Rapid analysis of matrix metalloproteinase-3 activity by gelatin arrays using a spectral surface plasmon resonance biosensor†

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We developed a novel assay system using an array-based spectral surface plasmon resonance (SPR) biosensor for a high-throughput analysis of matrix metalloproteinase (MMP)-3 activity. Gelatin arrays were fabricated by immobilizing gelatin, a MMP-3 substrate, on amine-modified gold arrays. MMP-3 activity was determined by monitoring the shift of SPR wavelength caused by gelatin proteolysis. The gelatinolytic activity of MMP-3, which caused a decrease of the SPR wavelength, was verified by SPR spectroscopy, atomic force microscopy, and fluorescence-based protein arrays. MMP-3 activity increased by three non-ionic detergents in a dose-dependent manner, and Brij-35 was most effective. 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, MMP inhibitors. Therefore, this new assay system using a spectral SPR biosensor is simple, label-free, and high-throughput, and is likely to have a strong potential for inhibitor screening.

Introduction

Matrix metalloproteinases (MMPs) are a family of Ca^{2+}- and Zn^{2+}-dependent enzymes that play a central role in many physiological processes, in tissue morphogenesis, tissue repair and angiogenesis. MMPs also have important functions in pathologic conditions by excessive degradation of ECM, such as rheumatoid arthritis, osteoarthritis, periodontitis, autoimmune blistering disorders of the skin and in tumor invasion and metastasis.[1–4] In addition, MMPs have been considered to be potential target molecules for a new class of antitumor agents.[5] A number of MMP inhibitors have been developed and tested in clinical trials for cancer therapy.[6] With the increasing of interest for identifying of prognostic markers and therapeutic targets against MMP-related disease, there has been much effort to develop analytical methods of MMP activity and its inhibitory effect by various inhibitors.[1] In early studies, the proteolytic activity of MMPs has been predominantly determined by zymography and ELISA,[7–9] but these assays are complex and time-consuming.[10] Recently, film *in situ* zymography has been applied not only to analyze MMP activity but to evaluate the inhibitory effects of MMP inhibitors.[6] In addition, fluorescence resonance energy transfer (FRET)-based approaches to detect the proteolytic activity of MMPs have been developed.[11–13] FRET is based on fluorescence quenching by energy transfer between fluorophore pairs allowing the detection of distance and orientation changes between a fluorescence donor and acceptor.[13] FRET-based techniques may be quite useful to investigate a variety of subcellular events, but the production of substrates is not easy since the efficiency of FRET depends on distance between the donor and acceptor.[13] FRET-based biosensors require complicated and costly chemical synthesis or genetic engineering of probes.[12] In addition, chemical labeling of substrates with various fluorophores may affect significantly enzyme activity.

As a non-labeling approach, SPR method is one of the favorite detection methods in protein array technology because it enables direct detection of molecular interactions without labeling or staining the proteins.[14] The principle of SPR biosensing is based on the changes in the refractive index on a thin metal surface induced by adsorption or desorption of molecules onto the metal. Thus, SPR biosensors are simple and can reduce non-specific interactions caused by conformational change of proteins induced by labeling with fluorophores or other probes. SPR biosensors can be classified into real-time SPR, SPR imaging, and SPR spectroscopy. The spectral SPR biosensor enables high-throughput analysis of molecular interactions in an array format and has more wide dynamic range than other SPR-based biosensors. It has been successfully applied for serodiagnosis of human diseases by seroconversion against the mumps virus and CRP based on immunoreactions.[15,16] Recently, an accumulating number of reports have demonstrated the potential applications of SPR biosensors for analysis of the activity of various enzymes secreted from cells or tissues.[17,18] However, most of these applications are based on changes of refractive index by binding of enzymes to substrates.[17,19] Only a few applications have been reported that analyze proteolytic activity of enzymes by using SPR imaging techniques.[20,21] Although the SPR imaging

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technique is sensitive to small variations around a resonance angle or wavelength, large signal variations cannot be analyzed.[22] To our knowledge, there is no report of an on-chip assay of proteolytic activity using an array-based spectral SPR biosensor.

In this paper, we present an array-based SPR biosensor system using gelatin arrays for a high-throughput analysis of MMP-3 activity. MMP-3 activity was determined by calculating the shift of SPR wavelength induced by the MMP-3-mediated degradation of the gelatin layer. A dose-dependent increase of gelatinolytic activity of MMP-3 was observed on gelatin arrays using the spectral SPR biosensor. This novel approach may have a strong potential for screening inhibitors, monitoring human diseases, and investigating cellular signaling mechanisms including MMPs, because this activity assay system is very simple, rapid, and label-free.

Experimental section

Chemicals and reagents

Octadecyltrichlorosilane, cyclohexane, 11-mercaptoundecanoic acid, carbon tetrachloride and type B gelatin were obtained from Sigma (St. Louis, MO). Brij-35 and Cy3 mono N-hydroxysuccinimide (NHS) ester were purchased from Pierce (Rockford, IL) and GE Healthcare (UK), respectively.

Preparation of functional catalytic domain of human MMP-3 (cMMP-3)

Catalytic domain (Phe^{100}-Pro^{273}) of human MMP-3 was expressed in *E. coli* as previously reported.[23] The insoluble pellet (inclusion bodies) of the cell lysate was refolded into a functional form of cMMP-3 by the procedures used for cMMP-14 preparation[24] with minor modifications. The pellet was extensively washed with an inclusion body washing buffer (50 mM Tris-HCl, pH 8.0, 2% Triton X-100, 2% sodium deoxycholate) and solubilized in a resuspension buffer (50 mM Tris-HCl, pH 8.5, 6 M urea, 30 mM 2-mercaptoethanol). Solubilized cMMP-3 was identified as a single band by SDS-PAGE (Fig. S1, ESI†). The cMMP-3 was diluted to 200 μg ml^{-1} with the resuspension buffer containing 150 mM 2-mercaptoethanol. The cMMP-3 was dialyzed against a reaction buffer without detergent (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$). The concentration of soluble cMMP-3 was quantified with Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Preparation of tissue inhibitors of metalloproteinase (TIMP)-1 secreted from fibroblast cells

TIMP-1 secreted from fibroblast cells was prepared as previously reported.[25] Briefly, human foreskin fibroblasts were maintained in DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 5% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Confluent fibroblast cells were incubated in serum-free medium for 5 days, and the supernatant was loaded onto a Q-Sepharose FF column (GE healthcare) equilibrated with 20 mM Tris-HCl, pH 7.4. The flow-through fraction was loaded onto a heparin-Sepharose FF column (GE healthcare) equilibrated with 20 mM Tris-HCl (pH 7.4), and bound protein was eluted with a linear gradient of 0–1 M NaCl. The fractions containing TIMP-1 were subjected to ultrafiltration with an Amicon YM-10 (Millipore), further purified with a Superdex G75 column (GE healthcare) equilibrated with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH7.4). The active fractions were pooled and dialyzed against the reaction buffer containing 0.001% Brij-35. Concentration of the fibroblast TIMP-1 was quantified with Micro BCA (bicinchoninic acid) Protein Assay Reagent Kit (Pierce).

Hydrophobic modification of gold arrays

Gold arrays were fabricated and modified chemically according to the procedures of Jung *et al.*[16] Briefly, gold arrays with 13 × 6 spots each of 2 mm diameter were fabricated by depositing Ti/Au (50/450 Å) films on pyrex glass, and were cleaned with a cleaning solution of $NH_4OH : H_2O_2 : H_2O$ (1 : 1 : 5, v/v) at 70 °C for 10 min. Then, hydrophobic glass surfaces were generated between gold spots according to a previous report[16] to prevent protein solutions from being mixed among the spots during incubation. The gold arrays were incubated with a mixture of hexadecane/carbon tetrachloride/octadecyltrichlorosilane (20 : 5 : 0.04, v/v) for 20 min, and washed with a mixture of hexadecane/carbon tetrachloride (20 : 5, v/v), carbon tetrachloride and ethanol in order.

MMP-3 activity assay on gelatin arrays

MMP-3 activity assay using a spectral SPR biosensor was performed on gelatin arrays as described in Fig. 1. Gold arrays were incubated with a mixed thiol solution of 0.1 mM 11-mercaptoundecanoic acid (MUA) and 0.9 mM 6-mercaptohexanol in ethanol for 16 h, and then washed with ethanol to remove the excess thiols.[26] The arrays were incubated with a mixture of 50 mM NHS and 200 mM N-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) for 10 min, washed with milli-Q water and incubated with 500 mM ethylenediamine (EDA) for 1 h. Amine-modified arrays were incubated with a gelatin solution (2 μl/spot), which was freshly prepared by dissolving 5 mg gelatin in 1 ml of phosphate buffer (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 7.4) and incubating at 37 °C for 2 h. For fabrication of gelatin arrays by covalent modification, the NHS/EDC-modified arrays (not incubated with EDA) were incubated with the gelatin solution at 37 °C for 2 h. The resulting gelatin arrays were washed with 0.1% Tween 20 in PBS and milli-Q water, and incubated at 37 °C for 1 h with reaction mixtures (2 μl/spot) containing various concentrations of cMMP-3 and detergents, including Brij-35, Tween 20 and Triton X-100, in the reaction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$). Then, the arrays were washed twice with 0.1% Tween 20 in phosphate buffered-saline for 5 min, rinsed with milli-Q water, flushed with N_2 gas to remove the water and immediately analyzed with a self-developed spectral SPR biosensor. Proteolytic activity of cMMP-3 was calculated using the following equation:

$$\Delta\lambda\ (\mathrm{SPR}) = \lambda_{\mathrm{gel}}\ (\mathrm{SPR}) - \lambda_{\mathrm{MMP}}\ (\mathrm{SPR})$$

where $\Delta\lambda$ (SPR) is the SPR wavelength shift, λ_{gel} (SPR) is the SPR wavelength of gelatin layers, and λ_{MMP} (SPR) is the SPR wavelength after incubation with MMP-3.

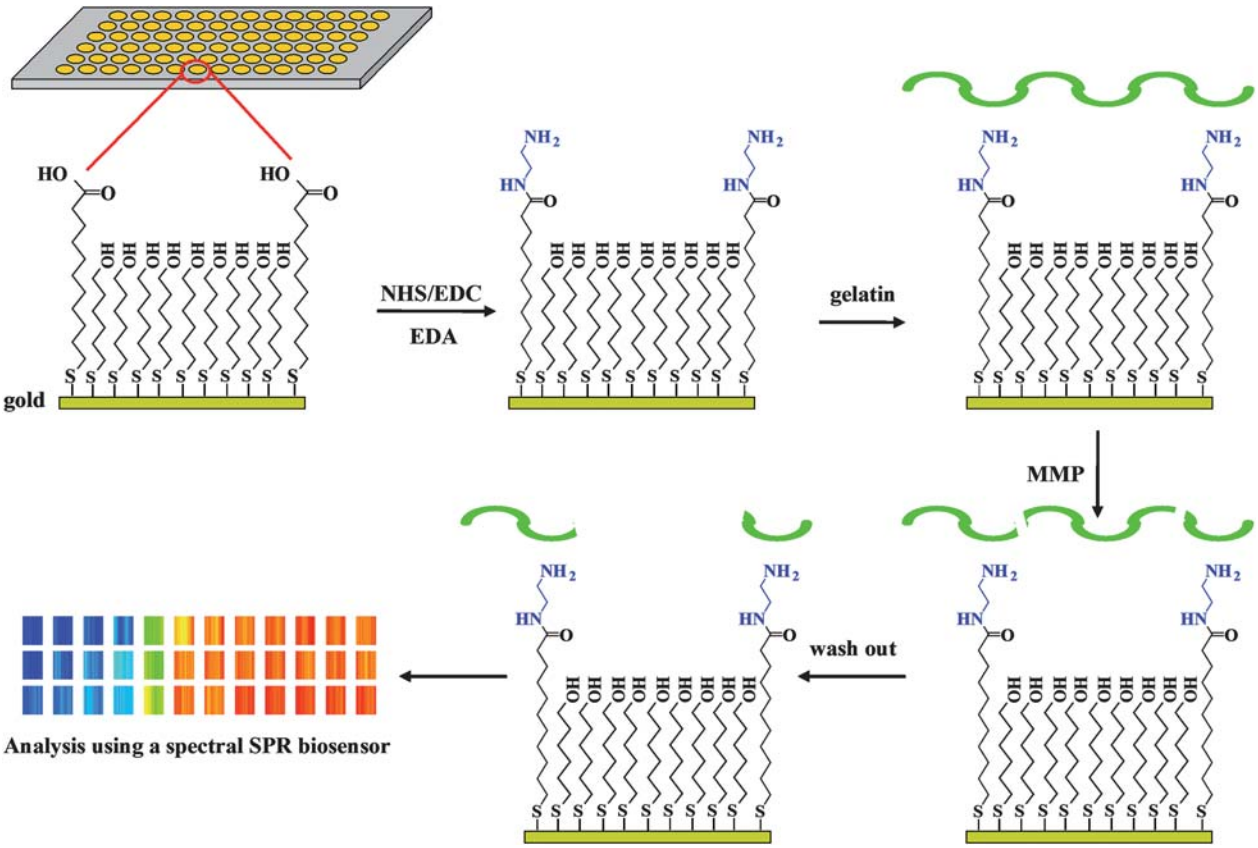

Fig. 1 Schematic diagram of MMP-3 activity assay on gelatin arrays using a spectral SPR biosensor.

Analysis of protein arrays with the line-scanning mode of a spectral SPR biosensor

The analysis of MMP activity on gelatin arrays was performed in the line-scanning mode of the spectral SPR biosensor according to a previous report.[26] Briefly, the spectral SPR sensor was configured using the Kretschmann geometry of the attenuated total reflection method. The arrays were coupled with a fused silica prism *via* index matching fluid and mounted on an x-y linear stage. Then, the array spots were automatically scanned every 100 μm along the central line by the line-scanning mode.

Analysis of gelatinolysis by MMP-3 using fluorescence arrays

Gelatin (2 mg ml^{-1}) was incubated at room temperature with 50 μg ml^{-1} Cy3-succinimidyl ester for 2 h, and the resulting Cy3-conjugated gelatin was purified with a 2.5 ml Sephadex G25 column. Cy3-gelatin (0.4 mg ml^{-1}) was immobilized onto the amine surface of well type arrays,[27] and the gelatin arrays were incubated at 37 °C with 10 μg ml^{-1} cMMP-3 in the reaction buffer (1 μl/spot) for various times. Then, the arrays were analyzed with a fluorescence scanner (ScanArray Gx, PerkinElmer).

Atomic force microscope imaging

Surface topology of gelatin layers before and after treatment of cMMP-3 was analyzed by AFM. AFM imaging was performed under air using a Nanoscope IIIa with an E type scanner (12 × 12 μm) (Digital Instruments, USA) in the contact mode. Cantilevers with Si_3N_4 oxide-sharpened tips (spring constant: 0.12 N m^{-1}) were used for the imaging. The applied force was varied from several to tens of nN. The film thickness of the gelatin layers was estimated by measuring the depth of an artificial hole that was made by puncturing the layer surface with the contact mode.

Results

Optimization of MMP-3 activity assay using the array-based SPR biosensor

For a very simple and sensitive analysis of MMP-3 activity in a high-throughput manner, we developed a novel assay system using gelatin arrays as a substrate of MMP-3, as illustrated in Fig. 1. Gelatin arrays were incubated with reaction mixtures containing cMMP-3, and analyzed in the line-scanning mode of the spectral SPR biosensor. MMP-3 activity was determined by calculating the net shifts of SPR wavelength obtained from the analysis. To determine the optimal concentration of gelatin for fabricating protein arrays, various concentrations of gelatin were applied to amine-modified gold arrays. As shown in Fig. 2A, the SPR wavelength shift, which represents the amount of bound gelatin to gold arrays, increased in a dose-dependent manner and reached saturation at 5 mg ml^{-1}. We then applied cMMP-3 (50 μg ml^{-1}) to gelatin arrays formed by immobilizing various concentrations of gelatin and the arrays were analyzed with the

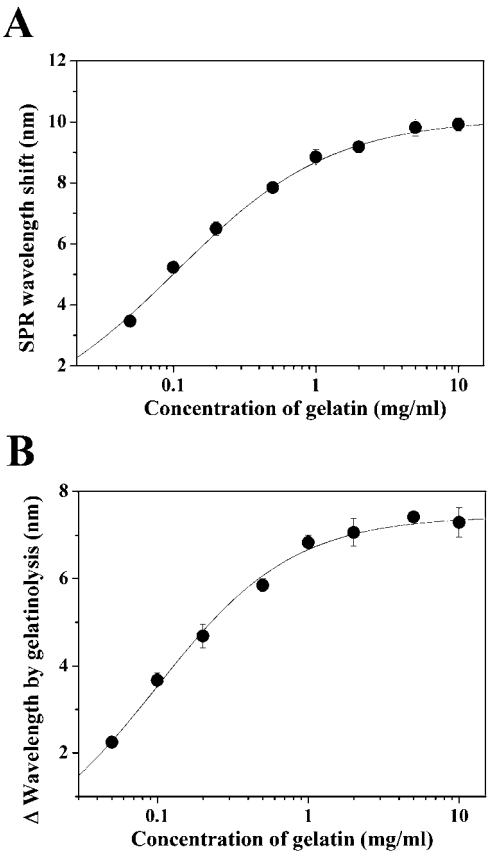

Fig. 2 Optimization of gelatin arrays for MMP-3 activity assay using the spectral SPR biosensor The indicated concentrations of gelatin were immobilized onto the amine-modified gold arrays (A), and the resulting gelatin arrays were incubated at 37 °C with 50 μg ml^{-1} cMMP-3 in the reaction buffer (B). Then, the arrays were analyzed with the spectral SPR biosensor as described in Materials and Methods. The results are expressed as means ± S.D. from three separate experiments.

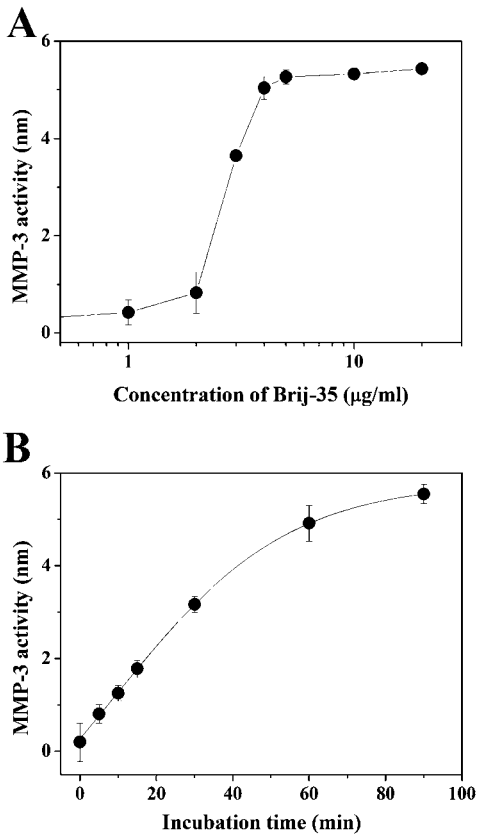

Fig. 3 Effect of Brij-35 concentration (A) and incubation time (B) on MMP-3 activity assay with the spectral SPR biosensor Reaction mixtures containing 10 μg ml^{-1} cMMP-3 were applied at 37 °C to gelatin arrays for 60 min in the presence of the indicated concentration of Brij-35 (A) or for the indicated time in the presence of 10 μg ml^{-1} Brij-35 (B). The resulting arrays were analyzed as described in Materials and Methods. The results are expressed as means ± S.D. from three separate experiments.

SPR biosensor. As shown in Fig. 2B, the wavelength shift induced by the proteolysis was dose-dependent, with the maximal shift observed when the arrays were coated with gelatin at 5 mg ml^{-1}. These results showed that 5 mg ml^{-1} gelatin was the optimal concentration in fabricating gelatin arrays for MMP-3 activity assay using the SPR biosensor.

It is known that Brij-35 is required for MMP-3 activity.[28] Thus, we determined the optimal concentration of Brij-35 for measuring gelatinolytic activity of MMP-3 using the spectral SPR biosensor. Reaction mixtures containing various concentrations of Brij-35 were applied to gelatin arrays and the resulting arrays were analyzed by the spectral SPR biosensor. MMP-3 activity was determined by calculation of the SPR wavelength shift of each array spot obtained from the line-scanning analysis. As shown in Fig. 3A, the gelatinolytic activity showed a dose-dependent increase, and it was saturated at 10 μg ml^{-1} Brij-35. In the absence of Brij-35, MMP-3 activity was nearly undetectable (0.11 ± 0.28 nm wavelength shift), however, there was a sudden rise in MMP-3 activity in the range from 1 to 5 μg ml^{-1} Brij-35. These results indicated that Brij-35 was essential for MMP-3 activity assay and the optimal concentration of Brij-35 was 10 μg ml^{-1} for measuring MMP-3 activity in the array format.

We then determined changes of proteolytic activity of MMP-3 on gelatin arrays according to incubation time. As shown in Fig. 3B, MMP-3 proteolytic activity increased in a time-dependent manner, and incubation time of 60 min was demonstrated to be sufficient to determine MMP-3 activity.

Verification of gelatinolytic activity of MMP-3 with SPR spectroscopy, AFM imaging and fluorescence arrays

Binding of a gelatin layer onto amine-modified gold arrays and proteolysis of bound gelatin were investigated by SPR spectroscopy using the spectral SPR biosensor. The binding of molecules to the gold surface causes the SPR spectrum to increase.[14] As shown in Fig. 4A, immobilization of gelatin caused an increase in the SPR spectrum, whereas incubation with MMP-3 decreased the spectrum. These results suggested that a gelatin layer was fabricated on the gold surface and then digested with cMMP-3.

The digestion of gelatin layers with cMMP-3 was confirmed by measuring the thickness of gelatin layers with AFM imaging before and after treatment with cMMP-3, according to the previous report.[16] We initially imaged the layer of N-(2-aminoethyl)-11-mercaptoundecanamide and 6-mercaptohexanol on the

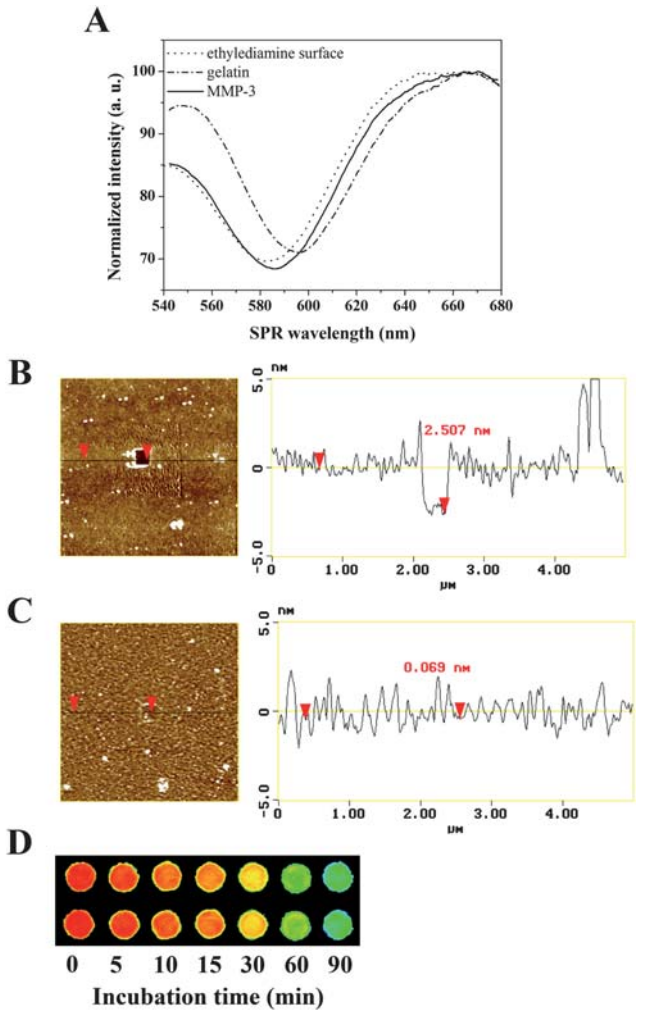

Fig. 4 Verification of gelatinolytic activity of MMP-3 by SPR spectroscopy, AFM imaging and fluorescence arrays (A) Shift of SPR spectra caused by successive binding of a gelatin layer and its hydrolysis with cMMP-3. (B,C) Film thickness of gelatin layers was measured before (B) and after (C) treatment of cMMP-3 by the contact mode of AFM as described in Materials and Methods. (D) Cy3-conjugated gelatin was immobilized onto the amine surface of well-type arrays, and then reaction mixtures containing 10 μg ml^{-1} cMMP-3 were incubated at 37 °C for the indicated times. The resulting arrays were analyzed with a fluorescence scanner.

gold surface. The surface was uniform; however, it was not possible to make any scratch on this layer by AFM. This result indicated that the mixed thiol layer would be a basal point in measuring film thickness of gelatin layers. Then, we determined the thickness of gelatin layer formed on the amine surface by scanning the artificial hole, made by puncturing the layer surface with the contact mode of AFM. As shown in Fig. 4B, the thickness of the gelatin layer was approximately 2.5 nm, whereas that after treatment with cMMP-3 decreased to be less than 0.1 nm (Fig. 4C). These results demonstrated that the gelatin layer was digested with MMP-3 activity, and confirmed the previous finding, the shift of SPR wavelength by MMP-3.

The degradation of gelatin layers by cMMP-3 was further confirmed using fluorescence-based gelatin arrays. The gelatin arrays were prepared by immobilizing Cy3-congugatd gelatin onto the amine surface. As shown in Fig. 4D, incubation with cMMP-3 decreased fluorescence intensity in an incubation time-dependent manner, demonstrating proteolytic digestion of the Cy3-conjugated gelatin layer by cMMP-3. Taken together, the gelatin layer on gold arrays was digested by proteolytic activity of MMP-3, which caused the shift of SPR wavelength.

Characterization of gelatin arrays with detergents for MMP-3 activity assay

Since Brij-35 was essential for MMP-3 activity assay using gelatin arrays (Fig. 3A), we investigated whether Brij-35 was more effective than other non-ionic detergents in activation of MMP-3. To test that, we determined MMP-3 activity in the presence of three non ionic detergents, Brij-35, Tween 20 and Triton X-100. As shown in Fig. 5A, the three detergents increased MMP-3 activity in similar dynamic ranges. Tween 20 and Triton X-100 showed similar dynamic ranges of MMP-3 activity; from 4 to 20 μg ml^{-1} and from 4 to 10 μg ml^{-1},

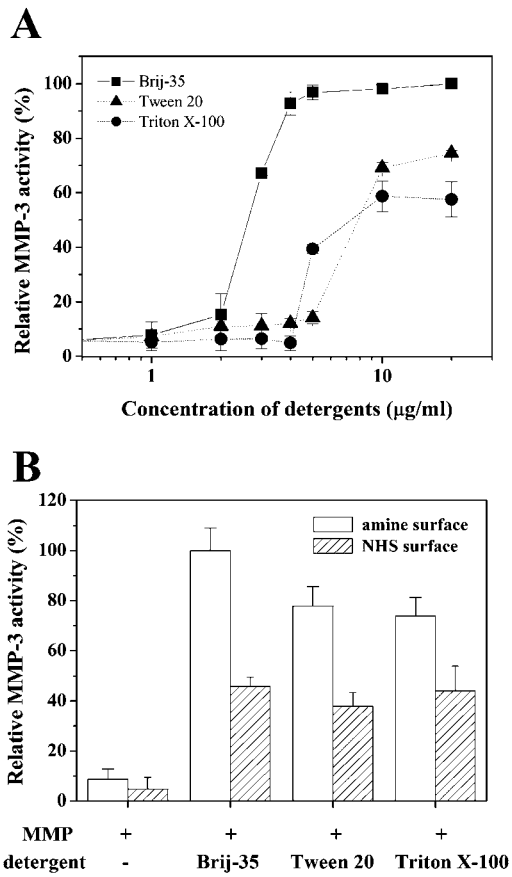

Fig. 5 Effect of detergents and gelatin modification on MMP-3 activity assay with the spectral SPR biosensor (A) Reaction mixtures containing 10 μg ml^{-1} cMMP-3 and indicated concentrations of Tween 20, Triton X-100 and Brij-35 were applied to gelatin arrays at 37 °C for 60 min. (B) Reaction mixtures containing 10 μg ml^{-1} cMMP-3, and 10 μg ml^{-1} Brij-35, Tween 20 or Triton X-100 were applied to gelatin arrays fabricated on amine surface or N-hydroxysuccinimide ester (NHS) surface for 60 min. The resulting arrays were analyzed as described in Materials and Methods. The results are expressed as means ± S.D. from three separate experiments.

respectively, which were slightly higher than dynamic range of MMP-3 activity (from 1 to 5 μg ml^{-1}) with Brij-35. However, maximal activity of MMP-3 with Brij-35 was lower than those with Tween 20 and TX-100. Thus, three detergents showed similarly short dynamic ranges of MMP-3 activity on gelatin arrays, and Brij-35 was most effective among the non-ionic detergents in activation of MMP-3.

We then performed MMP-3 activity assay using gelatin arrays fabricated by two different approaches, physical adsorption (amine surface) and covalent modification (NHS surface). In the presence of Brij-35, MMP-3 activity on the NHS surface was less than half of that on the amine surface (Fig. 5B), indicating that gelatin layers fabricated by physical adsorption were more suitable for the array-based MMP-3 activity assay. In the presence of Tween 20 and Triton X-100, decreased activities of MMP-3 were also observed with gelatin arrays fabricated by covalent modification. However, in the absence of detergent, MMP-3 activity was very low on both surfaces. These results demonstrated that gelatin arrays physically adsorbed onto the amine surface were appropriate for MMP-3 activity assay.

Dose-dependent MMP-3 activity and its inhibition by TIMP-1 and GM6001

To analyze MMP-3 activity in a high-throughput manner, we applied various concentrations of cMMP-3 onto gelatin arrays, and the arrays were analyzed by the line-scanning mode of the spectral SPR biosensor. The MMP-3 activity is shown as color spectra from blue to red (Fig. 6A), which were constructed by the shift of SPR wavelength. As shown in Fig. 6B, gelatinolytic activity of MMP-3 was dose-dependent with the evident increase in gelatin digestion at 50 ng ml^{-1} cMMP-3 ($p < 0.01$), and almost saturated at 20 μg ml^{-1} cMMP-3. This result showed that detection range of this array-based SPR biosensor is from 50 ng ml^{-1} to 20 μg ml^{-1} cMMP-3. Further, this demonstrated that gelatin arrays were appropriate for a rapid analysis of proteolytic activity of MMP-3.

We performed further MMP-3 activity assay with fluorescence-based gelatin arrays, fabricated by immobilizing Cy3-conjugated gelatin on the amine surface. In this assay, MMP-3 activity was determined by measuring decrease in fluorescence intensity. As shown in Fig. 6A and 6C, MMP-3 activity was dose-dependent with the evident increase at 500 ng ml^{-1} ($p < 0.05$) cMMP-3. These results indicated that intact gelatin arrays based on the array-based SPR biosensor was more sensitive for a high-throughput analysis of MMP-3 activity than fluorescence-conjugated gelatin arrays.

We then investigated inhibitory effects of TIMP-1 and GM6001, MMP inhibitors, on MMP-3 activity using the array system. Various concentrations of TIMP-1 and GM6001, mixed with 25 nM (0.5 μg ml^{-1}) cMMP-3, were applied to gelatin arrays and the resulting arrays were analyzed by the line-scanning mode (Fig. 7). TIMP-1 and GM6001 inhibited MMP-3 in a dose-dependent manner. These results demonstrated that gelatin arrays based on a spectral SPR biosensor were appropriate for a rapid analysis of proteolytic activity of MMP-3 and MMP-3 inhibitors.

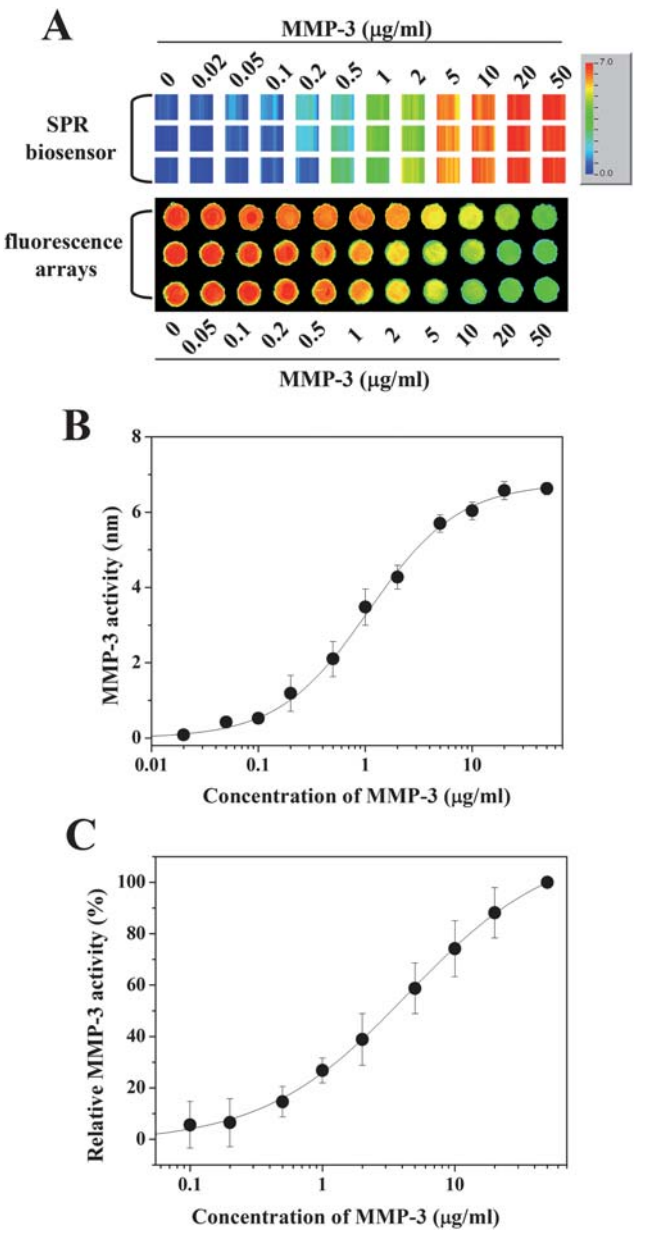

Fig. 6 Analysis of dose-dependent MMP-3 activity using intact gelatin arrays or Cy3-conjugated gelatin arrays. Reaction mixtures containing the indicated concentrations of cMMP-3 were applied at 37 °C to intact gelatin arrays or Cy3-conjugated gelatin arrays in the presence of Brij-35 for 60 min. Then, arrays were analyzed in the line-scanning mode of the spectral SPR biosensor or a fluorescence scanner as described in Materials and Methods (A). Dose-dependent activities of MMP-3 on intact gelatin arrays (B) and Cy3-conjugated gelatin arrays (C) were expressed as means ± S.D. from three separate experiments.

Discussion

In this report, we present a novel approach to the high-throughput analysis of MMP-3 activity using native gelatin substrate and an array-based spectral SPR biosensor. MMPs are Ca^{2+}-and Zn^{2+}-dependent enzymes and excessive or inappropriate expression of MMPs may contribute to pathogenesis, including rheumatoid arthritis, tumor invasion, metastasis, and central nervous system diseases.[28] Recently, there has been a great demand for novel high-throughput methods to analyze MMP activity, because such methods will be crucial for identifying prognostic markers and therapeutic targets for

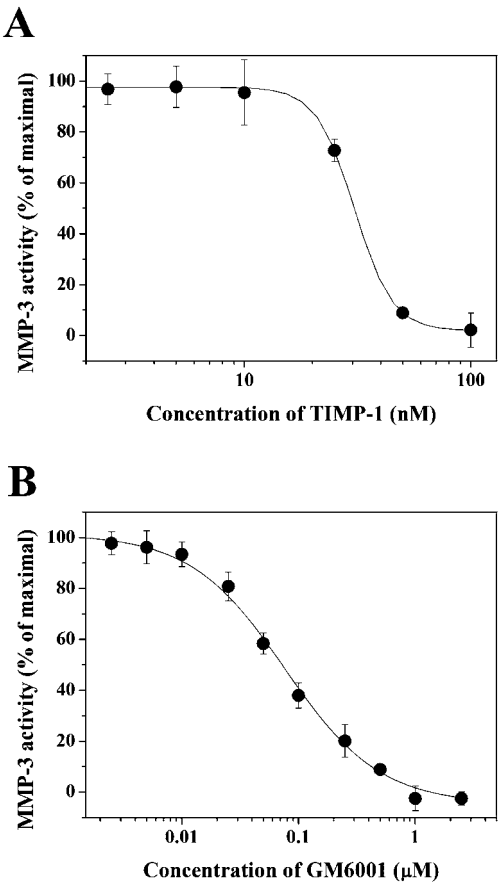

Fig. 7 Inhibition of MMP-3 activity by TIMP-1 and GM6001 on gelatin arrays Reaction mixtures containing the indicated concentrations of TIMP-1 or GM6001 in 0.5 μg ml^{-1} cMMP-3 were applied at 37 °C to gelatin arrays in the presence of Brij-35 for 60 min. Then, arrays were analyzed in the line-scanning mode of the spectral SPR biosensor as described in Materials and Methods. MMP-3 activities (% of maximal) in the presence of TIMP-1 (A) and GM6001 (B) were expressed as means ± S.D. from three separate experiments.

MMP-related diseases.[1] In early studies, the enzyme activity of MMPs was generally determined by zymography and chromatography.[12] Recently, fluorescence-based techniques including FRET have been developed that improved the sensitivity of the analysis and allowed for rapid analysis. However, fluorescence-based techniques require additional procedures for chemical or genetic engineering of probes, which is complex and expensive.[12] Our new methodology utilizes intact gelatin arrays, which were prepared on amine-terminated surface by physical adsorption, for high-throughput analysis of MMP-3 activity.

In general, MMPs contain a zinc-coordinated catalytic domain, and a low concentration of Brij-35 was used to improve the refolding of the catalytic domain and retain activity.[28] In this study, we observed that MMP-3 had negligible gelatinolytic activity in the absence of Brij-35 (Fig. 3A). As previously reported, the gelatinolytic activity of MMP-3 was dependent on the concentration of Brij-35, and MMP-3 activity increased substantially with addition of 2–5 μg ml^{-1} Brij-35 (Fig. 5). MMP-3 activity also rapidly elevated in short dynamic ranges of Tween 20 and Triton X-100. These results implied that detergents may contribute to catalytic reaction of MMP-3 using two-dimensional gelatin substrates. However, maximal activity of MMP-3 with Brij-35 was higher than that with Tween 20 or Triton X-100. These results demonstrated that Brij-35 is appropriate and essential for determination of gelatinolytic activity of MMP-3 with the array-based spectral SPR biosensor.

Covalent immobilization of ligand molecules onto surface is very common strategy in protein array technologies. Covalent immobilization is generally used to analyze various molecular interactions on array because it provides very reliable reactions between array surface and ligand molecules. However, covalent modification of enzyme substrates such as covalent immobilization or fluorescence labeling of substrates may affect enzyme activity. MMP-3 activities determined using gelatin layers fabricated by covalent modification were 46∼60% of those determined on gelatin layers prepared by physical adsorption. We assumed that covalent modification may affect the cleavage sites of gelatin, or covalently bound gelatin molecules may be not detached from gold arrays after being cleaved by MMP-3. In addition, the array-based SPR biosensor using intact gelatin arrays showed higher sensitivity than fluorescence-based protein arrays using Cy3-conjugated gelatin. Thus, MMP-3 activity might be affected by covalent modification of gelatin layers.

In this paper, we determined MMP-3 activity by calculating the net shift of SPR wavelength caused by MMP-3 digestion of the array-conjugated gelatin using the line-scanning mode of the spectral SPR biosensor. Proteolytic degradation of gelatin layers with MMP-3 was verified by SPR spectroscopy, AFM imaging and fluorescence-based protein array approach. Spectroscopy using the spectral SPR biosensor clearly demonstrated the backward shift of SPR spectrum by incubation with MMP-3. The proteolysis of gelatin layers with MMP-3 was confirmed by directly determining the film thickness of gelatin layer with AFM. The thickness of non-treated gelatin layer was approximately 2.5 nm, whereas that of MMP-3-treated gelatin layer decreased to the basal level (Fig. 4B and 4C). Although an artificial hole was made on the MMP-3-treated gelatin layer, the thickness was too thin to measure. The shift of SPR spectrum by MMP-3 treatment was well correlated with the decrease of film thickness of the gelatin layer after treatment with cMMP-3. The degradation of gelatin layers by MMP-3 was further supported by experiments with Cy3-conjugated gelatin arrays. These results demonstrated that the gelatin layer on arrays was degraded by MMP-3.

Native gelatin arrays based on the spectral SPR biosensor were successfully applied to investigate the inhibitory effect of TIMP-1 and GM6001, MMP inhibitors, on MMP-3 activity. With 25 nM (0.5 μg ml^{-1}) cMMP-3, half maximal inhibitory concentrations of TIMP-1 and GM6001 were 30.4 and 71.5 nm, respectively, consistent with previous reports[29,30] demonstrated that TIMP-1 has a higher affinity to MMP-3 than GM6001. A similar inhibitory concentration of GM6001 (88.7 nM) was also obtained with additional inhibitor studies using 10 nM cMMP-3 and a fluorescence peptide substrate in solution rather than the array surface (data not shown). Thus, these gelatin arrays have a strong potential for a rapid analysis of various inhibitors, although it is necessary to establish an optimal assay condition.

In summary, we developed an array-based SPR biosensor using intact gelatin arrays to analyze MMP-3 activity in

a high-throughput manner. This system was optimized and successfully applied to the rapid analysis of dose-dependent MMP-3 activity and of inhibitory effects of TIMP-1 and GM6001.

Conclusion

In this paper, we developed a native gelatin array-based assay system using a spectral SPR biosensor to analyze MMP-3 activity in a high-throughput manner. This novel approach is simple, label-free and rapid, and can be applied to any proteolytic enzymes along with substrate arrays as well as other gelatin-degrading MMPs. This system thus has a strong potential for the use in inhibitor screening and activity-based proteomics by determination of net MMP activity.

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