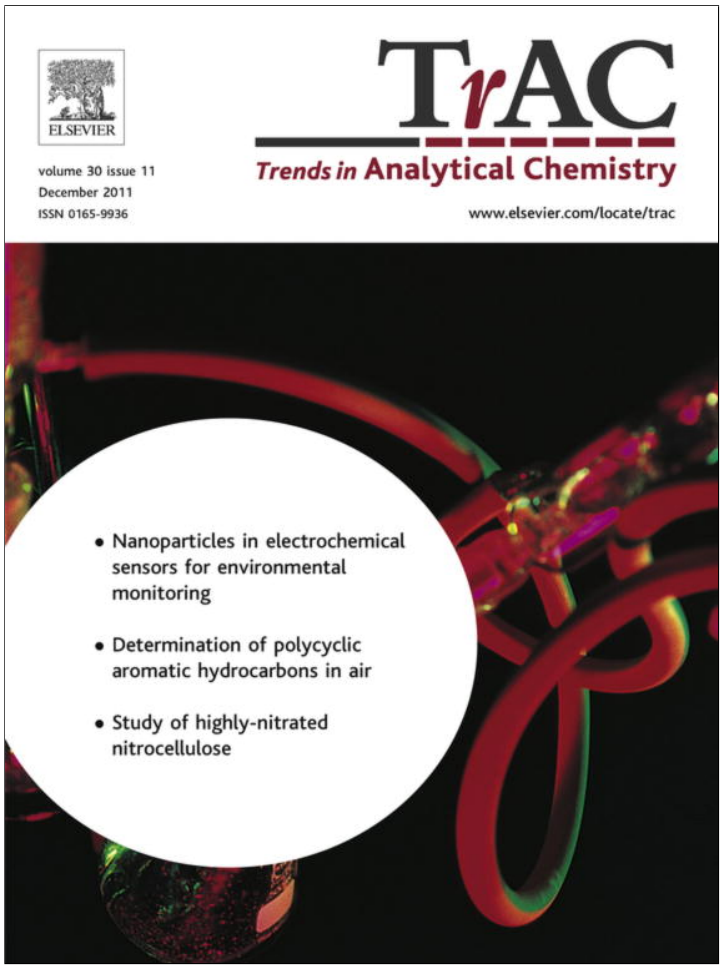

Assays for determination of matrix metalloproteinases and their activity

Sona Krizkova, Ondrej Zitka, Michal Masarik, Vojtech Adam, Marie Stiborova, Tomas Eckschlager, Grace J. Chavis, Rene Kizek

Matrix metalloproteinases (MMPs) are involved in many physiological and pathological processes. Due to their ability to cleave and to remodel components of surrounding tissues, MMPs may affect cell migration, differentiation, growth, inflammatory processes, neovascularization, wound healing, apoptosis, the uterine cycle and many other actions within the body, including those needed for tumorigenesis and other diseases.

MMPs can therefore be used as potential markers for detecting various cancers, neurodegenerative, and immune and cardiovascular diseases. Numerous MMP assays were developed for clinical and research purposes, but far more attention has been devoted to understanding their biological functions.

Due to differences in methodology, results obtained in various laboratory settings are difficult to compare because of the lack of standards and analytical methods of validation. Limits of detection of particular methods used for identifying MMPs are also disputable.

Enzymatic, immunochemical and fluorimetric methods are particularly suitable for clinical use. *In-vivo* imaging methods offer many potential advantages in cancer research and diagnostics. Other methods are subject to investigation [e.g., phage display, multiple-enzyme/multiple-reagent assay system (MEMRAS) and activity-based profiling].

Keywords: Activity-based profiling; Cancer; Disease marker; Enzymatic method; Fluorimetric method; Immunochemical method; *In-vivo* imaging; Matrix metalloproteinase (MMP); Multiple-enzyme/multiple-reagent assay system (MEMRAS); Phage display

Abbreviations: ABPP, Activity-based proteomic probes; Adp, D,L-2-amino-3-(6,7-dimethoxy-4-coumaryl)propionic acid; Amp, D,L-2-amino-3-(7-methoxy-4-coumaryl)propionic acid; APMA, p-aminophenylmercuric acetate; CPSA, Chronopotentiometric stripping analysis; Dabcyl, 4-(4-methylaminophenylazo)benzoic acid; Dnp, Dinitrophenol; ECM, Extracellular matrix; EDANS, Tryptophan, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid; ELISA, Enzyme-linked immunosorbent assay; FITC, Fluorescein isothiocyanate; fTHP, Fluorescently-labeled triple-helical peptide; ICAT, Isotope-coded affinity tag; iTRAQ, Isobaric tag for relative and absolute quantification; MCA, 7-methoxycoumarin; MEMRAS, Multiple-enzyme/multiple-reagent assay system; MMP, Matrix metalloproteinase; MRI, Magnetic resonance imaging; MT-1-MMP, Membrane type 1 matrix metalloproteinase; MS, Mass spectrometry; NMA, N-methylanthranyl acid; OIM, optical imaging; PET, Positron-emission tomography; pNA, p-nitroaniline; proUKCOL, pro-urokinase; SDS, Sodium-dodecylsulfate; SDS-PAGE, Sodium-dodecylsulfate polyacrylamide gel electrophoresis; SPECT, Single-photon emission computed tomography; TAPI-2, TNF-alpha protease inhibitor 2; TIMP, Tissue inhibitors of matrix metalloproteinases; TNBSA, 2,4,6-trinitrobenzensulfonic acid

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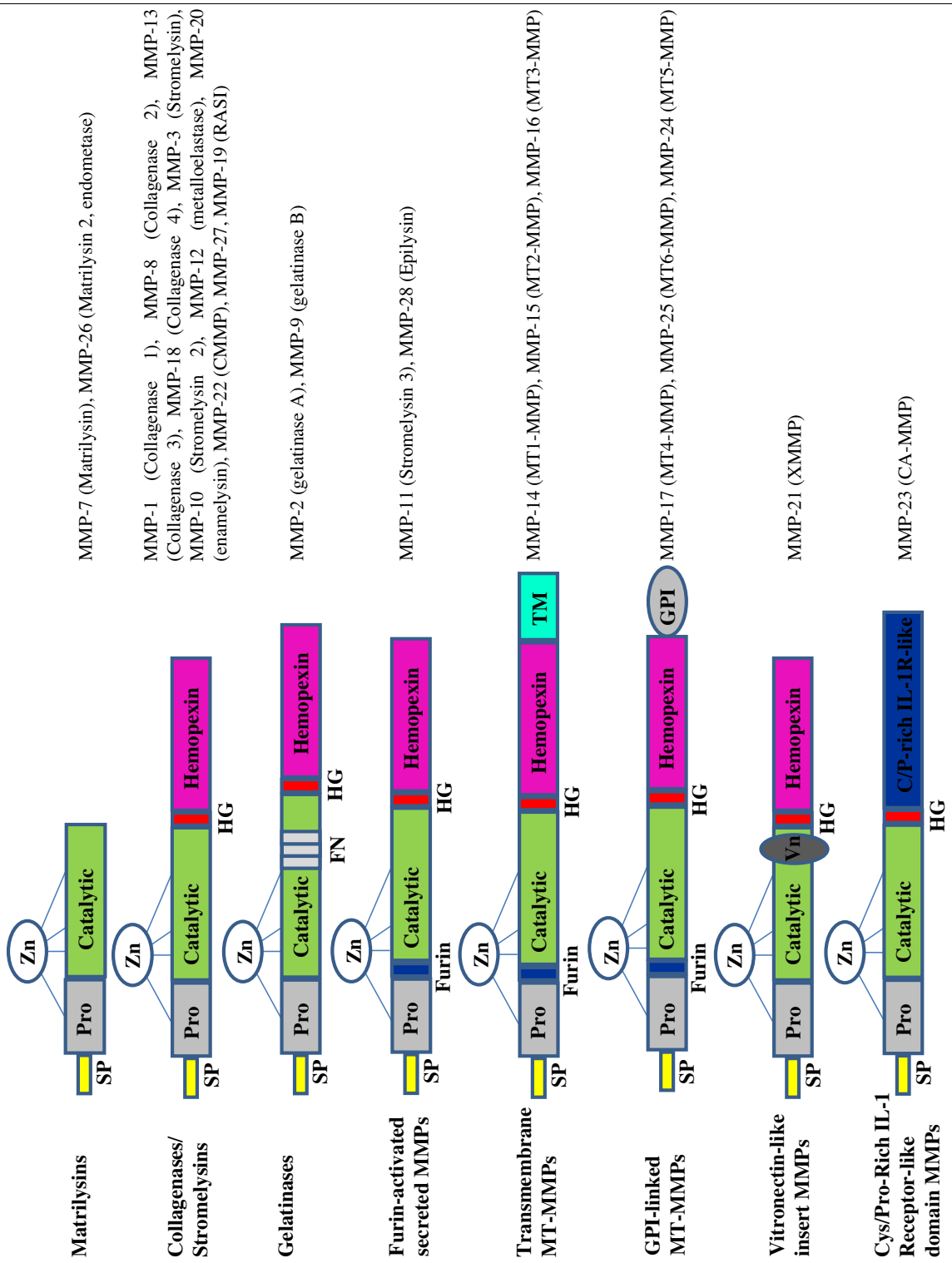

Figure 1. Classification and domain structure of matrix metalloproteinases (MMPs). Most MMPs contain a signal peptide (necessary for secretion), a propeptide [a catalytic domain that binds zinc (Zn^{2+})], a hinge region (HG), and a hemopexin carboxy (C)-terminal domain. In the catalytic domain, MMP has a Zn^{2+} binding site and a binding site for the specific substrate. Matrilysins lack a hemopexin domain. Gelatinases contain fibronectin type II modules (FN) that improve the efficiency of collagen and gelatine degradation. Furin-activated secreted MMPs (MMP-11 and MMP-28) have a recognition motif for furin-like serine proteinases within their catalytic domain for intracellular activation. This motif is also found in the vitronectin (Vn)-like insert MMPs (MMP-21) and the MT-MMPs. MT-MMPs have an additional transmembrane-binding domain (TM). Most MMPs are secreted, but six membrane-type MMPs (MT-MMPs) have been identified and they are anchored by a transmembrane domain or a glycosyl-phosphatidylinositol (GPI) linker.

1. Introduction

Matrix metalloproteinases (MMPs) were discovered in 1962 by Gross and Lapiere, who studied the degradation of triple-helical collagen during a tadpole-tail metamorphosis [1]. More than 20 classes of MMPs have been identified in humans, and they are classified according to the pre-synthetic region on chromosomes and substrate specificities. They are labeled MMP-1 to MMP-28 [2], which are classified into sub-groups according to functionality (i.e. collagenases, stromelysins, matrilysins, gelatinases, membrane-associated MMPs and MMPs with no group designation).

All MMPs require zinc and calcium ions to support their enzymatic activity. The enzyme itself is divided into several domains, which differ in dependence on MMP sub-type. However, most MMPs are composed from N-terminal propeptide, catalytic domain, hinge region and C-terminal hemopexin domain [3]. Matrilysins lack C-terminal hemopexin domain and HG region, membrane-type MMPs (MT-MMPs) have a C-terminal transmembrane domain and GPI-linked MT-MMPs contain a C-terminal GPI domain. In the gelatinases, fibronectin (FN)-like type II repeats are also present in the catalytic domain. The differences in MMPs structures are summarized in Fig. 1.

MMPs are often subject to study due to their roles in numerous physiological and pathological processes [4,5]. The best-known physiological role of these proteins is cleaving and rebuilding of connective tissues (e.g., collagen and elastin, components of extracellular matrix providing structural support to the animal cells and also performing various other important functions).

A number of matrix and non-matrix proteins are potential substrates for MMPs [6]. MMPs are able to cleave and remodel components of surrounding tissues, and this is important for cell migration, differentiation, growth, inflammatory processes, neovascularization, wound healing, apoptosis, the uterine cycle, embryonic development and ovulation [7]. MMPs produced by endothelial cells also play a complex role in angiogenesis [8].

Furthermore, MMPs are involved in plenty of pathological processes (e.g., arthritis, Alzheimer disease, atherosclerosis, vascular disease, gastritis ulcer disease, central nervous system diseases, cirrhosis, and pro-angiogenic activities in malignant tumors).

This review summarizes analytical, bio-analytical and molecular-biological methods used for studying MMPs from various points of view, including clinical applications.

2. Analysis

MMPs are used as markers for some malignant tumors, including colorectal, thyroid, bladder and breast cancer, and other disorders (e.g., neurodegenerative, immune and cardiovascular diseases) [9]. Assays for determination of MMPs for clinical and research purposes are summarized in several reviews, in which different methods and applications are discussed [10–14]. Literature surveys show great interest in studying the biological functions of MMPs, but methodological differences make it difficult to compare results from various studies and to draw well-founded conclusions from their results. Lack of methods for analytical validation is one of the challenges faced in MMP detection and determination in clinical research. There are many unresolved methodological issues in the clinical detection and determination of MMPs, the most important being

(1) whether samples should be taken from whole blood plasma or serum;
(2) whether total content of MMPs or selected MMP should be determined; and,
(3) whether to measure the levels of single or several MMPs or their proenzymes [15].

Since the last review on MMP assays by Cheng et al. in 2008 [11], the assays used have been updated and new methods and analytical approaches developed, especially in imaging, mass-spectrometric (MS) techniques and biosensors. Enzymatic, immunochemical and fluorimetric methods are commonly used techniques in clinical research. *In-vivo* imaging methods are of particular interest in cancer research and diagnostics [16]. Immunochemical methods precede enzymatic methods, but they are unable to distinguish between active and inactive MMPs in zymogen form [10]. Fluorimetric methods using fluorescently-labeled substrates show low limits of detection (LODs), but they allow MMP activity to be determined quantitatively and target MMP sequences to be studied [11]. A number of other methods are subjects of interest [e.g., phage display, Multiple-Enzyme/Multiple-Reagent Assay System (MEMRAS) and activity-based profiling] (Table 1). Methods used in MMP detection and determination are summarized and discussed in the following sections.

3. Fluorimetric methods

Fluorimetric methods use fluorescently-labeled substrates for detection and/or determination of various MMPs. Implementation of microplate-based screening (excluding so-called real-time zymography) enables analysis of a large number of samples. The availability of various fluorescent probes including near-infrared (NIR) fluorescent probes [17–19] makes it possible simultaneously to detect and to quantify several different types of MMPs.

The use of fluorescently-labeled substrates is beneficial for monitoring MMP activities in real time without stopping the reaction. Fluorescein isothiocyanate (FITC) is a fluorescent label commonly used in detecting enzy-

Table 1. Summary of methods used for detection and determination of matrix metalloproteinases (MMPs)

Method		Analyte	Limit of detection (ng)	Ref.
Enzymatic methods (Bioassays)				
Labeled substrate	Biotinylated gelatine	MMP-2,9	0.016 MMP-2	[120]
	Succinylated gelatin	MMP-2,9	21–12.5	[30]
Zymography	Gelatine	MMP-2,9	0.12	[121]
	Casein	MMP-3,10,7	0.013	[56]
	Collagen	MMP-1,13	1.6	[56]
	In situ	MMP-2,7,9	–	[122]
	Reverse	TIMP 1,2,3	0.49	[123]
	Real-time	MMP-2,9,3	0.02	[123]
		TIMP 1,2,3	0.49	[56]
		MMP-1,13	0.013	[56]
	In vivo	all MMPs	–	[122,124]
Immunochemical methods				
Immunocapture assay		MMP-2,9	0.1	[63]
Western blotting		all MMPs	units of ng	[125]
ELISA		MMP-1,2,3,7,8,20 TIMP-2	0.01–10	[66,67,126,127]
uTIINE assay			–	[11,73]
Fluorimetric methods				
FITC		MMP-2,9, TIMPs	0.064	[56]
fTHP		MMP-1,2,3	–	[26,128]
MDPF		MMP-2,9, TIMPs	0.002–0.02	[123]
Other methods				
Radioisotopic methods			10	[129]
Phage display			–	[75]
MEMRAS			–	[81]
ABPP			0.01	[82,83,130]
Electrochemical methods			0.046	[85]

Abbreviations: MMP, Matrix metalloproteinase; TIMP, Tissue inhibitor of MMP; FITC, Fluorescein isothiocyanate, fTHP, Fluorogenic triple-helical peptide, MDPF, 2-methoxy-2,4-diphenyl-3(2H)-furanone, MEMRA, Multiple-enzyme/multiple-reagent system, ABPP, Activity-based proteomic probes

matic activity of MMPs. In un-degraded substrate, the fluorescence is quenched due to the proximity of the labels. After substrate degradation, the fluorescent molecules are released, causing the fluorescence to increase at 521 nm. The increase in fluorescence is proportional to the degree of substrate degradation and thus to MMP activity [20]. This method requires a thermosensitive ''over-labeled'' substrate [21]. Excessive FITC binding to protein structure can cause its aggregation, loss of biological function, decreased solubility and changes in conformation [22,23]. The resulting relaxed structure of collagen may affect the results due to the substrate preferences of collagenases [24].

Fluorescent peptide-based substrates are popular in activity-based protein-profiling assays. The peptidic bonds of substrate are labeled by one pair of chromophores, a reporter and a quencher. Degradation of the peptidic bonds of the substrate causes their separation, which leads to the increase of fluorescence. Fluorophore-quencher methods are suitable for studying the specificity and the inhibitors of MMPs [25]. Fluorophore-quencher pairs commonly used in MMP chromogenic substrates comprise 5-((2-aminoethyl)amino)-naphthalene-1-sulfonic acid (EDANS), 4-(4-methylaminophenylazo)benzoic acid (Dabcyl), 7-methoxycoumarin (MCA), D,L-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (Amp), D,L-2-amino-3-(6,7-dimethoxy-4-coumaryl)propionic acid (Adp) or N-methylanthranylic acid (NMA)2,4-dinitrophenol (Dnp). Moreover, fluorescently-labeled triple-helical peptides (fTHPs), which mimic the behavior of native collagen, can be used to study triple-helicase activity of the collagenases [26].

An example of using charge-changing fluorescent peptide substrates is the whole-blood assay for elastase, chymotrypsin, MMP-2, and MMP-9 activity. Degradation of these substrates leads to formation of positively-charged fluorescent product fragments upon cleavage by the target proteases. Using a simple electrophoretic format, the fragments are rapidly separated, concentrated, and detected directly from a whole-blood sample with LODs of 100–200 ng/mL [27]. Fluorescently-labeled substrates are widely used for imaging MMPs.

4. Enzymatic methods

4.1. Use of modified substrates

Gelatine is a substrate for certain MMPs (e.g., MMP-2 or MMP-9) [28]. Following gelatine, modifications are the most frequently used in MMP assays.

Biotinylated gelatine is degraded by MMPs. The fragments are captured to the microplate surface and visualized with horseradish peroxidase (HRP) conjugated with avidin. HRP activity is then detected using a chromogenic substrate. The absorbance of the colored end product is proportional to the amount of remaining gelatine, which is inversely proportional to gelatinase activity. The LOD of this method is 0.016 ng of MMP-2 per mL [11,29].

Another assay is based on the use of succinylated gelatine as substrate and measurement of primary amines exposed by hydrolysis of the substrate by gelatinases. Gelatine is treated with succinic anhydride to block all free amino groups on the protein. Hydrolysis of peptide bonds in the succinylated gelatine by proteolytic attack exposes primary amines. The exposed primary amines are detected by reaction with 2,4,6-trinitrobenzene sulfonic acid (TNBSA). The colored product of this reaction is proportional to gelatinase activity. The estimated method LOD is 20 ng of MMP-2 and 12.5 ng of MMP-9 per mL [11,30].

These methods are suitable for crude and purified samples of tumor tissue and inhibition studies (Table 1), but they are unable to distinguish and to separate isoenzymes with gelatinase activity. Array-based biosensors employing modified gelatine are usable for this purpose. A simple, label-free, and high-throughput array-based spectral surface plasmon resonance (SPR) biosensor for a high-throughput analysis of MMP-3 activity was developed. Gelatine arrays were fabricated by immobilizing gelatine, a MMP-3 substrate, on amine-modified gold arrays. MMP-3 activity was determined by monitoring the shift of SPR wavelength caused by gelatine proteolysis. The array-based SPR biosensor was successfully applied to the rapid analysis of dose-dependent MMP-3 activity and its inhibition with tissue inhibitors of metalloproteinase 1 and GM6001 [31]. A chip-based approach to measure MMP-3 activity using Cy3-conjugated gelatine arrays was proposed by Hong et al. [32].

4.2. Zymography

Zymography is a simple quantitative method, which can be used for direct determination and studying of spatial distribution of MMPs and their inhibitors [33]. Zymography involves the electrophoretic separation of proteins under denaturing (SDS, sodium-dodecylsulfate), but non-reducing, conditions through a polyacrylamide gel containing substrates (e.g., gelatine, casein or collagen). The cleavage of the substrate occurs after renaturation of the resolved proteins by the exchange of the SDS for a non-ionic detergent (e.g., Triton X-100) [34–36]. Proteolytic activities of MMPs are then detected as clear bands after staining with Coomassie Blue [37]. The duration of the proteolytic reaction of an unknown sample must be optimized with respect to sample type. Absence of a universal standard contributes to the lack of inter-laboratory reproducibility and transferability of results [38]. The best established standard is human capillary blood due to its availability [39]. Despite the problems with standardization, this method is widely used in tissue-sample analysis [38,40].

Gelatine zymography was used to identify the activities of MMP-2 and MMP-9 in blood and plasma of patients poisoned by lead(II) ions [41]. This technique was also used to determine MMP-2 and MMP-9 activities during tissue degrading and remodeling. Tissue degrading and remodeling were mimicked by treating rabbit corneas with 1 M sodium hydroxide [42]. Overexpressed MMP-2 and MMP-9, during the development of glioma in rat nervous system, was detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymography [43]. MMP-8, which is found in human saliva, was studied as a potential marker for diagnosis and monitoring of periodontitis [44]. SDS-PAGE zymography was also utilized for determining gelatinase B (MMP-9) activity [45].

Casein [46] and collagen [47] zymography can also be used for MMP characterization. This allows further study of MMPs and their target substrates with superior LODs. The use of casein degradation as an assay for enzyme activity, specifically stromelysins 1 and 2 (MMP-3 and MMP-10) and matrilysin (MMP-7), exhibited significantly enhanced sensitivity compared with gelatine. Casein zymography has an LOD at least two orders of magnitude less than gelatine zymography. With casein zymography, greater sensitivity is achieved with a smaller amount of substrate. However, casein migration increases residual background noise, reducing resolution of certain isoenzymes, specifically latent and activated forms of matrilysins with molecular weights of 20 kDa and 29 kDa. The background may be adjusted by prerunning the gel. This method responds well to 0.1 pg of pro-collagenase activated by treatment with p-aminophenylmercuric acetate (APMA), while gelatinases or stromelysins have significantly lower LODs (5 ng) [11].

Other variations in zymographic analysis involve studying the presence and the localization of MMPs and their inhibitors' activity in cells, tissues and the whole organism.

In-situ zymography was developed for localization of MMP activity in tissue slices or cells. A photographic emulsion containing gelatine, or another fluorescence-labeled substrate, is brought into contact with a tissue section or cell preparation. The degradation of substrates by activated MMPs is detected using light or fluorescence microscopy. MMP activity appears as white spots against a black background, which consists of undegraded substrate. Degradation of the fluorescent substrate causes loss of fluorescence and MMPs are observed as black spots against a fluorescent background. This method is successful in detecting and localizing MMPs in various tissues [48,49].

In-vivo zymography studies MMP activity at the level of the whole organism. This method is based on injection and *in-situ* degradation of fluorescently-labeled collagen [50]. Native substrates emit weak fluorescence due to quenching caused by intramolecular proximity of fluorescent labels. The increase in fluorescence occurs only after release of the labels from the substrate as a result of degradation. MMP activity was determined in embryos of zebrafish (*Danio rerio*) using this method [51,52]. Moreover, *in-vivo* zymography was utilized to study the effects of Prinomastat on MMP activity [53].

Reverse zymography is used to analyze the activities of tissue inhibitors of metalloproteinases (TIMPs) in complex biological samples. TIMPs are major endogenous tissue regulators of MMPs with molecular weights of 21–30 kDa. Four homologous TIMP proteins have been identified using reverse zymography (i.e. TIMP-1, TIMP-2, TIMP-3 and TIMP-4) [54]. This method involves electrophoresis on SDS-PAGE gel containing MMP and its substrate (e.g., gelatine). During electrophoresis, MMP is inactive because the electrophoresis buffer inactivates it. After electrophoresis is finished, the gel is incubated in MMP-activating buffer. During incubation, protein is degraded and only bands where TIMPs are present are intact. Molecular-weight and semi-quantitative analysis are performed on the undegraded TIMPs.

Incorporation of purified recombinant human gelatinase A or B generated a standardized method and improved the overall sensitivity for detection of TIMP-1, TIMP-2 and TIMP-3 [55]. It is often difficult to estimate the duration of proteolytic reaction in an unknown concentration of TIMPs in a sample, so it is necessary to determine an optimal incubation time and to perform multiple repetitions of the analysis. Experimental details were well described by Oliver et al. [55].

Real-time zymography and reverse real-time zymography are based on electrophoretic separations and incubation with fluorescently-labeled substrates, specifically FITC-labeled collagen [56]. Compared to other methods, real-time zymography does not require enzymatic reactions to be stopped to obtain results, thus making possible multiple evaluations of similar assays. Using various labeled substrates (e.g., FITC-labeled gelatine and casein labeled with Texas red dye) allows simultaneous detection of MMP-2, MMP-3 and MMP-9 [57]. Total time of analysis does not exceed 5 h.

5. Immunochemical methods

5.1. Blotting techniques

Western blotting is used to detect proteins by an antibody after electrophoretic separation and blotting onto a membrane. This method is used for MMP analysis in clinical [58] and research [59] studies. However, this method is rather time consuming, as it requires the availability of antibodies and the inhibition of proteolytic activity of analytes during protein blocking and immunochemical reactions.

Checkerboard immunoblotting (CBIB) was developed for the high-throughput quantification of multiple inflammatory mediators in gingival crevicular fluid (GCF) samples. Monoclonal antibodies against GCF interleukin (IL)-1 beta and MMP-8 are immobilized to the surface of membranes. Biotinylated antibodies are used to detect bound antigens in a checkerboard format. Signals are developed using chemiluminescence and quantified using software for array analysis [60].

5.2. Immunocapture assay and enzyme-linked immunosorbent assay

Immunocapture assay is based on the use of specific antibodies against MMPs and the ability of 4-aminophenylmercuric acetate to activate proteolytic enzymes. This specific technique is suitable for screening of large numbers of compounds or samples. MMPs are first immobilized on the surface of microplates by using specific antibodies. After activating immobilized MMPs with 4-aminophenylmercuric acetate, modified pro-urokinase (proUKCOL) with an inserted artificial activation amino-acid sequence recognized by MMPs (ArgProLeuGly+IleIleGlyGly, where + indicates the cleavage site) is activated by proteolysis. The activity of urokinase is determined using a chromogenic substrate (e.g., pyro-Glu-Gly-Arg-p-nitroaniline. The rate of p-nitroaniline (pNA) release is measured photometrically at 405 nm [61]. This method was developed to detect MMP-2 [62] and MMP-9 [63], with an LOD of 15 pM [11].

Enzyme-linked immunosorbent assay (ELISA) is one of the most widely-used molecular biological methods. Many protocols have been optimized to detect MMPs, pro-MMPs, TIMPs, and MMP-TIMP complexes. The protocols differ in types of antibody used, arrangement and time management. Monoclonal antibodies for most of the above-mentioned proteins (complexes) were prepared recently. To detect immobilized MMPs, labeled peptide substrate (e.g., by methoxycoumarin) is used [64].

Table 1 summarizes various methods. ELISA was used to study the mechanism of MMP-9 action during skin inflammation [65], MMP expression in ischemic heart disease [66], degradation of extracellular bone matrix in osteoporosis by osteoprotegerin and MMP-2 [67] and to determine levels of TIMP1 and MMP-9 in exhaled breath condensate in children with bronchietactis [68].

5.3. Immunocytochemistry and immunohistochemistry

Immunocytochemistry and immunohistochemistry are used to localize a protein of interest in cells and tissues, using specific antibodies along with a fluorescent or an enzyme label. Studies confirmed that the overexpression of certain MMPs increased the invasivity of carcinoma

cells [69]. MMPs are detected in immersion-fixed paraffin-embedded tissue sections using polyclonal antibodies. Tissues are further stained using specific staining kits and counterstained with hematoxylin.

Immunohistochemistry on paraffin and cryostat sections at the light-microscopy level clearly demonstrated the presence of MMPs and TIMPs along the invasive pathway extending from placental cell columns to maternal tissues. Light microscopy does not allow more detailed studies due to the methodological problems:

(1) fixation-dependent denaturation of antigens; or,
(2) loss of enzyme because of diffusion.

Immunocytochemistry at the electron-microscopy level shows a noticeable co-distribution of several matrix substrates with their corresponding cleaving enzymes. This correlation seems to be more specific than the specificities of MMPs found in *in-vitro* systems (e.g., MMP-3 *in vitro* degrades nearly all extracellular matrix molecules, whereas, *in vivo*, it is specifically associated with the fibronectin fibrils. Possibly the spectrum of substrates degraded *in vivo* is very much smaller, due to binding of limited enzyme quantities to their preferential substrates, than found under *in-vitro* conditions [70]. Combined use of antibodies with sophisticated imaging techniques and nanoparticles (NPs) and microparticles opens new possibilities in immunocytochemical and immunohistochemical MMP analysis (for more information, see Section 7). For example, localization and distribution of MMP-2 and -9 in human dentin organic matrix were studied by employing correlative field-emission in-lens-scanning electron microscopy (FEISEM) and transmission-electron microscopy (TEM). Dentin specimens were submitted to pre-embedding or post-embedding immuno-labeling techniques using primary monoclonal anti-MMP-2 and anti-MMP-9 antibodies and exposed to a secondary antibody conjugated with gold NPs (GNPs) [71].

A novel electrochemical immunosensor for the detection of MMP-3 was constructed based on vertically aligned single-wall carbon nanotube (SWCNT) arrays. Detection is based on a sandwich immunoassay comprising horseradish peroxidase (14–16 labels) conjugated to a secondary antibody and/or a polymer bead loaded with multi-enzyme labels. Results provided an LOD of 0.4 ng/mL (7.7 pM) for the 14–16 label sensor protocol and 4 pg/mL (77 fM) using a multiply enzyme-labeled polymeric bead-amplification strategy in 10 μL of calf serum [72].

5.4. Detection of neo-epitopes in collagen Type II (uTIINE assay)

Type II collagen degradation by MMPs is associated with development and progression of osteoarthritis. Neoepitopes in type II collagen are detected using antibodies recognizing epitopes containing free carboxyl or amino ends [11]. Collagenase activity is proportional to the number of linked antibodies. Elevated levels of collagen fragments (uTIINE assay) in urine are linked to osteoarthritis progression. In addition, quantitative determination of collagen neoepitopes can be used as a detection tool for cartilage degeneration. [73].

6. Other methods

In radioisotopic analysis, MMP activity is determined by monitoring radioisotope-labeled degradation of MMP substrates (^{14}C, ^{3}H, ^{125}I). This method has been used to study MMP-2 and MMP-9, but its use has been ended due to the availability of more sophisticated instrumentation and methods of analysis [28].

Phage display, a method for the study of protein-protein, protein-peptide, and protein-DNA interactions, uses bacteriophages [74]. M13 bacteriophages and filamentous phages are most frequently used [75,76]. During the phage-display DNA encoding, the protein or the peptide of interest is inserted into the pIII or pVIII gene encoding a phage-surface protein. The phage gene and inserted DNA hybrid are then transfected into *E. coli* bacterial cells.

Two different ways of phage display have been introduced to search and to decode large peptide libraries quickly:

(1) the monovalent system was used to study substrate specificity of membrane type 1 MMP (MT-1-MMP) [77,78]; and,
(2) the polyvalent system was used to optimize rapidly the substrates for stromelysine, matrilysin and human collagenase 3 [79].

Both systems are based on random insertion of hexameric domains into surface-protein gene III of phage fTC [11]. Phages carrying the recombinant protein are then incubated with MMPs. If the inserted sequence is a substrate for MMPs, the N-terminal domain of the protein is cleaved off. For removal of the phages without substrate sequences, immunoaffinity chromatography employing antibodies raised against the N-terminal domain is used. Phages bearing substrate sequences are then amplified and further analyzed (Fig. 2A). An example of phage display use is the selection of substrate peptides for MMP-2 with subsequent study of the reaction mechanisms of enzyme-peptide interactions [80].

Achieving high selectivity and substrate specificity is critical in MMP-isoenzyme detection. The requirement for selectivity and absolute substrate specificity may be compromised using a multiple-enzyme/multiple-reagent system (MEMRAS) [11]. This method measures the activity of MMPs in liquid using fluorogenic substrates with different selection profiles for various MMPs. This approach is advantageous in detecting MMP activity in biological samples, such as body fluids [10,81]. Measuring reaction rates as a change of fluorescence in time

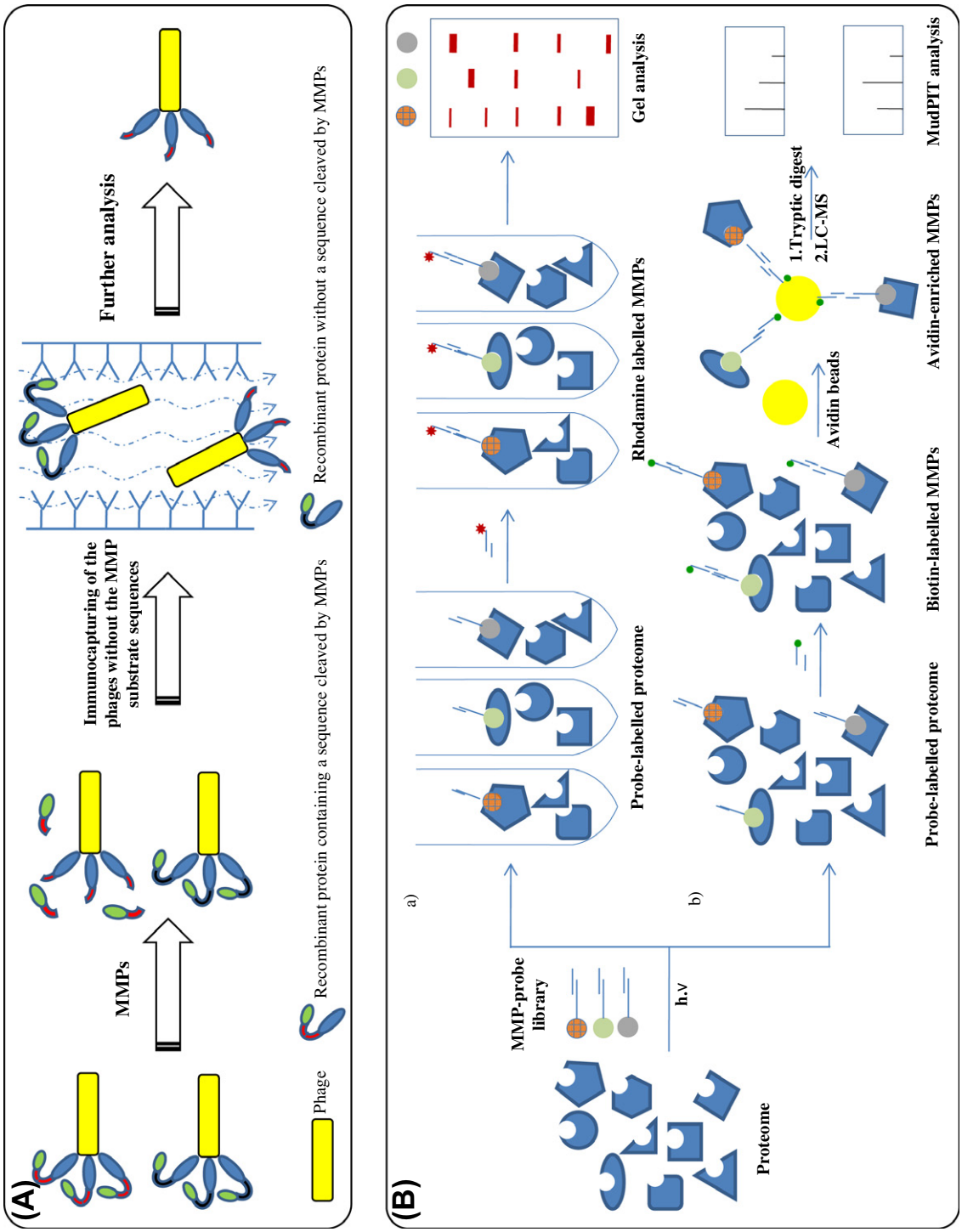

Figure 2. (A) Phage display using matrix metalloproteinases (MMPs) for analysis of phages carrying recombinant proteins. (B) Application of Rh-HxBP probes for proteome analysis of results by (a) gel-based or (b) multidimensional-protein identification technology-based (MudPIT) activity-based proteomic probes (ABPP). In gel-based ABPP, individual Rh-HxBP-treated proteomes reacted with a rhodamine-reporter tag and separated by SDS-PAGE. Labeled MMPs are then visualized by in-gel fluorescence scanning. In ABPP-MudPIT, proteomes are treated with the Rh-HxBP cocktail probe and reacted with a biotin reporter tag. Probe-labeled metalloproteases are then captured on avidin beads, digested with trypsin and analyzed by multidimensional liquid chromatography coupled to mass spectrometry.

for each substrate leads to a set of simultaneous equations with unknown MMP concentrations. Solving the system of equations determines detection of single-MMP activities. This method was applied to determine collagenase 3 and gelatinase activities in a mixture using two substrates with different cleavage profiles for collagenase and gelatinase [81].

Activity-based proteomic probes (ABPPs) are active locally-controlled probes used for detecting enzymatic activity in whole-proteome analysis. ABPPs usually contain binding groups, which interact with active sites of a particular class of enzymes, a reactive group for covalent labeling of the enzyme active site, and a reporter group (e.g., fluorophores or biotin) for visualization and affinity purification of labeled enzymes. A probe for detecting MMP activity is conjugated with rhodamine (Rh-HxBP) by incorporating a zinc-chelating hydroxamate moiety. Fig. 2B shows a detailed mechanism for conjugation.

In gel-based ABPP, individual Rh-HxBP-treated proteomes are reacted with a rhodamine reporter tag and separated by SDS-PAGE. Labeled MMPs are then visualized by in-gel fluorescence scanning. In ABPP-multidimensional-protein identification technology (MudPIT), proteomes are treated with an Rh-HxBP cocktail probe and reacted with a biotin reporter tag. Probe-labeled metalloproteases are then captured on avidin beads, digested with trypsin and analyzed by multidimensional liquid chromatography mass spectrometry (LC-MS). Since the probe interacts with active MMP sites, it is possible to distinguish among zymogenes, active MMPs and MMP-inhibitor complexes. HxBP-Rh is used to identify increased MMP-expression levels in invasive tumor cells and to evaluate the effectiveness of MMP inhibitors. ABPP showed a sensitivity of 3 nM for MMP-2 in selected tissues [82].

Another method based on activity profiling uses sorbents with group-specific inhibitors for the selective enrichment of a sample prior to standard proteomic procedures. This is known to increase the sensitivity of this method, which was used to enrich synovial fluid samples from rheumatoid arthritis patients [83]. The use of an MMP inhibitor, TNF-alpha protease inhibitor 2 (TAPI-2), showed strong enrichment in the samples. The yield was 98.8% for MMP-1, MMP-7, MMP-8, MMP-10, MMP-12 and MMP-13, and 96.1% for MMP-9 [83].

In the 1930s, Heyrovsky described so called pre-natrium catalytic wave, but its use for analytical purposes failed at that time. In the early 1990s, Palecek et al. showed the possibility of using the catalytic signal for electroanalytical determination of proteins [84].

Chronopotentiometric stripping analysis (CPSA) measures hydrogen evolution from the supporting electrolyte catalyzed by the presence of a protein, especially cysteine residues. The measured signal is called ''peak H''. It can be assumed that collagen is cleaved into smaller fragments by MMP-9, which are conveniently accessible to the electrode surface, and that results in a higher signal. This method is very sensitive, with LODs in the sub-nM range. Some disadvantages include high standard deviations of results and the long time of analysis at higher stripping currents. In spite of the advantages of this technique, MMPs have not been investigated using CPSA. Recently, Huska et al. used this technique to study collagen cleavage by MMP-9 [85]. The LOD was approximately 100 pM.

A multiplexed, particle-based flow cytometric assay was used to identify plasma MMP-7 associated with cancer-related death among patients with bladder cancer [86].

7. Imaging methods

Utilizing MMPs as targets for *in-vivo* imaging is a relatively young approach and much has been done over the past decade to develop probes for MMPs. MMP imaging has been limited to optical imaging method (OIM), positron emission tomography (PET), single-photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI) [16]. Imaging of MMPs in cancer has many potential applications. It takes advantage of the catalytic nature of proteinases as means to enhance the sensitivity of screening methods for early cancer detection [16,17].

Optical imaging (OIM) is capable of evaluating a number of *in-vivo* processes with the mechanism of contrast generally requiring the accumulation of a fluorescent reagent at the target site. A multitude of fluorescently-labeled probes have been developed to target cell-surface receptors, enzyme biodistribution, protein function and gene regulation [87]. The main objective of OIM is to accumulate fluorophores at a targeted region that, upon excitation, emit photons. OIM utilizes the ability to probe tissue with light for minimal/non-invasive detection of cancer. This technique has a practical application mainly due to the fluorescent probes emitting in the NIR spectrum where tissue has both low absorption and reduced scattering. Fluorogenically-labeled substrates that have been designed are quenched due to the proximity of the fluorophores, or utilize Förster resonance-energy transfer (FRET) to quench the fluorescent signal that is then enhanced upon proteolytic cleavage [88].

Positron-emission tomography (PET) is a highly sensitive, quantitative molecular imaging method, use of which in clinical and experimental medicine is based on the assessment of radiolabelled tracer molecules. A radioactive isotope usually has a short half-life and decays by emitting a positron. For *in-vivo* assessment, positron-emitting isotopes are produced in a cyclotron, chemically linked to a probe/tracer molecule, and injected intravenously.

Single-photon emission computed tomography (SPECT) is based on the radiolabelled tracer principle, but it utilizes gamma radiation from isotopes with a longer half-life. Using a simple gamma camera, SPECT can measure the biodistribution of small concentrations of biomolecules *in vivo* and quantify the molecular kinetic processes [16]. An activable MMP-14 targeted SPECT imaging probe was developed and used for MMP-14 imaging in transfected human breast-cancer cells [89].

Magnetic resonance imaging (MRI) has become another possibility in *in-vivo* imaging of MMPs. However, this method is not frequently used for this purpose. Lepage et al. hypothesized that cleavage of MRI-contrast agents by extracellular proteinases could be used to detect and to image biochemical processes (e.g., the expression of enzymes in the microenvironment of tumors and/or metastases) [90].

Due to the multiple roles of MMPs in pathological processes, engineered magnetic NPs based on MMP action can be used for MRI, therapy and diagnostics [91]. The use of very small iron-oxide particles (VSOPs) as protease-activable nanosensors in sensitive probes for molecular MRI was recently reviewed [92].

In conclusion, OIM using sensor and reference probes provides the opportunity for quantitative measures of proteolytic activity. However, these methods are relatively insensitive compared to PET or SPECT imaging. They are negatively influenced by the amount of tissue that has to be penetrated, and the equipment required is not readily available in clinics.

Although PET/SPECT radiotracers circumvent many of these problems and could be more readily used in human subjects, the agents are difficult to synthesize and are not yet proved to be effective indicators of proteolytic activity. The MRI approach evades the synthesis and clinical translation problems, but the quantitative aspects have still to be demonstrated [16].

Fluorescently-labeled or DQ (dye-quenching) substrates were also used for imaging of MMP activity in real-time and to study tumor-associated cell migration both *in vivo* and in cell cultures [93].

Molecular imaging aims to enable personalized medicine via imaging-specific molecular and cellular targets that are relevant to diagnosis and treatment of particular diseases. By providing *in-vivo* read-outs of biological detail, molecular imaging complements traditional anatomical imaging modalities to allow:

(1) visualization of important disease-modulating molecules and cells *in vivo* {e.g., tumor microenvironment [94] or angiogenesis [95]};
(2) serial investigations to visualize evolutionary changes in disease attributes {e.g., atherosclerosis [96]}, and,
(3) evaluation of the *in-vivo* molecular effects of biotherapeutics [97].

In-vivo NIR fluorescence imaging employing an MMP-activated probe was explored as a non-invasive method for imaging MMPs after cerebral ischemia in a mouse model of stroke [98] or detecting colon tumors at a very early stage in murine models [99].

NP systems in various unique configurations are highly effective at detecting protease activity both *in vivo* and *in vitro*. Recent advances in protease-responsive nanosensors and conventional modern methods for monitoring protease activity were summarized by Wesler et al. [100]. Hybrid *in-vivo* FMT-CT (fluorescence molecular tomography – computed tomography) imaging of protease activity with customized nanosensors was tested as a robust and observer-independent tool for non-invasive assessment of inflammatory murine atherosclerosis [101].

An optical biosensing platform using GNPs covered with gelatine modified with mercaptohexan-1-ol (MCH) was developed to detect proteinases. This system was tested for MMP-2 and trypsin. After digestion with proteinase (either trypsin or gelatinase), the GNPs lose shelter and MCH increases the attraction force between the modified GNPs. As a result, the GNPs gradually move closer to each other, resulting in GNP aggregation. It can then be monitored by the red shift of surface-plasmon absorption, and the visible color change of the GNPs is from red to blue. A linear correlation was determined with MMP-2 activity at concentrations of 50–600 ng/mL [102].

Protease-specific nanosensors were developed for *in-vivo* imaging by MRI. Upon specific protease cleavage, the NPs rapidly switch from a stable, low-relaxivity, stealth state to become adhesive, aggregating high-relaxivity particles [103]. An *in-vivo* visualization of MMP activities by MRI and fluorescence of dendrimeric NPs coated with activable cell-penetrating peptides (ACPPs) was reported, labeled with Cy5, gadolinium, or both. It was possible to detect residual tumor and metastases as small as 200 μm, and these can be excised under fluorescence guidance and analyzed histopathologically with fluorescence microscopy [104].

Also, MMP-sensitive gold nanorods were tested for simultaneous bioimaging and photothermal therapy of cancer [105].

7.1. Mass spectrometry

New rapid techniques were required to identify and to quantify proteinase substrates in more complex biological samples and on a system-wide basis [106,107]. These came with the development of quantitative mass-spectrometry (MS)-based proteomic techniques {e.g., isotope-coded affinity tag (ICAT) labeling [108]}. ICAT labeling involves differential tagging by reductive alkylation of proteins containing cysteine residues with chemically identical biotin tags that differ in isotopic

composition and therefore mass. This allows MS quantification of the relative abundance of the labeled proteins within two samples.

ICAT was used in a cell-based substrate discovery screen of MMP-14 that led to the identification of 14 novel MMP-14 candidate substrates, only two of which were extracellular matrix (ECM) proteins [109]. ICAT analysis has proved to be a powerful tool for revealing new functions for MMPs in processes (e.g., angiogenesis). Apart from facilitating angiogenesis through ECM degradation, identification of novel MMP-2 substrates uncovered the role of MMP-2 in the proteolytic release of proangiogenic vascular endothelial growth factor from inhibitory complexes with connective tissue growth factor and heparin affin regulatory peptide (HARP) that led to stimulation of neovascularization [110].

The main disadvantage of ICAT is that only proteins containing cysteines are analyzed. The number of cysteine-containing peptides per protein is limited, reducing the confidence in protein identification and quantification. To avoid these problems, an alternative labeling approach, isotope tags for relative and absolute quantification (iTRAQ) was developed [111]. In iTRAQ analysis, proteins from complex biological samples are digested with trypsin and free amines are labeled with isobaric tags, thus ensuring that all proteins within the sample can potentially be represented and also improving the peptide coverage of those proteins.

Tandem MS (MS^2) analysis provides both sequence identification of peptides and quantification via the unique mass signature of the tags. Another advantage of this technique is that there are now eight unique iTRAQ tags, allowing eight different conditions to be analyzed in a single experiment. Further, iTRAQ analysis was used to uncover the diverse substrate degradome of MMP-2 in a cellular context. With increased peptide coverage, it was possible to perform ''peptide mapping'' in order to predict the location of cleavage sites within the native cellular substrate for 6 out of 23 novel MMP-2 substrates identified [112].

High-performance LC coupled with MS (HPLC-MS) was also used for detecting MMP activity [113,114]. Alternative quantitative proteomic analysis of cell-based MMP substrates includes a label-free technique using ultraperformance LC electrospray ionization high/low-collision-energy MS was used for analysis of metastatic prostate-cancer cell line in which MMP-9 expression was knocked down by RNA interference [115]. Relative quantification between samples was achieved by comparing peak intensity, with each mass peak defined by exact mass and retention time. Of 20 novel MMP-9 potential substrates identified, only four were ECM related.

Interestingly, there was significant overlap of potential MMP-9 substrates identified by this label-free approach and the MMP substrates identified by proteomic techniques using isotope tags. A peptide-mapping procedure similar to that described before was used to infer cleavage sites *in vivo*, but validation was performed employing nested synthetic peptides instead of native proteins [112]. This approach could lead to wrong conclusions, as the exact cleavage sites might differ in the folded protein state.

Other proteomic screens for MMP substrates in complex biological fluids have utilized 2-dimensional polyacrylamide gel electrophoresis and in-gel digest. These approaches are easily used in many laboratories, but lack throughput and sensitivity and have low discovery rates. Two-dimensional differential in gel electrophoresis of bronchoalveolar fluid from knock-out mice compared to wild type mice identified three MMP substrates, which were chemotactic proteins involved in lung inflammation [116].

An immunoaffinity LC-tandem MS (LC-MS^2) method was developed for the quantification of the zinc endopeptidase MMP-9 from mouse serum. Sample preparation for the assay included magnetic-bead-based enrichment using an MMP-9 antibody and was performed in a 96-well plate format using a liquid-handling robotic platform. The method was employed to measure MMP-9 concentrations in 30 mouse-serum samples [117].

8. Nanoparticle-based assays

A new strategy for highly-sensitive, rapid protease assay has been developed by mediating proteolytic formation of oligonucleotide duplexes and using the duplexes for signal amplification. In the presence of MMP-2, fragmentation of the intact DNA-peptide on GNPs by hydrolytic cleavage of a peptide bond within the substrate allows diffusion of DNA away from the GNP. It is followed by the formation of a DNA/RNA heteroduplex, which leads to digestion of RNA by RNase H. Because of high quenching efficacy of GNP to the fluorophore in RNA and multiple digestions of the RNA, the fluorescence signal recovery is amplified. This method permits the assessment of MMP-2 activity at concentrations as low as 10 pm within 4 h. Compared with the reported protease nanosensors using quantum dots, GNP, and magnetic NPs with the same peptide sequence, the assay time of this method is six-fold faster and the LOD is 100-fold more sensitive [118].

Using p-type Si-nanowire-based field-effect transistors (FETs) for biological applications was proposed. A combination of electron-beam lithography and a lift-off process was utilized to fabricate individual 50-nm-thick Si-nanowire FETs, and the conductance of the Si-nanowire FET depended upon the existence of negatively-charged streptavidin binding to a biotin with a peptide and MMP-9, cutting the peptide [119].

9. Outlook

MMPs are an important class of enzymes that play crucial roles in physiological and pathological states. Due to the complexity of their pathways, activators, inhibitors and substrates, it is difficult to study the specific role that metalloproteinases play in complex biological matrices. Lack of standards and validated analytical methods complicates the comparison of results obtained from different research facilities. Enzymatic, immunochemical and fluorimetric methods are most suitable for clinical research. *In-vivo* imaging methods have potential benefits in cancer research and diagnostics (Table 1). There are a number of other methods {e.g., phage display, the multiple-enzyme/multiple-reagent assay system (MEMRAS) and activity-based profiling}, which are of interest and currently being used to investigate MMPs.

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