biotinylated DNA aptamer is incubated with the sample for 30 min. The detection is then achieved on the devices containing streptavidin-AuNPs on its pad and antiOPN pAb adsorbed in the test line, while a complementary sequence with which the aptamer was elongated is adsorbed in the control line. It was adequate for qualitative purposes with a visible double line appearing in 5 min above 10 ng/mL in spiked serum [90].

3.3.2.2. Electrochemical assays

Electrochemical approaches are also possible. In contrast to common direct impedimetric assays, a competitive sensor with potential for multiplexing was described for TN. The specific Ab was immobilized on a carboxylated SAM. Since the binding of TN did not significantly change the analytical signal, that is, the electron transfer resistance, they used an anti-IgG Ab labeled with peroxidase to bind the unoccupied anti-TN Ab. Further amplification through the formation of an insoluble product allows the TN determination. The array is regenerable with piranha solution and might be used up to 20 times without loss of performance [91]. Using label-free techniques such as FIS or field-effect transistors, OPN was also determined. In the former case, the immobilization of the mAb was oriented using a hexapeptide specific of the Fc with better affinity than protein A [92]. The FET assay used SWCNTs to immobilize the mAb achieving detectabilities several orders of magnitudes below the commercial ELISA and the OPN levels in serum, which allows high dilution of the sample minimizing interferences [93].

There are aptamers for all these five matricellular proteins except SPARC, though not all have been used to detect the biomarker in biological fluids of cancer patients. As in the case of antibodies, aptamers can also be employed in aptahistochemical assays on tissues. Anti-OPN RNA aptamer was originally obtained protected from endonuclease degradation by using 2F'-ribonucleotides. The aptamer with sequence 5'-CGGCCACAGAAUGAAAAACCUCAUCGAUGUUG-3' and K_d=18 nM was used unmodified in an impedimetric direct sensor [94]. The LOD, 240 ng/mL, was surpassed

by a homogeneous electrochemical assay using the same aptamer though 2 nt shorter in its 5' end and elongated with two extra nucleotides in its 3' end. The aptamer, hybridized with the complementary sequence labeled with MB, is displaced in the presence of OPN. The addition of exonuclease I breaks the labeled strand releasing the MB that is entrapped and preconcentrated on a host macromolecule, cucurbit[7]uril, attached to the electrode surface (Figure 5b). The electroactivity of MB is then proportional to the OPN concentration after measuring by square wave voltammetry [95].

DNA is less susceptible to degradation and then more suitable for diagnostic applications. With this aim an anti-OPN DNA aptamer was developed with a completely different sequence 5'-TGTGTGCGGCACTCCAGTCTGTTACGCCGC-3 as expected. The K_d was lower than the RNA counterpart (1.1 nM). A signal-off voltametric sensor with a still high LOD was developed [96]. The performance was greatly enhanced using FIS on a nanomaterial consisting of nanohybrid of Ti_3C_2Tx MXene and phosphomolybdic acid within polypyrrole. The detectability in the subfg/mL level allows 100-fold dilution of serum samples [97].

In comparison with collagen fragments, the concentration of ECM glycoproteins in plasma and serum is not so low. Some approaches exhibit a level of detectability that allow high dilution of the samples. This is a good news to limit a battle horse of most sensors, surface fouling, but makes the methods less suitable for point of care, simple devices.

3.4. Proteoglycans

Proteoglycans (PGs) are considered as a subclass of glycoproteins whose carbohydrate units consist of long linear chains of alternating residues of hexosamine (D-glucosamine or D-galactosamine) and either uronic acid (D-glucoronic or L-iduronic) or a neutral sugar (D-galactose), often sulfated, referred to as glycosaminoglycans or GAGs. These chains are covalently linked to the core protein by an oligosaccharide of a structure different from the repeating units. PGs are important components of the ECM contributing to the organization and physical properties thereof, although they are also found on the surface of the cells and within them [98]. Matrix proteoglycans are classified into two main groups according to their protein core

structure: (i) hyalectans that interact with hyaluronan and lectins (i.e. hyaluronan- and lectin-binding proteoglycans) and (ii) small leucine-rich proteoglycans or SLRPs containing a central region of leucine-rich repeats. Unlike cellular PGs, such as glypicans, which are sparking interest in the analytical chemistry community, just a handful of bioassays for the detection of matrix PGs have been reported so far.

Their anionic nature, large size and extensive glycosylation along with the inherent complexity of ECM complicate the analysis of proteoglycans. The determination of these potential cancer biomarkers is traditionally carried out by using ELISAs and immunohistochemical staining methods. However, they are multistep tedious methods that need to be conducted by well-trained personnel. Alternative proposals for matrix PGs determination reported so far are discussed below.

Unfavorable cancer prognosis is correlated with overexpression of chondroitin sulfate proteoglycans or CSPGs. Commercial colorimetric ELISA kits respond to proteoglycans in the pg/mL range. Trying to reduce the analysis time, reagent consumption, and promote portable devices, different strategies for the transduction of antibody-antigen binding event have been described. In this line, a platform with 28 gold microelectrodes to perform immunoassays for a melanoma-associated CSPG (MCSPG) has been designed [99]. It involved a detection antibody functionalized with Au-Ag alloy nanoboxes for enhanced SERS signaling. The application of an electric field to mix nanovolumes favors the specific entrapment of the proteoglycan and improves sensitivity, also in fortified small volumes of plasma samples [99]. On the other hand, electrochemical detection methods are particularly attractive since they are less-expensive, easier to miniaturized, and able to work with cloudy samples. Label-free approaches are of special interest because of their simplicity, and they often make use of nanomaterials to achieve the sensitivity demanded. An impedimetric direct immunosensor has been recently developed for CSPG-4 detection in cell line culture media and lysates. For that, indium tin oxide surfaces functionalized with a nanocomposite film of polyaniline and graphene has been used for covalent immobilization of the monoclonal antibody to the amine group on the aniline, mediated by the bifunctional reagent glutaraldehyde. The specific binding of the proteoglycan generates an insulating layer, thus increasing the electron transfer resistance. The assay is completed in only 2 hours and requires just 5 microliters of sample [100]. Likewise, the interesting properties of graphene have been applied to the development of a

graphene field-effect transistor functionalized with an antibody that recognizes CSPG-4, by using a heterobifunctional cross-linker that establishes π-π interactions with graphene and reacts with primary amines from the antibody. Changes in the electrical properties resulting from the specific recognition of the proteoglycan are reported. This strategy implemented in spiked undiluted serum leads to a detection limit of 0.01 fM, 5 orders of magnitude lower than that described for a conventional colorimetric assay [101].

Relying on the type and context of cancer analyzed, opposite roles (promoter or suppressor) on tumor progression have been reported for the small SLRP decorin. In the case of bladder cancer, the levels of decorin and two other proteins (interleukin-8 and vascular endotelial growth factor) in urine have been reported as a potential biomarker panel for non-invasive diagnosis of bladder cancer relapse. Their simultaneous quantification has been conducted by sandwich immunoassays developed onto chips in combination with colorimetric detection that could be recorded with a simple office scanner [102]. To improve the signal, different detection labels (nanoparticles and enzymes) have been comparatively evaluated. The best analytical performance was obtained with the traditional redox enzyme HRP and TMB as enzyme substrate, and with oxidized carbon nanoparticles. Both approaches reliably operate in synthetic urine, although the strategy involving oxidized carbon nanoparticles leads to a shorter analysis time (4 versus 5 hours) without affecting sensitivity.

Hyalectans (hyaluronan- and lectin binding proteoglycans) constitute another family of ECM proteoglycans, which includes aggrecan, versican, neurocan and brevican. They contain a N-terminal domain that binds to hyaluronan (HA), also known as hyaluronic acid, a non-sulfated GAG present in the ECM whose overexpression has been related to different cancer types. Although unable to trigger an immune response, its specific binding to numerous proteins and proteoglycans has promoted the development of different enzyme-linked sorbent assays (ELSAs) for its detection, which use proteins with a HA-binding domain as selective receptors. Both sandwich and competitive formats have been reported, the latter being more suitable due to their independence of HA size. In order to simplify the procedure and reduce the sample volume required by these heterogeneous tests, a competitive inhibition assay onto nanobeads combined with luminescent detection has been recently proposed [103]. Hyaluronan coated nanobeads including a photosensitizer (phthalocyanine) are mixed with the sample containing

natural HA as well as a second type of nanobeads modified with a HA-binding protein also incorporating a chromophore (europium ions). The formation of the affinity complex between both partners immobilized onto the beads comes phthalocyanine and europium into close proximity. The excitation of the phthalocyanine at 680 nm generates singlet oxygen molecules that give rise to a series of chemical events near to the chromophore, which produces chemiluminescent emission at 570 nm. The free unlabeled HA present in the sample competes with its immobilized counterpart, resulting in a decrease in the emitted light proportional to the amount of HA in the sample. This approach exhibits an improved sensitivity with a detection limit of 30 ng/mL. Nevertheless, a previous digestion of the biological fluid to remove interfering HA-binding proteins and lipids is mandatory. These issues might be circumvented by substitution of the immobilized protein partner by an artificial receptor as an aptamer whose selection, although challenging, has already been reported for other GAGs with submicromolar binding affinity [104].

4. Conclusions and future outlook

The need to identify new ways to diagnose cancer at an early stage has led to enormous efforts over the past few decades to discover cancer biomarkers for use in clinical practice. Among these, circulating proteins or protein modifications related to the aberrant ECM remodeling associated with tumorigenesis open exciting possibilities to develop clinical tests that provide useful medical information. As the scientific community gains a deeper understanding of the various processes that associate dysregulated ECM remodeling and cancer, analytical chemists must develop new technologies for the detection of these potential biomarkers fulfilling the analytical requirements for a robust clinical biomarker assay [105], thus accelerating the rate of translation to the clinical acceptance.

This article presents some of these molecular biomarker candidates, providing a description of the currently available methods and technologies for their detection. The discovery of new biomarkers, usually through proteomic technologies, should be followed by the development of highly sensitive detection assays (these molecules could be found in plasma or serum samples at concentrations in the range of pg/mL to ng/mL) with high accuracy and precision, which allow its use in diagnostic accuracy studies [106]. In addition, for their eventual transfer to the routine clinical practice, the assays should be robust, cost-effective, and high throughput capable. In most cases, the

choice is to develop an ELISA, which requires to obtain either monoclonal or polyclonal antibodies with suitable specificity. Competitive ELISA is used for small peptides, while sandwich ELISA is the most convenient alternative for whole proteins, even though this means having two antibodies that recognize different epitopes. However, immunoassays have some limitations, mainly related with cross-reactivity of the reagent antibodies, with the subsequent interference from other isoforms of the marker or even more distantly related molecules. Sandwich ELISA is also adversely affected by the so-called "hook effect" i.e. at high analyte concentrations, a decrease in signal with concentration may be observed. This effect is of particular concern in the case of cancer biomarkers for which a variation in concentration of several orders of magnitude is expected, depending on the pathological state. Furthermore, not all aberrant forms of proteins of clinical interest are easily recognized by antibodies. The large investment required to obtain suitable antibodies means that antibodies are not available for many of the discovered biomarkers. In consequence, the lack of selective reagents is limiting the verification of these novel biomarker candidates. As alternative, other affinity reagents such as aptamers could be developed. These *in vitro* generated affinity reagents can be tailored to specific moieties in the biomarkers that are altered during tumor progression, thus improving their clinical specificity [107,108]. Other benefits of aptamers compared with antibodies are their low production costs, their low batch-to-batch variability and their easy of modification for immobilization and detection. Additionally, aptamers can be easily combined with powerful signal amplification strategies [109]. New aptamer projects need to be developed, placing especial emphasis on targets that are not currently accessible to antibodies, to overcome the challenges associated to the starting steps in the process of translating ECM-related biomarkers from discovery into cancer diagnostics.

Another important gap in the development of analytical assays useful for the clinical verification studies of the potential biomarkers herein presented is the lack of calibration standards. The use of recombinant proteins is the most common option and its concentration is stablished by weight. Different tools of genetic engineering can be used to obtain proteins of human origin in various hosts such as bacteria, fungi, insect or mammalians. In many cases, the extensive processing and post-translational modifications that human proteins undergo in the body are not considered, and consequently the production of correctly modified, folded and functional proteins is not

guaranteed. This problem is combined with the high production costs of these proteins, which negatively affects the final cost of the assay. In any case, for the test results to be robust and comparable in different laboratories, it would be necessary to obtain certified standards made in matrices similar to the study samples.

The new analytical assays should demonstrate their reliability for the intended application, with rigorous assessment of their analytical performance. It is very important to assess the reproducibility and precision at clinically relevant concentration levels, over a reasonable period of time, and using different reagent lots. Other parameters that should be evaluated are linearity, working range, LOD and selectivity of the assay. The definition of LOD as the smallest concentration of the analyte that can be reliably distinguished from zero [110] is rather ambiguous and when reporting LOD values it is necessary to specify the approach that was used for its estimation. Most of the LOD estimation approaches are only useful for linear relationships between signal and concentration, and in consequence they cannot be used when the linear relationship is stablished between signal and the logarithm of concentration. In these cases, a narrow concentration range where linearity occurs or a visual evaluation of LOD are recommended. In addition to the LOD, the lowest reportable concentration should also be documented by independent analysis of a suitable number of samples with a concentration near the LOD. Finally, the validation in samples that closely represent the population in which the clinical test would be developed is highly recommended.

Only with appropriate and well-validated analytical assays, which are robust, reliable and relatively simple to operate, will be possible to initiate the necessary steps to take the ECM-related biomarkers from the research into the clinical laboratory. This will also require the collaboration with clinicians, who provide the clinical specimens more suitable for the validation of the assays, and clinical laboratories for evaluating the new tools in the real-life practice. From a clinical perspective, these new candidates will most likely be included in diagnostic and prognostic panels to provide information to add or replace what is already available from existing assays, with the final objective of demonstrably improving patient outcomes.

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Compliance with ethical standards

Conflict of interest: The authors declare no conflicts of interest

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