Journal of Chromatography B, 878 (2010) 1777–1783

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

On-line chromatographic screening of matrix metalloproteinase inhibitors using immobilized MMP-9 enzyme reactor

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ARTICLE INFO

Article history:
Received 12 February 2010
Accepted 28 April 2010
Available online 4 May 2010

Keywords:
Matrix metalloproteinase
MMP-9
Immobilized enzyme reactor
High throughput screening

ABSTRACT

Matrix metalloproteinase 9 (MMP-9) plays an important role in cancer invasion and metastasis and has been an attractive target for therapeutic intervention of cancer metastasis. However, considering the high cost and intricacy associated with the expression, isolation and purification of the recombinant enzyme for the screening of drug candidates, alternative methods that explore the recycling of enzymes become desirable. In this study, a new immobilized enzyme reactor (IMER) containing human recombinant MMP-9 enzyme was developed and characterized for the on-line screening of MMP-9 inhibitors. The MMP-9 IMER containing active unit of the enzyme (U = 0.08 μmol/min) on the disk was inserted into a HPLC system connected to a UV–vis detector for on-line chromatographic screening. The resulting conjugated enzyme was shown to be an active and stable catalyst for the hydrolysis of MMP-9 chromogenic thiopeptide substrate Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5. The kinetics profile of the enzyme in IMER and free solution were determined and compared. Selected reversible MMP inhibitors, N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH), doxycycline and minocycline were further characterized using the MMP-9 IMER and free enzyme solution assays. Our system demonstrated applicability as a rapid and cost-effective screening tool for inhibitors of the MMP-9 enzyme.

1. Introduction

Combinatorial chemistry is an important development in drug discovery that has significantly increased the rate in synthesizing compound libraries for pharmacological screening. Identification of potential leads from combinatorial libraries demands a high throughput screening (HTS) platform that can select potential effectors of pharmacological activity against disease targets for further optimization and development. While the multiwell robotic screening platform has revolutionized HTS in many areas, other ligand-based HTS methodologies, such as the "target protein-immobilized" flow-through systems are less explored [1–3]. Enzymes are versatile catalysts that mediate diversified metabolic and pharmacological processes in a biological system. Naturally, enzymes are attractive targets for drug therapy because of their essential roles in pathophysiology [4]. By their nature, enzymes are not consumed during biochemical reactions that they catalyze. However, enzymes are difficult to recover as a soluble component from a reaction mixture for repetitive usage *in vitro*. Immobilization of enzymes is therefore explored as an attempt to stabilize and prolong the activity and reusability of the catalyst [5]. In the last 3 years, immobilized enzyme reactors (IMERs) coupled to separation modules have been developed to screen enzyme inhibitors [6,7], profile drug metabolism [8], map proteins [1,9], and analyse enantiomers [10] in a rapid and automatic fashion.

Matrix metalloproteinases (MMPs) constitute a family of more than 20 structurally and functionally related zinc-dependent enzymes in humans [11]. MMPs are classified mainly into five groups, namely collagenase, gelatinase, stromelysin, matrilysin, and membrane-type MMPs, based on their structures and functions. MMPs are involved in remodeling of several components of the extracellular matrix (ECM), and many are recognized as potential drug targets in cancer and inflammation [11,12]. Among these MMPs, MMP-9 was determined to possess gelatinase activity and played an important role in cancer invasion and metastasis [12,13]. Direct evidence for the involvement of MMP-9 in tumor growth and invasion was revealed in a study where MMP-9 knockout mice demonstrated reduced melanoma tumor progression and angiogenesis [14]. Therefore, MMP-9 has become an attractive target for therapeutic intervention of cancer metastasis. Some widely known MMP inhibitors are chemically modified tetracyclines, bisphosphonates, or compounds isolated from natural sources [11,15]. At present, MMP inhibitors are undergoing structural activity rela-

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doi:10.1016/j.jchromb.2010.04.040

tionship (SAR) study or clinical trials for the development of new drugs.

Considering the high cost and intricacy associated with the expression, isolation and purification of recombinant enzymes for the screening of drug candidates, alternative methods that explore the recycling of enzymes become desirable. Monolithic disk, under trademark convective interaction media (CIM®), has been used as a novel stationary phase to generate enzyme reactors in many studies [8,16,17]. The CIM® disk is an amine-activated monolithic support obtained by reacting the native epoxy groups with ethylene diamine (EDA). It is used for affinity chromatography or bioconversion by immobilizing proteins, peptides and other ligands through a crosslinking reaction with a suitable bifunctional reagent, for example glutaric dialdehyde. The CIM® disk is characterized by its short monolithic length and the specially engineered highly porous structure that offers high-speed analysis and low back pressure in HPLC system. In the present study, an active model enzyme, MMP-9, was covalently immobilized onto glutaraldehyde derivatized EDA CIM® disk to obtain a IMER. The MMP-9 IMER was placed in a liquid chromatographic system where on-line chromatographic studies were performed for the rapid screening of model MMP inhibitors.

2. Materials and methods

2.1. Materials

EDA CIM® disks (3 mm × 12 mm I.D., 0.34 mL internal volume) were purchased from Separations (Ljubljana, Slovenia). 5,5-Dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent), glutaraldehyde 70% aqueous solution, calcium chloride, Brij-35, HEPES, sodium cyanoborohydride, doxycycline and minocycline were purchased from Sigma (Singapore). MMP-9 colorimetric drug discovery kit comprising the human recombinant MMP-9, its chromogenic thiopeptide substrate Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5 and an inhibitor N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH), was purchased from Enzo Life Sciences International (Plymouth Meeting, PA). All solutions were prepared with Milli-Q water obtained from a Millipore Direct-Q3® UV water purification system (Millipore S.A.S., Molsheim, France). The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for HPLC.

2.2. Instrumentation

All HPLC experiments were performed on an Agilent 1100 system equipped with an Agilent 8453 diode array spectrophotometer (slit width of 2 nm, data pitch of 0.5 s and 37 °C) (Agilent Technologies, Singapore). For routine analyses the detector wavelength was set at 412 and 450 nm. The chromatographic analyses on MMP-9 enzyme reactor were performed at column temperature of 37 °C. The mobile phase at pH 7.5 comprised 50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35 and 1 mM DTNB. Parallel non-chromatographic enzymatic assays were performed using a TECAN Infinite M200 plate reader (Tecan, Austria).

2.3. MMP-9 enzyme immobilization

The EDA CIM® disk was inserted into the housing and connected to the HPLC system. The unit was washed with a mobile phase of 50 mM phosphate buffer, pH 7.5 for 30 min at the flow rate of 1 mL/min. The disk was removed and placed into a 50 mL beaker. The beaker was charged with 20 mL of a 5% glutaraldehyde solution in 50 mM phosphate buffer, pH 6.0. The disk was stirred in the solution for 4 h and treated subsequently with 10 mL of 0.1 M cyanoborohydride solution in phosphate buffer (50 mM, pH 6.0) for 3 h at room temperature (23 ± 1 °C) to reduce the Schiff base that was formed *in situ*. An enzyme solution was prepared by diluting an aliquot of 10 μL of MMP-9 enzyme (2.68 U/μL) to 1 mL using the assay buffer. The glutaraldehyde-treated disk was then incubated with the above enzyme solution for 18 h at room temperature. After immobilization, the enzyme solution was analysed with the Ellman's assay in order to determine the unreacted enzyme units. The disk was washed with 50 mM phosphate buffer, pH 6.0, and was treated again with 10 mL of 0.1 M cyanoborohydride solution in phosphate buffer (50 mM, pH 6.0) for 3 h at room temperature. The unreacted aldehyde group was subsequently blocked using 0.2 M monoethanolamine solution in phosphate buffer (50 mM, pH 6.0) at room temperature for 6 h. The disk was further reduced with 0.1 M cyanoborohydride solution in phosphate buffer (50 mM, pH 6.0) for 3 h at room temperature. Finally, the MMP-9 enzyme-immobilized EDA CIM® disk was washed, inserted into a housing unit, connected to the HPLC system and conditioned for 1 h with a mobile phase consisting of phosphate buffer (100 mM, pH 7.4) at a flow rate of 1 mL/min. For the storage, the MMP-9 IMER was stored at 4 °C in phosphate buffer (100 mM, pH 7.4) spiked with 0.2% sodium azide to prevent microbial growth.

2.4. Determination of MMP-9 IMER activity

For the assay, a thiopeptide substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5) containing a thioester bond was utilized. Hydrolysis of the thioester bond of the substrate by the MMP-9 enzyme produced a sulfhydryl group. The free sulfhydryl group reacted subsequently with DTNB to form 5-mercapto-2-nitrobenzoic acid (TNB) which was in turn detected by its absorbance at 412 nm (ε = 13,600 M^{-1} cm^{-1} at pH 6.0 and above) [18]. In summary, the activity of MMP-9 was determined indirectly by measuring the formation of TNB obtained from the reaction between Ellman's reagent and the enzymatically derived sulfhydryl group from thiopeptide substrate. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the hydrolysis of 100 pmol of thiopeptide substrate per min at 37 °C, which was equivalent to the stoichiometric formation of the correspondent TNB.

The MMP-9 IMER was inserted into a housing unit and flushed with 100 mM phosphate buffer solution pH 7.5 for 0.5 h. The disk was subsequently equilibrated using the complete assay buffer at pH 7.5 consisting of 50 mM HEPES (0.1 M, pH 8.0), 1 mM Ellman's reagent, $CaCl_2$ (10 mM) and 0.05% Brij-35 at 1.0 mL/min and the eluent was monitored using UV–vis detection at 450 nm. Aliquots of 5 μL of thiopeptide substrate in DMSO were injected into the HPLC system at increasing concentration (1–50 mM). Each eluate, containing differential levels of TNB, was collected over the first 3 min after each injection. The absorbance of each eluate (A_{412}) was measured at 412 nm using UV–vis spectroscopy. According to the Beer–Lambert law, the amount of hydrolyzed substrate S was calculated from the absorbance by applying the following equation:

$$S\,(\text{mol}) = \frac{3 \times 10^{-3} \times A_{412}}{\varepsilon \times 1} \quad \text{where } \varepsilon = 13,600\,\text{mol}\,\text{cm}^{-1}$$

Therefore, the rate of each enzymatic reaction V (mol/min) with regards to the on-line system was calculated by dividing the amount of hydrolyzed substrate S by the contact time (0.34 min). A correlation between absorbance values at 412 nm and relative chromatographic peak areas acquired at 450 nm was plotted to verify the accuracy of using chromatographic peak areas as surrogate measurement for the enzymatic reaction.

A Michaelis–Menten plot approximately describes the kinetics of the immobilized enzyme. To determine the maximum rate of the enzyme reaction, a series of experiments is carried out with

varying substrate concentration [S] and the rate of product formation V is measured. The Michaelis–Menten plot was obtained by plotting the rate of enzymatic reaction V against substrate concentration [S]. A Lineweaver–Burk plot was also obtained by plotting of enzyme kinetics in a double-reciprocal manner to derive K_m and maximum rate of the enzyme reaction V_{max}. From V_{max}, the immobilized active units of the enzyme MMP-9 was determined by applying the following relationship: one active unit was defined as the amount of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute at 37 °C.

$$U\,(\mu\text{mol min}^{-1}) = \left(\frac{V_{max}}{\varepsilon}\right) \times 3 \times 10^{-3} \times 10^{6}$$

2.5. Screening for MMP-9 inhibitory activity

Test inhibitors (NNGH, doxycycline and minocycline) were prepared and stored as stock solutions in DMSO at a concentration level of 1.3 mM. Each assay solution was prepared by mixing an equal amount of the respective stock solution of test inhibitor with the thiopeptide substrate (20 mM) to obtain a mixture containing the test inhibitor and substrate at 0.65 and 10 mM, respectively. A 5 μL aliquot of substrate solution (10 mM) was injected into the MMP-9 IMER HPLC system and the integrated area of the chromatographic peak eluting at t_R = 0.374 min was determined (A_o). Each assay solution containing the test inhibitor and substrate was then injected into the HPLC system and the peak area was similarly recorded (A_i). The percentage inhibition of the enzyme activity due to the presence of test inhibitor was calculated using the following equation:

$$\text{percentage inhibition}\,(\%) = 100 - \left(\frac{A_i}{A_o} \times 100\right)$$

where A_i is the peak area calculated in the presence of inhibitor, while A_o is the peak area obtained with the substrate solution in the absence of the inhibitor.

2.6. Non-chromatographic enzymatic assay of MMP-9

The assay was performed according to the protocol as provided in the MMP-9 colorimetric drug discovery kit. Briefly, an assay buffer at pH 7.5 containing 50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35 and 1 mM DTNB was used as provided in the kit. Each test inhibitor (NNGH, doxycycline and minocycline) and substrate were diluted 200 and 25 times to the concentration levels of 6.5 μM and 1 mM, respectively, using the assay buffer. The enzyme was diluted 60 times using assay buffer and warmed to reaction temperature (37 °C). The assay was started by mixing the appropriate volumes of assay buffer, MMP-9 enzyme, inhibitor solution or blank in each well of 96-well plate and pre-incubating the plate for 60 min to facilitate interaction between the inhibitor and enzyme (Supplementary Table 1). The enzymatic reaction was initiated subsequently by adding 10 μL of thiopeptide substrate to the incubation mixture. The final substrate concentration in each well was 100 μM. The absorbance in each well was continuously read at 412 nm using a Tecan microplate reader with 1 min time interval for 10 min. The OD *versus* time for each well was plotted and the reaction velocity was determined by the slope of the line which fit to the linear portion of the data. Triplicate experiments were performed. The reaction rates were compared and the percentage inhibition due to the presence of each of the test inhibitors (NNGH, doxycycline and minocycline) was calculated using the following equation:

$$\text{percentage inhibition}\,(\%) = \frac{V_{inhibitor} - V_{blank}}{V_{control} - V_{blank}} \times 100$$

3. Results and discussion

3.1. Enzyme immobilization

An important factor in the preparation of the IMER for its on-line application in drug screening is the type of its support. The support must be inert and stable in the HPLC system to retain its structure and ensure substrate accessibility to interact with the enzyme active sites. While various supports for IMER are commercially available or specifically developed in-house, most of them belong to the following three categories: silica derivatized matrices, immobilized artificial membrane (IAM) and monolithic chromatographic supports [19]. While silica-based derivatized matrices are the most commonly used supports, monolith is a relatively new stationary phase providing important advantages with regards to chromatographic IMER as compared to traditional microparticulate sorbents, such as silica-based matrices and IAM [20]. In contrast to conventional supports, the only voids in a monolithic unit are the interconnected pores, including the pores of polymer particles and cracks between the particles. This characteristic provides unique advantages to monolith such as fast kinetics, high reactivity, and high throughput application. When connected to HPLC system, the large pores of the monolithic materials allow high-speed analysis and low backpressure. Similar observation was found in an earlier IMER research on human recombinant acetylcholinesterase inhibition where a short EDA CID® disk was used as support to replace the original chromatographic column (50 mm × 4.6 mm I.D.) containing epoxy silica so as to reduce the long elution time and decrease the high concentration of saturating substrate [16]. The EDA CID® disk is an amine-activated monolithic support generated by reacting the native epoxy group with ethylene diamine. The reductive amination, a major strategy in biomacromolecular conjugation, has been used for MMP-9 immobilization on the monolithic support. During the process, an aliphatic dialdehyde was attached covalently and directly to the amino groups of the enzyme as well as amine-containing matrices. The scheme of the MMP-9 immobilization via reductive amination on the EDA CIM® disk is illustrated in Fig. 1. The amine terminal spacer in the disk was reacted with 5% glutaraldehyde. One aldehyde end formed an imine linkage with the matrix while the other end remained free and available for the subsequent coupling to the amine-containing MMP-9 enzyme. Sodium cyanoborohydride was used to selectively reduce the formed imine while sustaining the free aldehyde group. The disk with terminal formyl groups was reacted subsequently with the amino groups on the MMP enzyme for 18 h. Another reduction was carried out after the enzyme immobilization using sodium cyanoborohydride to promote reduction of Schiff bases to form stable secondary amine bonds. Before using this disk in the HPLC procedure, excess unreacted formyl terminal was blocked using ethanolamine.

Under the described conditions, the yield of immobilized enzyme was found to be 4.7%, by determining the activity of MMP-9 before and after its immobilization using the non-chromatographic enzymatic assay. However, the yield was found to be 2.5% *via* determination of protein loss before and after the reaction using the Tecan microplate reader at 280 nm. The difference in the measured yields of enzyme immobilization might be related to the decrease in enzymatic activity upon overnight incubation at room temperature (23 ± 1 °C).

3.2. MMP-9 IMER HPLC system

The amount of immobilized enzyme unit was further assessed using a HPLC system. After the enzymatic reaction using MMP-9 enzyme reactor, the thiopeptide was cleaved and the products did not present significant UV absorbance for detection. However, one of the hydrolyzed products (HS-LeuLeuGly-OC_2H_5) possessed

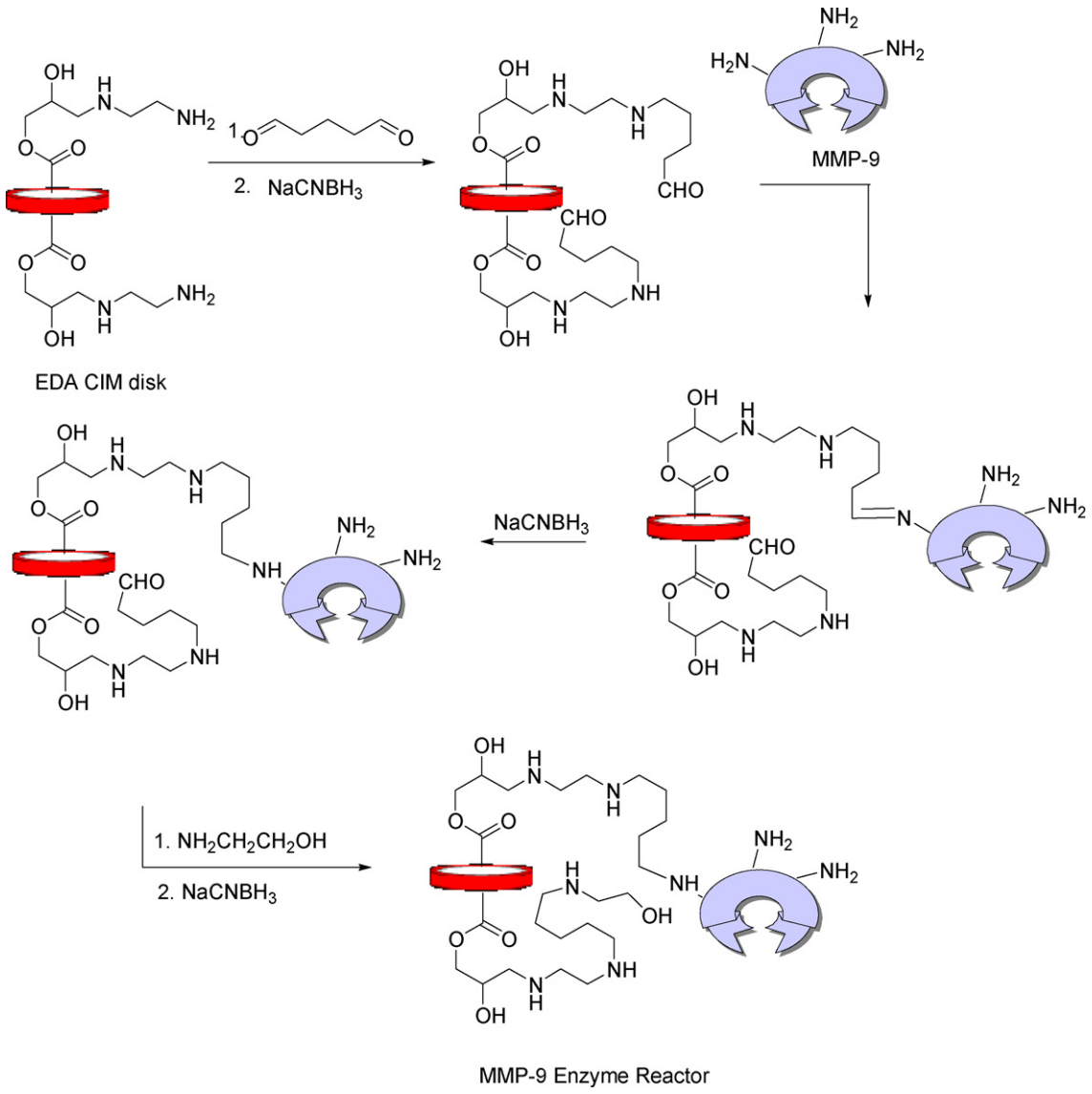

Fig. 1. Procedures for immobilizing MMP-9 enzyme onto EDA CIM® disk.

a sulfhydryl group and reacted with DTNB to form 5-mercapto-2-nitrobenzoic acid (TNB) that was in turn detected at 412 nm (Fig. 2). In the HPLC system, detection of analytes at higher wavelength (greater than 400 nm) was found to be more selective as a greater degree of interference was observed when the detection was performed at lower wavelengths (200–300 nm). Thus, the evaluation of enzymatic activity was performed by the selective detection of TNB at 412 nm. In addition, the mobile phase was prepared by mixing DTNB (1 mM) with the complete assay buffer for free enzyme where $CaCl_2$ (10 mM) was included for its essential role in maintaining the structural integrity and optimal activity of MMP-9 in the free and immobilized enzyme assay systems [21,22]. In order to quantify the amount of TNB produced by the enzyme reactor system, a series of the calibration curve was obtained by using different concentrations of substrate in the system. The concentration of the injected substrate ranged from 1 to 50 mM, while the injection volume was fixed at 5 μL. The flow rate was chosen to be 1 mL/min, which was equal to the optimal flow rate discovered by an independent research where another enzyme-immobilized disk and DTNB were used [16]. A linear relationship between the absorbance of eluent at 412 nm and the substrate concentration was observed (Fig. 3A, $y = 0.0003x + 0.0017$, $R^2 = 0.9926$). It could be explained that the immobilized enzyme was not saturated even with the highest substrate concentration tested. At the same time, the peak area of TNB for each injection was also integrated and correlated to the absorbance of eluent at 412 nm. The correlation between the peak area of TNB and absorbance was also found to be linear (Fig. 3B, $y = 3E^{-5}x + 0.0013$, $R^2 = 0.9856$). These results suggested collectively each chromatographic peak area of TNB was well correlated to its level in the enzyme reactor. Importantly, we further confirmed that the correlation between peak area of TNB and substrate concentration was also linear (Fig. 3C, $y = 10.1430x + 12.7650$, $R^2 = 0.9972$).

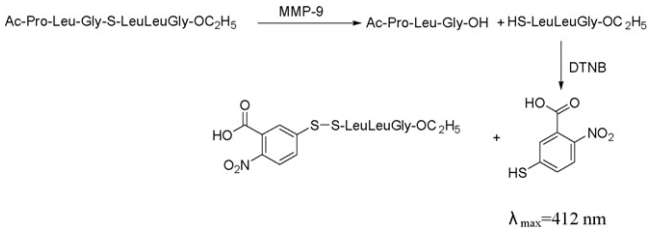

Fig. 2. Enzymatic reaction catalyzed by MMP-9 and Ellman's reaction resulting in the formation of 5-mercapto-2-nitrobenzoic acid.

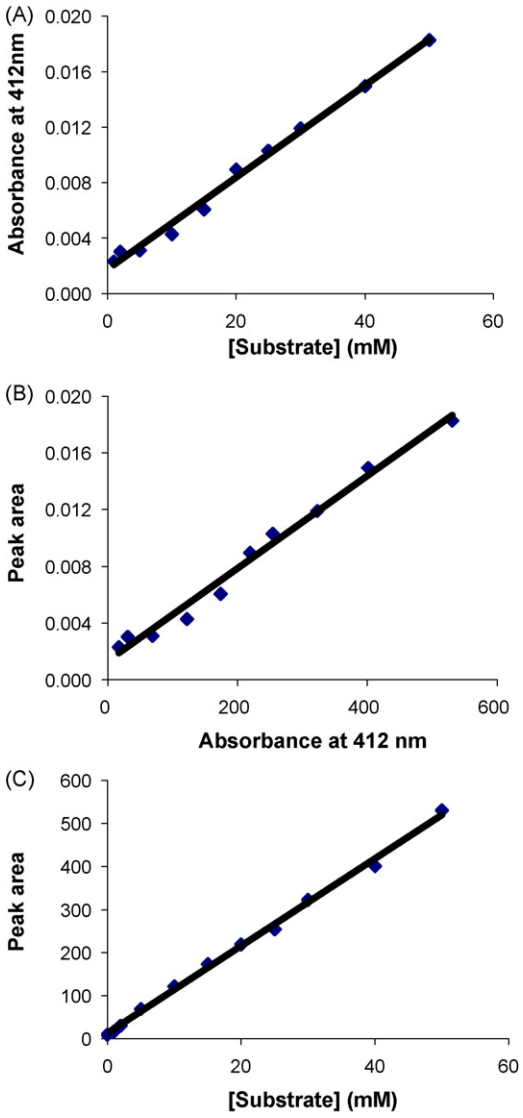

Fig. 3. Correlation plots. (A) Absorbance measured at 412 nm of eluates *versus* substrate concentration. (B) TNB peak area *versus* absorbance measured at 412 nm of eluates. (C) TNB peak area *versus* substrate concentration.

Based on these cross-correlations, we established that the chromatographic peak area of TNB could be extrapolated to its *in situ* concentration. With this important establishment, the chromatographic peak area of TNB was applied for the calculation of MMP-9 enzyme activity.

The kinetic parameters of the immobilized MMP-9 enzyme were determined from velocity measurement by varying the thiopeptide substrate concentration (1, 2, 5, 10, 15, 20, 25, 30, 40, and 50 μM). A Michaelis–Menten plot was obtained by plotting the enzyme reaction velocity *versus* the injected substrate concentration (Fig. 4A). Apparently, the highest substrate concentration in this study was below its saturation concentration K_m. Due to the solubility limit of the substrate in DMSO (50 mM), it was not possible to generate the saturation curve in the Michaelis–Menten plot. Within the experimented concentration range of the substrates, the enzyme active sites failed to reach saturation and both V_{max} and K_m appeared to be infinite graphically. In order to estimate V_{max} and K_m, the double-reciprocal Lineweaver–Burk line was plotted (Fig. 4B, $y = 0.7676x + 2.7531$, $R^2 = 0.9943$). The K_m and V_{max} were 0.2788 M and 0.3632 ΔAU/min, respectively. The active unit of the enzyme (U) on the disk was calculated to be 0.08 μmol/min from

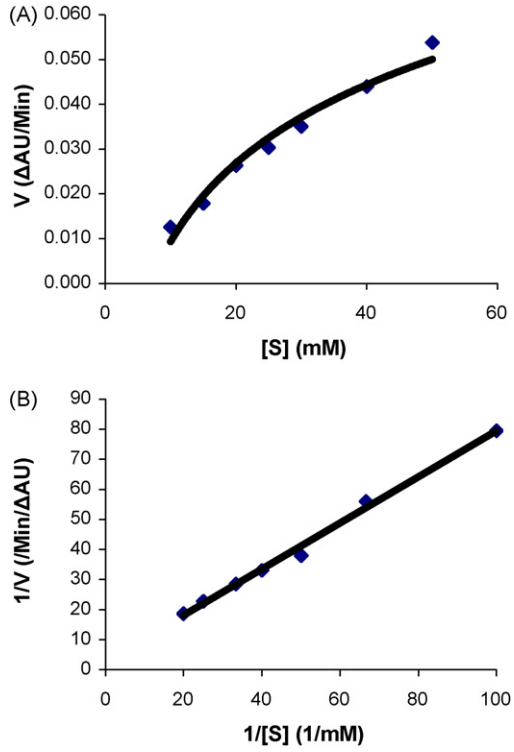

Fig. 4. Kinetics for determining enzymatic activity of MMP-9 IMER: (A) Michaelis–Menten kinetics and (B) Lineweaver–Burk plot.

the equation mentioned in Section 2. The K_m and V_{max} of the free MMP-9 enzyme in solution were also determined (Fig. 5) to be 5.50×10^{-5} M and 65.8 pmol/min, respectively. Since the amount of enzyme present in solution was significantly different from that immobilized on the disk, their K_m values and associated V_{max} were found to be different.

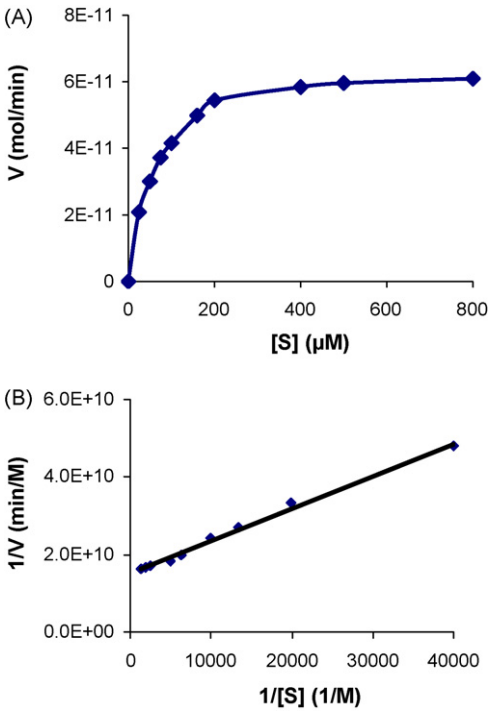

Fig. 5. Kinetics for determining enzymatic activity of solution-based MMP-9 enzyme: (A) Michaelis–Menten kinetics and (B) Lineweaver–Burk plot.

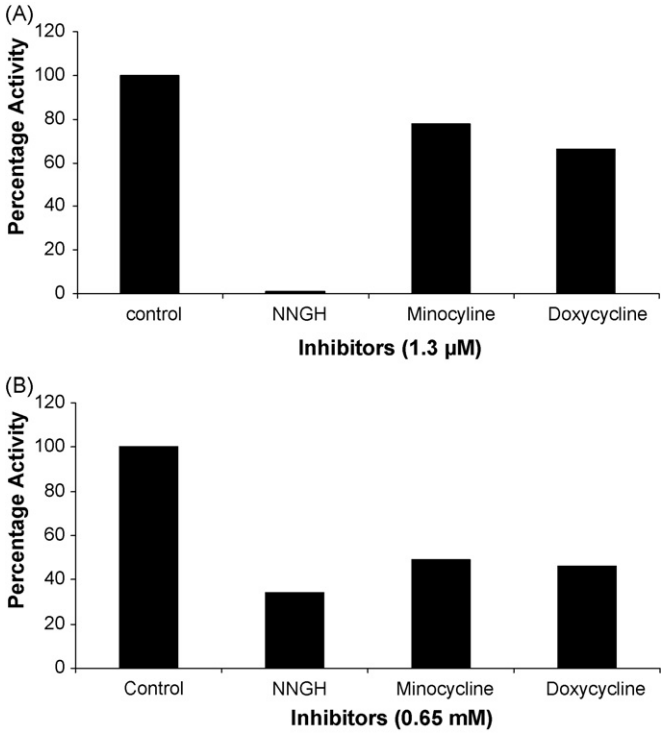

Fig. 6. Screening of MMP-9 inhibitors using (A) solution-based MMP-9 enzyme and (B) MMP-9 IMER.

3.3. Screening for MMP-9 inhibitory activity

The inhibition of MMP-9 using both free enzyme and IMER was examined. Three known inhibitors, NNGH (standard inhibitor provided in the kit), doxycycline and minocycline (nonspecific MMP inhibitors) were used to test the systems. The inhibitory activities of these model inhibitors were first screened using conventional enzyme assay in solution (Fig. 6A). The inhibitory activity of NNGH was highest among the compounds tested in this study. At the concentration of 1.3 μM, NNGH almost inhibited all the catalytic activity, while the nonspecific inhibitors, doxycycline and minocycline, only inhibited less than 50% of the enzyme activity. The inhibition potency ranking was NNGH > doxycycline > minocycline.

The inhibition screening was further performed using the MMP-9 IMER (Fig. 6B). The inhibition curves were obtained by injecting both substrate (10 mM) and inhibitor (650 μM) into the HPLC system. The reduction of the respective peak area was observed and recorded (Fig. 7). The screening result showed that the NNGH as expected was the strongest inhibitor among the tested compounds with the reduced peak area of 34.1%. The inhibition potency ranking was NNGH > doxycycline > minocycline, which corroborated with the findings based on the conventional assay. These result confirmed the feasibility of on-line chromatographic screening of MMP inhibitors using immobilized MMP-9 enzyme. One key advantage of the on-line screening system was the significant reduction in the screening time. The cycle time for each on-line screening was 5 min as compared to traditional enzymatic assay where more than 1 h was needed for incubation and detection. The second key advantage was that only a very small amount of enzyme (U = 0.08 μmol/min) was needed for its immobilization onto the monolithic disk. This is pertinent from a cost perspective and it might reduce batch-to-batch variations related to the enzyme. The third key advantage of on-line screening was that the IMER could be reused many times without compromising on its stability. Considering the high cost and difficulty to express and purify human recombinant enzyme, the technique presented in the present paper was found to be complementary to the existing technique in screening MMP inhibitors in a medium-throughput manner. The current study extended the pioneer work of Bartolini and Andrisano [6,7,16] in applying IMER on-line inhibition study on cholinesterases to MMP enzyme. The success of the current study proved that the EDA CIM® disk and the immobilization technique could be applied to a broader range of enzymes for the rapid on-line screening of enzyme inhibitors.

While the paper presented the proof-of-concept in immobilizing enzyme for drug screening, the success of the current platform for application in drug development will require careful improvement in terms of sensitivity in detecting enzyme inhibitors. The current set-up utilized a relatively high concentration of test inhibitors (650 μM) and may not be completely feasible for early drug screening where only a small quantity of each drug candidate is available. This sensitivity hurdle however may be mitigated through assay miniaturization such as the use of

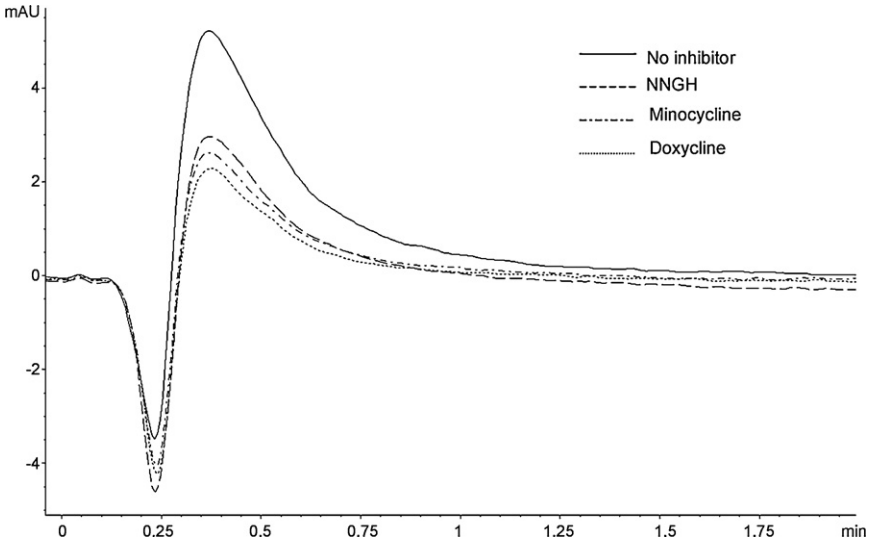

Fig. 7. Overlaid chromatograms obtained after injection of substrate and inhibitors.

a smaller enzyme reactor coupled to capillary or nano liquid chromatographic system where the total system volume is significantly reduced.

4. Conclusion

In this study, human recombinant MMP-9 enzyme was immobilized onto EDA CIM® monolithic disk via a covalent bond. The MMP-9 IMER was inserted into a housing unit and connected to a HPLC system for a medium-throughput screening of potential MMP-9 inhibitors. The preliminary study showed that the immobilization rate was 2.5% and the immobilized enzyme unit was 0.08 μmol/min. This system demonstrated applicability as a rapid and cost-effective screening tool for inhibitors of the MMP-9 enzyme. The MMP-9 IMER is a potential tool to be developed further for the primary screening of MMP inhibitors derived from combinatorial and natural product libraries.

Acknowledgements

This work was supported by the National University of Singapore grant (R-279-000-249-646) for the initiative on advanced membranes for pharmaceutical and biomedical applications. The authors are grateful to Professor Neal Chung and Associate Professor Chan Sui Yung for facilitating the grant support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.04.040.

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