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Aqueous zymography screening of matrix metalloproteinase activity and inhibition based on colorimetric gold nanoparticles

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ABSTRACT

An optical gold nanoparticles (AuNPs)-based method was fabricated for the rapid detection of matrix metalloproteinase (MMP) activity and screening potential MMP inhibitors without sophisticated instruments. The diagnosis platform was composed of AuNPs, particular MMP substrates and 6-mercapto-1-hexanol (MCH). The functionalized AuNPs were subjected to specific MMP digestion, and the MMP found the substrate on AuNPs, such that the AuNPs lost shelter and MCH increased the attraction force between AuNPs. Consequently, AuNPs aggregation and a color change from red to purple with increasing MMP concentration were observed. The surface plasmon resonance (SPR) of the formed AuNPs allowed for the quantitative detection of MMP activity. A sensitive linear correlation existed between the absorbance and the activity of the MMPs, which ranged from 10 ng/mL to 700 ng/mL in NTTC buffer and plasma samples. The proposed colorimetric method could be accomplished in a homogeneous solution with one-step operation in 30 min and has been successfully applied to the determination of particular MMP activity in plasma samples, in which the results are consistent with substrate zymography. This technology may become a simple platform for parallel screening a number of inhibitors and offer an alternative method to studying the efficiency of inhibitors for suppressing MMP activity. The absorbance ratio at 625 nm and 525 nm (A_{625}/A_{525}) confirmed the efficiency of the inhibitors as observed in substrate zymography. The IC_{50} of ONO-4817 and galardin for MMP-1, MMP-2 and MMP-7 determined by the proposed colorimetric method was similar to the results of substrate zymography.

1. Introduction

Enzymes analytical detection is a key tool in enzymology, extremely important for the quality and quantity of enzyme activity. Among enzymes, matrix metalloproteinases (MMPs), a family of zinc-dependent endoproteinases secreted by both normal and transformed cells. Most MMPs are synthesized as inactive zymogens and must be enzymatically activated to become active. Furthermore, the in vivo activity of MMPs is regulated by their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs play an important role in both normal and pathological processes, and a deregulation of the balance between MMPs and TIMPs is a characteristic of diverse pathological conditions, such as cardiac disease and cancer (Schulz, 2007; Lin et al., 2009; Kupai et al., 2010). Therefore, analyzing MMPs and TIMPs in pathological conditions can serve as a benchmark for reliable outcomes in clinical trials. In recent years, the standard assays that are generally used for detecting MMP activity include substrate zymography (Gerlach et al., 2007; Chen et al., 2008), enzyme immunoassay (Takahashi et al., 2004; Kanesaka et al., 2006), and fluorogenic substrates (Faust et al., 2008; Zhang et al., 2008; Yi et al., 2010). One of the widely used techniques for the detection of MMP activity is substrate zymography. In zymography, proteins are separated by electrophoresis under denaturing, non-reducing conditions. This process may also be used to separate MMPs from TIMPs. The activity of the MMPs is revealed by an absence of protein staining in the region where the substrate has been digested. However, there are still many problems in using zymography for clinical diagnosis: (1) the two-step staining/destaining method is not reliable and is difficult to reproduce, and (2) a majority of MMPs are secreted as latent proenzymes interacting with the TIMPs. In addition, proteases rarely act alone but function in a "proteinase web"; pathologic conditions are always associated with the abnormal expression of more than one MMP. As previously mentioned, applying substrate zymography to analyze MMPs in clinical samples is not sufficient; therefore, the development of a parallel, rapid, cost-effective method for MMP analysis is extremely urgent.

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Recently, gold nanoparticles (AuNPs) have received great attention in the development of a visual sensing method for clinical diagnosis (Chen et al., 2009a,b; Griffin et al., 2009). The use of AuNPs as a colorimetric reporter relies on their unique surface plasmon resonance (SPR), with the colors red and blue corresponding to their dispersion or aggregation state, respectively. Based on this principle, several colorimetric assays have been developed for the detection of DNA (Chen et al., 2009a,b; Mao et al., 2009; Vlachou et al., 2010), proteins (Gupta et al., 2010; Bonomi et al., 2011), ions (Jiang et al., 2010; Li et al., 2010) and cancerous cells (Kang et al., 2010; Lu et al., 2010).

In our previous study, an optical AuNPs method was established and applied to gelatinase activity analysis. Accordingly, we established the AuNPs platform as aqueous zymography and utilized substitute engrafted substrates onto AuNPs, rather than polyacrylamide gel, to assay blood MMPs. As mentioned in our previous research (Chuang et al., 2010), proteins can be grafted onto the surfaces of colloidal AuNPs and retain their native structure; therefore, MMPs could recognize specific structure and unwind protein conformation, and then degrade their substrates, which provides a more correct way to analyze proteinase activity. MMP that digest grafted substrate would disrupt the stability of the AuNPs and result in a change in the SPR properties of the AuNPs; therefore, the MMP activity could be determined by examining the SPR properties. On the basis of these advantages, an optical AuNPs platform could allow for performing rapid and numerous assays to detect MMP activities and MMP inhibitors within quite a short time and with a high-throughput.

2. Materials and methods

2.1. Chemicals

Sodium citrate was obtained from Merck (Darmstadt, Germany). Tris–HCl was purchased from Chemicon (Invitrogen, San Diego, LA, USA). Agarose and 10× Tris–Borate–EDTA buffer were purchased from Amresco (Cleveland, OH, USA). Recombinant MMP-1, -2 and -7, sodium chloride (NaCl), calcium chloride ($CaCl_2$), 6-mercaptohexan-1-ol (MCH), trisodium citrate ($Na_3C_6H_5O_7$), Triton X-100, Tris–HCl, type A gelatin, casein, hydrogen tetrachloroaurate (III) ($HAuCl_4{\cdot}3H_2O$), and p-aminophenylmercuric acetate (APMA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Type I collagen was purchased from Advanced BioMatrix (San Diego, CA, USA), and twice-distilled water was obtained through a Milli-Q system (18 MΩ cm; Millipore, Bedford, MA, USA). ONO-4817 ($C_{22}H_{28}N_2O_6$) was purchased from Tocris (Ellisville, MO, USA), and Galardin (Ilomastat, GM6001; $C_{20}H_{28}N_4O_4$) was purchased from USBiological (Swampscott, MA, USA).

2.2. Synthesis and modification of AuNPs

Approximately 13 nm diameter AuNPs were prepared by citrate reduction of $HAuCl_4{\cdot}3H_2O$ according to the reported procedure. An aqueous solution of $HAuCl_4{\cdot}3H_2O$ (50 mL, 1 mM) was brought to a vigorous boil with stirring in a conical flask, and then 5 mL of 38.8 mM $Na_3C_6H_5O_7$ solution was added rapidly. The solution was boiled for another 15 min, during which time its color changed from pale yellow to deep red. The solution was cooled to room temperature with continuous stirring. The sizes of the AuNPs were verified by dynamic light scattering (DLS) (BI-200SM; Brookhaven Instruments, Holtsville, NY, USA) and zeta potential (Delsa Nano C; Beckman Coulter, LA, USA). Citrate-stabilized AuNPs appeared to be nearly monodisperse, with an average size of 13 ± 1.2 nm. A UV–vis absorption spectrophotometer (Apices Scientific, Boston, MA, USA) was used to measure the absorbance of the AuNPs in citrate solution (Chen et al., 2009a,b).

2.3. Modification of AuNPs/MCH–substrate

Before the modified processes, the synthesized AuNPs were analyzed by a UV–vis absorption spectrophotometer; the particle concentrations were according to Beer's law using an extinction coefficient ($\varepsilon_{spheres}$, 520 nm = 0.344/nmol cm), and the particles were modulated to a concentration to 5 nM. The substrate and MCH were modified onto the AuNP surfaces according to the following procedures. For the preparation of AuNPs/MCH–gelatin and AuNPs/MCH–casein, an aliquot of the aqueous AuNPs solution (950 μL) was mixed with an aqueous native substrate (0.1%, 50 μL) and incubated at 37 °C for 2 h. Then 1 mM MCH (10 μL for AuNPs/MCH–gelatin and 5 μL for AuNPs/MCH–casein) was added to the solution to block the surface space to avoid peptide absorption on the AuNPs and increase the attraction among the AuNPs, the mixture was incubated at 37 °C for another 2 h. The mixture was then centrifuged for 6 min at 12,000 × *g* to remove the excess substrate and MCH. After removal of the supernatants, the AuNPs/MCH–substrate colloids were resuspended in 200 μL MMPs reaction buffer, NTTC buffer (50 mM NaCl, 50 mM Tris–HCl, 5 mM $CaCl_2$ and 0.05% triton X-100, pH 7.5). The concentration of the modified AuNPs was adjusted to 5 nM for further use in the proteinase activity assay. In contrast to gelatin and casein, type I collagen fibroses at pH < 7 such that the AuNPs would be encapsulated by collagen. To keep the structure of type I collagen, the environment of bare AuNPs should be trimmed to weak acid. To prepare the AuNPs/MCH–collagen, first, the aqueous AuNPs solution (950 μL) was mixed with 0.1 N HCl to adjust the pH to a weak acid. Afterward, an aqueous native of type I collagen (2.5 mg/mL, 50 μL) was added and incubated at 37 °C for 2 h. Then 5 μL of 1 mM MCH was added to the solution, and it incubated at 37 °C for another 2 h. The mixture was then centrifuged for 6 min at 11,000 × *g* to remove the excess substrate and MCH. After the centrifuge/wash cycle, the AuNPs/MCH–collagen colloids were resuspended in 200 μL NTTC buffer.

2.4. Electrophoresis analysis of substrate-modified AuNPs

Gel electrophoresis analysis modified from Hanauer's protocol (Hanauer et al., 2007) was used to check the change in diameter and charge of substrate-modified AuNPs. Agarose gels were prepared and immersed in 0.5× TBE buffer (Tris–Borate–EDTA buffer, prepared by diluting 10× stock solutions). The gels were run in a horizontal electrophoresis system (Mini-Sub Cell GT; Bio-rad, Corston, UK) for 30 min at 110 V in 0.5× TBE buffer. After electrophoresis, gel images were taken by a digital camera and processed with only small linear contrast adjustments to obtain a true representation of the visual gel appearance.

2.5. MMP activity assay by substrate-modified AuNPs

The AuNPs/MCH–substrate were used to evaluate the enzymatic activity of MMPs. In a typical experiment, 50 μL of known concentration MMP were added to a solution containing 200 μL of AuNPs/MCH–substrate, and the mixture was incubated at 37 °C. All of the analysis of the MMP activities were carried out by measuring the absorbance of the solution with a UV–vis absorption spectrophotometer and shown in Fig. S1. While MMP-2 digested the substrate of AuNPs/MCH–gelatin, the absorption band around 525 nm decreased gradually, and concomitantly, a new broad absorption above 625 nm emerged and its intensity increased by prolonging the reaction time, and at 60 min reached the a maximum. However, while MMP digested AuNPs/MCH–gelatin over

60 min, dramatic particles aggregation would interfere colorimetric detection. Therefore, we controlled the reaction time within 60 min and recorded spectral profiles and calculated the ratios of absorbance at 625 nm and 525 nm (A_{625}/A_{525}) after a 60 min reaction time.

2.6. MMP activity assay by zymography

The MMPs activity assay was also performed according to our previously described zymography method. Briefly, MMPs were activated by APMA and mixed with 2× zymography sample buffer (0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 0.005% bromophenol blue), incubated for 10 min at room temperature, and then loaded into each lane of a SDS-PAGE (10%) gel containing 0.1 mg/mL gelatin. After electrophoresis, the gel was washed twice for 30 min in zymogram renaturing buffer (2.5% Triton X-100) with gentle agitation at room temperature to remove SDS and incubated at 37 °C for 8 h in reaction buffer (50 mM Tris–HCl pH 7.4, 200 mM NaCl, and 5 mM $CaCl_2$). After staining with Coomassie brilliant blue, MMP activity was identified as clear zones against blue background. In this study, zymography was used as the standard method to confirm the reliability of the optical AuNPs-based platform for the determination of the MMP samples.

2.7. Detection of MMPs in plasma samples

In order to evaluate the potential application of our optical AuNPs-based method, known concentrations of MMPs (including MMP-1, MMP-2, and MMP-7) were added to plasma to test the performance of the optical AuNPs-based assay in plasma. Blood samples from three healthy male volunteers were simultaneously collected into heparin coated plastic tubes for preparation of plasma samples. The tubes were centrifuged within 10 min after centrifuged at 1600 × *g* for 15 min at 4 °C. The supernatants were stored at −80 °C until analysis. To imitate the diagnosis of a blood sample, a well-known dose of MMP were mixed into a plasma sample, and each sample contained 0, 10, 50, 100, 200, 300, 400, 500, 600, or 700 ng/mL of additive MMPs. The mean levels of MMP-1, MMP-2 and MMP-7 in normal/cancer patient plasma range from 10 ng/mL to 500 ng/mL (order of appearance reflects their relative concentration) (Jung et al., 1996; Kai et al., 1998; Kim et al., 2008).

2.8. Assay for the efficiency of MMP inhibitors by an optical AuNPs-based method

For a drug screening assay of MMPs, the procedure was similar to the proteinase activity assay. Two well-known MMP inhibitors were choose in this study, the K_i values of ONO-4817 for each MMP were obtained as follows: MMP-2 (0.73 nM), MMP-7 (2500 nM) and MMP-1 (1600 nM); the K_i values of Galardin for each MMP were obtained as follows: MMP-1 (0.4 nM), MMP-2 (0.5 nM) and MMP-7 (41 nM).

To study the inhibition effect of MMP inhibitors, a total volume of 50 μL of the reaction mixture containing MMP (50 ng/mL) and AuNPs/MCH–substrate (5 nM) in the absence and presence of different concentrations of inhibitor was incubated at 37 °C for 30 min. Finally, the mixture solution was transferred into a 96-well plate, the color changed, and the UV–vis absorption spectra were collected by a SpectraMax 190 UV–vis spectrophotometer. The A_{625}/A_{525} ratio of the AuNPs/MCH–substrate was used to quantitatively estimate the inhibitor activity.

2.9. Assay for the efficiency of MMP inhibitors by zymography

The conditions of zymography electrophoresis for inhibitor screening were the same as previously narrated, except for the composition of the developing buffer. After washing twice in renaturing buffer and replacing the solution with developing buffer for 30 min, the gel rinsed in fresh developing buffer, to which different concentrations of inhibitor were added, and the gel was incubated at 37 °C for 48 h. Finally, the gel was stained with Coomassie brilliant blue staining solution and destained with the destain buffer.

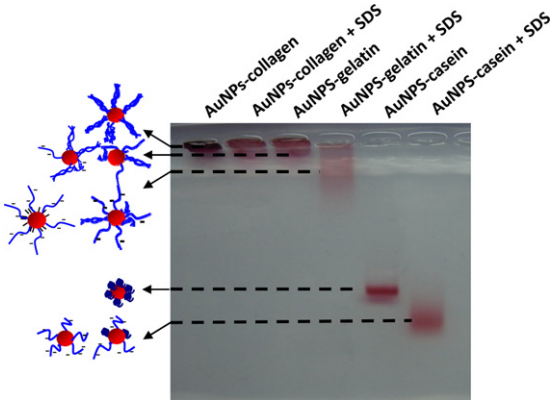


Fig. 1. Electrophoresis mobility of modified AuNPs–substrate conjugates. The coating consists of type I collagen, gelatin or casein with different modified processes. Type I collagen functionalized particles are retarded in the well. The gelatin functionalized particles are retarded, which moves slightly toward the negative electrode, while the casein functionalized ones move faster toward the negative electrode. All of SDS treatment particles move dexterous compare with references.

3. Results and discussion

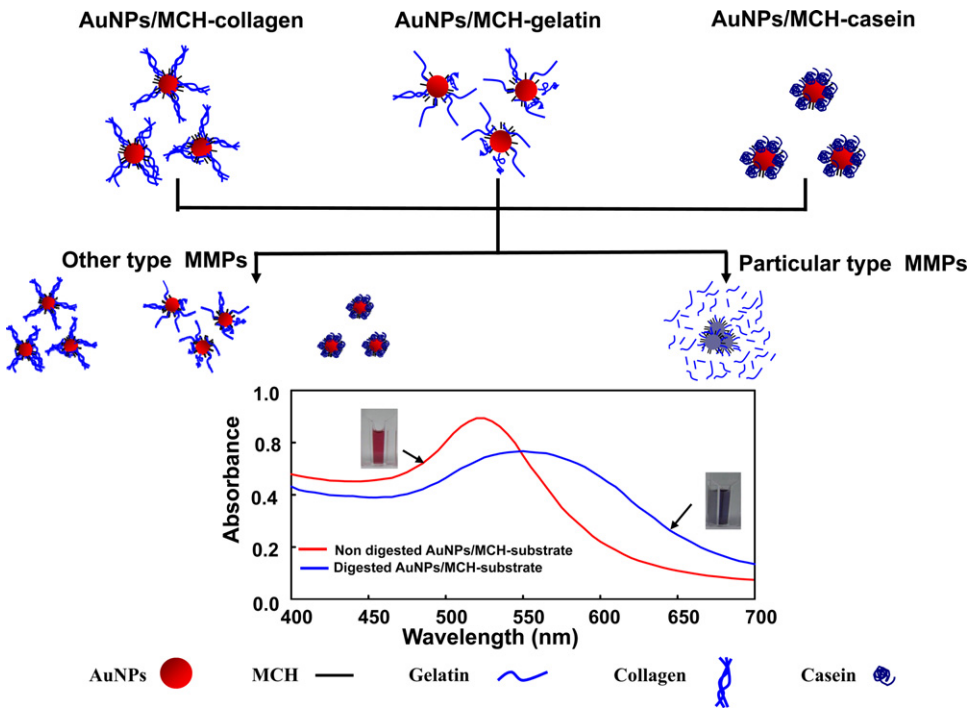
3.1. Establishment of a colorimetric biosensing platform using AuNPs

In our previous report, AuNPs had been applied as a colorimetric biosensing method for the detection of proteinases such as trypsin and MMP-2. As with zymography, all types of AuNPs/MCH–substrates originate from AuNPs/MCH–gelatin (Supporting Scheme S1). The techniques are the same, except that the substrate is selected based on the type of MMPs to be detected. The foundation of our design, as shown in Scheme 1, all of the functionalized-AuNPs displayed the wine-red color and were suspended in NTTC buffer. Upon enzymatic degradation of the functionalized-AuNPs, a significant color change from red to purple occurred and was followed by a red-shift in the UV–vis spectrum (see schematic illustration in Scheme 1). Moreover, the AuNPs/MCH–substrate system was tested for the interaction of pH and temperature to study the stability of the colorimetric platform. (Fig. S2). These results proved that the AuNPs/MCH–substrate was relatively stable before proteinase digestion.

3.2. Characterization of modified AuNPs

We next subjected the functionalized-AuNPs to electrophoresis, DLS and zeta potential assays to characterize the properties of modification.

Gel electrophoresis was applied to confirm the different AuNPs–substrates in accordance with their modified processes. We found that the grafted substrate affected the sizes and charges of the AuNPs and decreased their mobility. Fig. 1 emphasizes the difference in the mobility of the AuNPs–substrates (lanes 1, 3, 5). We noted that AuNPs modified with casein had the fastest mobility and the mobility of the AuNPs were always retarded by the addition of gelatin and collagen. The size and surface charge (zeta potential) of the AuNPs were also measured to confirm the properties of the AuNPs–substrates (Table 1). In the DLS measurements, colloid



Scheme 1. Schematic illustration of the AuNPs-based optical biosensing platform used to assay particular MMPs activity. Schematic illustration for the interaction between MMPs and their particular AuNPs/MCH–substrate. When substrate was digested by specific MMPs, the AuNPs/MCH–substrate lost shelter to cause aggregation and MCH enhance the attraction among AuNPs. And aggregation of AuNPs/MCH–substrate results in a color change from pink red to violet blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

AuNPs revealed hydrodynamic diameters of 13.60, 28.37, 149.30 and 19.69 nm for bare AuNPs, AuNPs–gelatin, AuNPs–collagen and AuNPs–casein, respectively. Although the length of both gelatin and collagen is about 260 nm, the gelatin is more flexible than trihelix collagen; therefore, the hydrodynamic diameter of AuNPs–gelatin is much smaller than AuNPs–collagen.

We also examined the zeta potentials of AuNPs–substrate to confirm the mobility of AuNPs. With these substrates immobilized on AuNPs in NTTC buffer (pH 7.4), the surface charges of the AuNPs would change. Upon gelatin incubation with the gold particles, the mean surface charge increased from −29.09 mV to 1.77 mV, and the same measurement for the AuNPs–collagen and AuNPs–casein increased to 4.44 mV and −22.58 mV, respectively. This experiment also provided indirect evidence of substrate denaturizing in zymography; 0.1% SDS was incorporated into the AuNPs–substrates to denature the grafted substrates for 2 h and then centrifuged/washed to remove residual SDS. Compared with the AuNPs–substrates pretreated with SDS (lanes 2, 4, 6 in Fig. 1), the AuNPs–substrates without SDS treatment had lower mobility (lanes 1, 3, 5 in Fig. 1). This result clearly indicates that residual SDS appends a negative charge to the AuNPs–substrates. Therefore, the protein structures are different from the original ones, and we suggest that the structures of MMP substrates inlaid in the SDS-PAGE are not native structures, especially for type I collagen (Lantz and Ciborowski, 1994). In addition, SDS also can activate some proteinase, such as cysteine proteinase and MMPs, both of them they are responsible for extracellular matrix remodeling and would lead to degradation gelatin and other substrate which was dispersed in solution. To optimize the diagnostic validity of plasmatic MMPs, the denaturing agent SDS should be avoided.

When the AuNPs/MCH–substrate was challenged with specific MMP, the AuNPs/MCH–substrate aggregation was followed by a red shift of their maximal absorption band, and the ratio of dispersed to aggregated AuNPs/MCH–substrate was be indicated by the ratio of the absorption value at 525 nm to that at 625 nm (A_{625}/A_{525}) (Guo et al., 2011; Kim and Chung, 2011; Li et al., 2011). Meanwhile, control experiments were performed under the same conditions using other MMPs. Fig. 2 shows that the A_{625}/A_{525} ratio increased from 0.2 to 0.4 after MMP-1 digested AuNPs/MCH–collagen and AuNPs/MCH–gelatin; the A_{625}/A_{525} ratio increased from 0.2 to 0.8 after MMP-2 digested AuNPs/MCH–gelatin and MMP-7 digested AuNPs/MCH–casein. For comparing the AuNPs platform with

Table 1
Summary of size and surface charge measurements on gold colloids.

Sample	DLS-average hydrodynamic diameter (nm)	Zeta potential (mV)
AuNPs	13.16	−29.09
AuNPs–gelatin	28.37	1.77
AuNPs–collagen	149.30	4.44
AuNPs–casein	19.69	−22.58

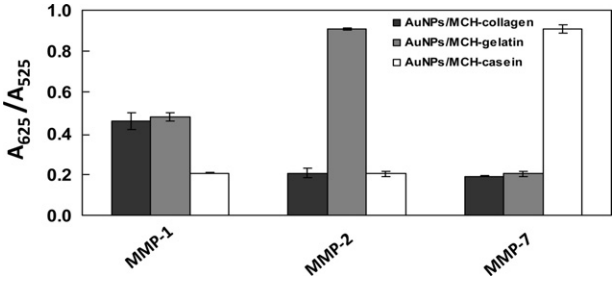


Fig. 2. Specificity of AuNPs/MCH–substrate used in the detection of MMPs activity. MMPs, including MMP-1, MMP-2 and MMP-7 were used to test the specificity of the AuNPs/MCH–gelatin, AuNPs/MCH–collagen and AuNPs/MCH–casein. The AuNP/MCH–gelatin, AuNPs/MCH–collagen and AuNPs/MCH–casein were used to determine the MMPs activity, and the ratios of A_{625}/A_{525} were calculated before and after MMP-1, MMP-2 and MMP-7 treatment. Each reaction included 10 (L MMPs (50 ng) and 240 (L of AuNPs/MCH–substrate. The experiment condition was controlled at 37 °C for 30 min.

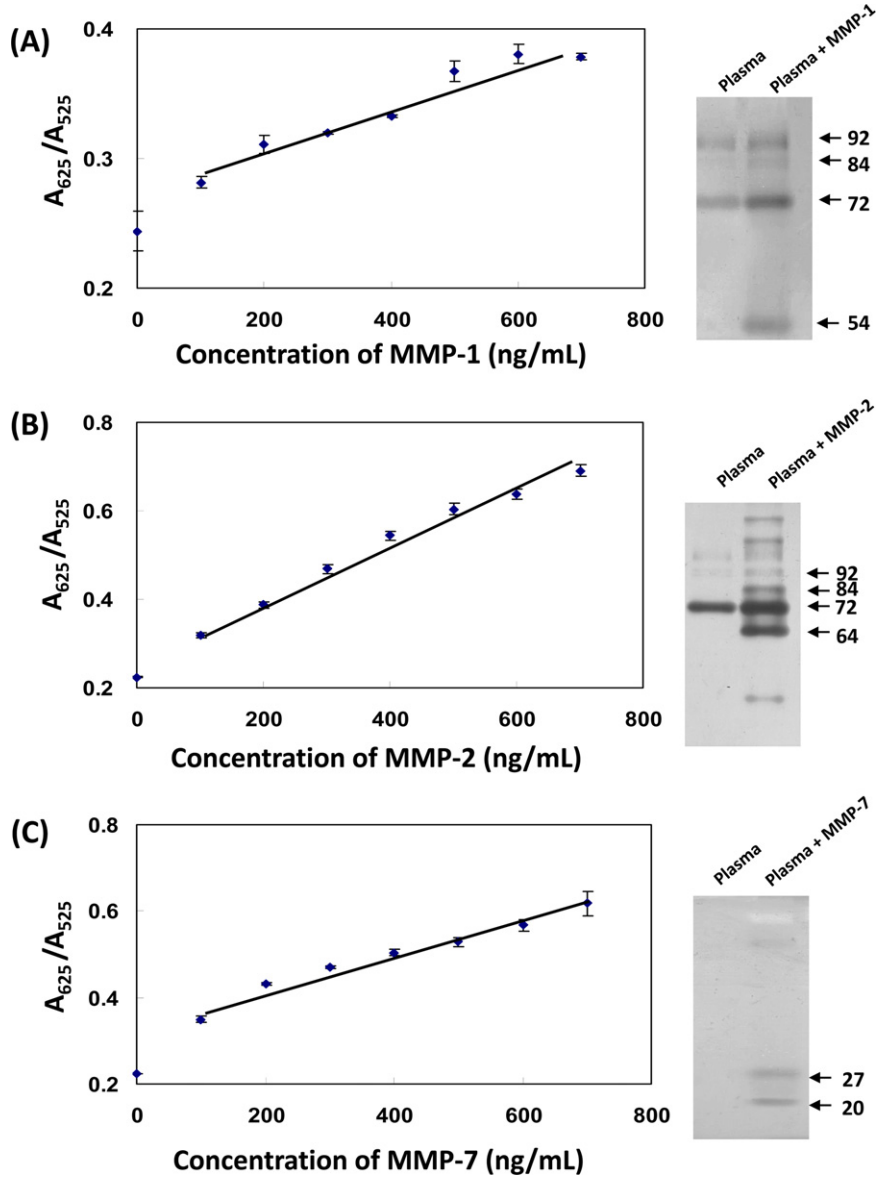


Fig. 3. The calibration curves of additive MMPs in plasma samples. Plasma was inoculated with MMPs including MMP-1 (A), MMP-2 (B) and MMP-7 (C) at concentrations of 0 (blank), 10–700 ng/mL. The mean of each value of dispersion and aggregation (A_{625}/A_{525}) was calculated from three independent measurements. Inset: the optical image of zymography gel represented MMPs activities of human plasma and human plasma added 50 ng of MMPs.

substrate zymography assay (Supporting Fig. S3), for the most part, the results were identical, except that the MMP-2 exhibited proteinase activity in the collagen zymographic gel. To summarize the results of Fig. 1 and Fig. 2, the substrates in zymographic gel did not maintain their native structure; in addition, collagen and gelatin possess similar primary structures. For these reasons, analyzing MMPs by substrate zymography may partly measure the MMP peptidase activity rather than the proteinase activity.

3.3. Analytical applications

To evaluate the reliability and applicability of the optical AuNPs platform to assay MMPs, a series of dilutions were analyzed simultaneously with substrate zymography and the optical AuNPs platform (Du et al., 2008). As shown in Fig. S4, a good correlation was obtained between the results of MMP-1, MMP-2 and MMP-7 using our method and substrate zymography. The correlation equations were $y_{\text{gelatin}} = 1.011x + 15.732$ (where y and x are the determination results of zymography and the optical AuNPs platform, respectively), $y_{\text{casein}} = 0.9463x + 17.422$ and $y_{\text{collagen}} = 0.9394x - 11.772$. The correlation coefficients, R^2, were 0.994, 0.987 and 0.967, respectively. Usually, 0.5–1 mg/mL of substrate is used in conventional zymography, while 50 μg/mL of substrate is used in our optical AuNPs-based method. The reason for this difference in substrate concentration is that AuNPs have a larger surface to volume ratio than SDS-PAGE, and thus, detection is much more sensitive than protein staining with Coomassie brilliant blue. This difference between substrate zymography and the optical AuNPs-based method may also contribute to the ability to detect lower amounts of MMP activity by optical AuNPs-based methods in a few minutes.

3.4. Detection of MMPs in plasma samples

Because MMP activity in pathological tissue may be reflected in body fluids, the measurement of MMPs in blood has been recommended as a useful diagnostic tool (Zucker et al., 1999). However, unlike zymography, the separation of the MMP–TIMP complex by

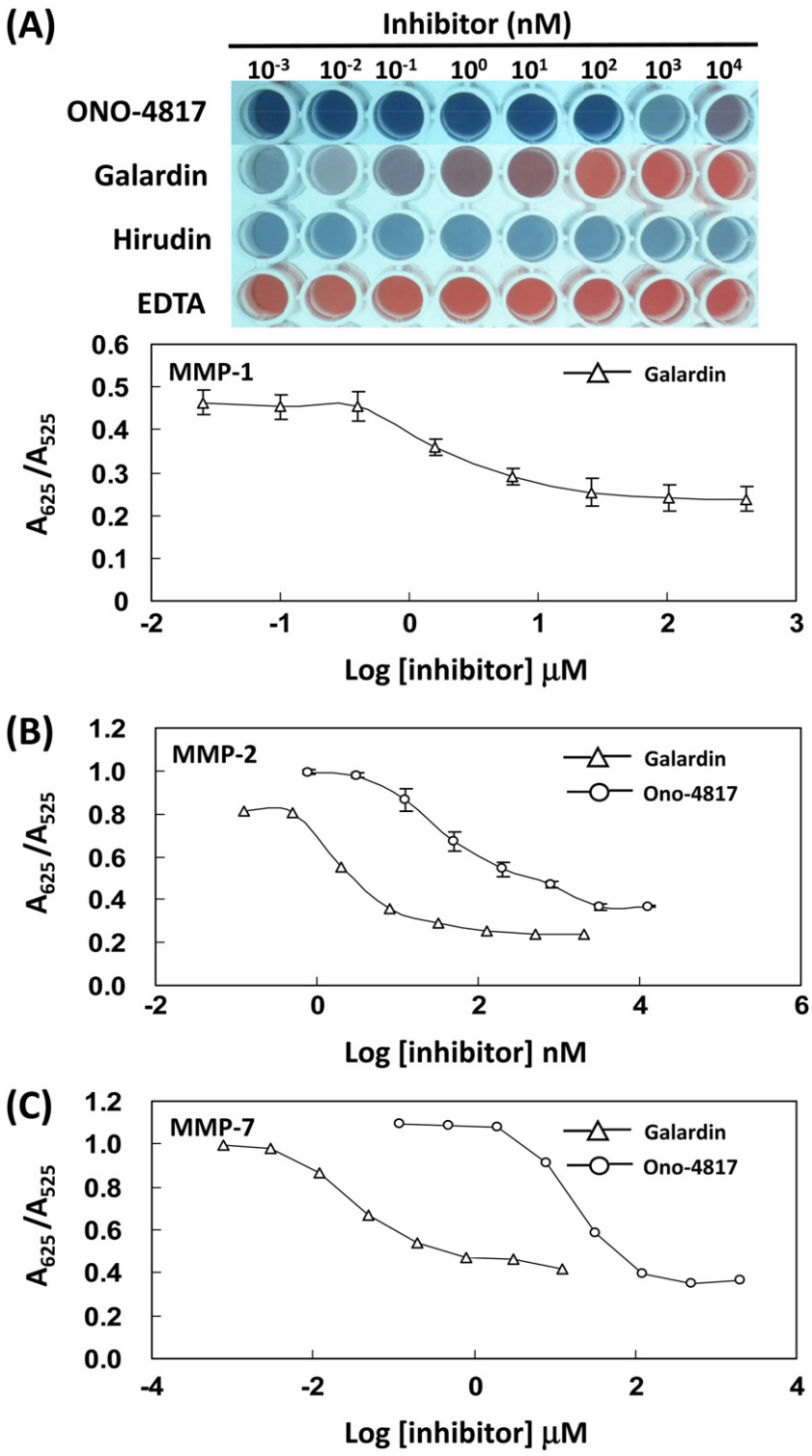


Fig. 4. Assay the efficiency of MMPs inhibitor by optical AuNPs-based assay. AuNPs/MCH–substrate were incubated with ONO-4817 and galardin and incubated at 37 °C for 30 min, then analyzed the ratio of A_{625}/A_{525} of AuNPs/MCH–substrate. Color changes of the AuNPs-based platform were determined in the absence and presence of MMP-1 inhibitors (A). Inhibition of MMP-2 by the candidate inhibitors (B). Inhibition of MMP-7 by the candidate inhibitors (C). Two MMPs inhibitors (ONO-4817 (-○-) and galardin (-△-)) were used as positive control. The concentration of MMPs was 50 ng/mL. Each value was derived from 3 independent detections and error bars mean SD ($n = 3$).

SDS polyacrylamide gel electrophoresis enables MMP activities to be determined, which is not possible in solution assays (Crabbe et al., 1993). In addition, α_2-macroglobulin, one of the major proteinase inhibitors in plasma, plays an important secondary role in backing up the primary function of the other inhibitors, including TIMPs (Travis and Salvesen, 1983; Ando et al., 1993; Jung et al., 2001). To overcome this predicament, the analytical steps of blood sampling should separate MMPs from the MMP–TIMP complex and avoid free MMPs binding with α_2-macroglobulin. In previous reports (Alby et al., 2002; Mannello, 2003; Löffek et al., 2011), heparin was recommended as the anticoagulant of choice to study circulating MMPs; an additional advantage of heparin is it might

interact with the hemopexin domain of MMPs and interfere with the proMMP–TIMP complex (Supporting Fig. S5), therefore the stronger digested zones can be observed in pro and activated MMP, the result was also found in studies on interaction between heparin and collagenase or metrilysin (Mannello et al., 2008), and result in activating MMP activity. To summarize what was mentioned before, we mixed heparin with additional MMPs and incubated the solution at 37 °C for 30 min to activate additional MMPs and avoid them being inhibited by α_2-macroglobulin.

Upon pretreatment, the UV–vis absorption spectra of AuNPs/MCH–substrate became broader and shifted to much longer wavelength (Supporting Fig. S6); furthermore the value of A_{625}/A_{525} and the concentrations of MMPs from 10 ng/mL to 700 ng/mL had a positive linear correlation, as shown in Fig. 3. In the present study, MMPs mixed with plasma obtained a higher A_{625}/A_{525} value than pure ones. There are two possible explanations for this phenomenon. First, the activated MMPs in plasma use their functionality and digest the AuNPs/MCH–substrate. Secondly, the additional MMPs cleave the propeptide form of pro-form MMPs and switch pro-form MMPs to activated-form MMPs (Itoh et al., 1995).

3.5. Assay the efficiency of MMP inhibitors

To validate a potential application of the colorimetric method of AuNPs, we also applied this method to evaluating the efficacy of different chemical compounds on inhibiting MMP activity (Lee et al., 2008; Dormán et al., 2010). We examined potential MMP family inhibitors ONO-4817, a kind of Zn^{2+} chelator (Yamamoto et al., 2003; Fisher and Mobashery, 2006) and galardin, a kind of peptidomimetics synthetic drug (Augé et al., 2004; Almholt et al., 2008), and chose hirudin as a negative control (Chang, 1983) and EDTA as a broad-spectrum inhibitor of metalloproteinase (Haas et al., 1998).

The AuNPs/MCH–substrate differed depending on the type of MMPs (250 ng/mL), and the presence of different concentrations of inhibitors was measured after the solutions were incubated at 37 °C for 1 h. With the presence of an efficient inhibitor in the AuNPs/MCH–substrate, no detectable color change occurred, and the solutions were indefinitely stable without showing signs of aggregation. These results can be explained because galardin and ONO-4817 can inhibit the activity of MMPs; thus, the aggregation of the AuNPs/MCH–substrate will become slow and result in less absorption variation (and less color change). Fig. 4A displays the inhibitor concentration-dependent color changes that were observed in ONO-4817 and galardin. However, these chemicals showed no effect on the stability of dispersive AuNPs/MCH–substrate (data not shown). For the quantitative analysis of the inhibitory effects on each MMP, the A_{625}/A_{525} ratio was plotted against the inhibitor concentration. The half maximal inhibitory concentration (IC_{50}; concentration of inhibitor that reduces enzyme activity to 50% of the activity of the native enzyme) was estimated by calculating the A_{625}/A_{525} ratio of the AuNPs/MCH–substrate. The IC_{50} values were 17.76 nM and 1.87 nM for MMP-2 and 40.45 μM and 16.07 nM for MMP-7 (Fig. 4B and C). Compared with MMP-2 and MMP-7, ONO-4817 cannot inhibit MMP-1, and IC_{50} of galardin toward MMP-was established to be 1.61 nM. Moreover, the efficiencies of ONO-4817 and galardin were also analyzed by zymography (Supporting Fig. S7). Each result obtained from zymography was consistent with the AuNPs-based optical biosensing method. The IC_{50} values for ONO-4817 and galardin were estimated to be 14.33 nM and 3.48 nM for MMP-2 and 60.67 μM and 5.27 nM for MMP-7. ONO-4817, as usual, cannot inhibit MMP-1, and the IC_{50} of galardin toward MMP-1 was established to be 2.37 nM. This IC_{50} value is different from each assay method. But, it is understandable because the reported IC_{50} values are affected by several parameters including the concentrations of enzyme and the substrate. In zymography, the MMP-inhibitor is separated by electrophoresis under denaturing conditions and cannot inhibit MMPs anymore (Ando et al., 1993; Kleiner and Stetler-Stevenson, 1994). To avoid interference by electrophoresis, the inhibitor was always included in the development buffer during proteinase reactions. Therefore, to reveal potent enzyme inhibition by zymography, the dose of inhibitor should be higher than when analyzing the inhibitor by the optical AuNPs-based method. Additionally, the two-step staining/destaining method of zymography is not reliable and is difficult to reproduce (Leber and Balkwill, 1997), which would increase inaccuracy for inhibitive efficiency. From these results, we suggest that our colorimetric system can be applied to the quantitative screening of MMP inhibitors.

4. Conclusions

We presented an optical AuNPs-based method of aqueous zymography for analyzing the activity of MMPs. The established method can be customized to specific experimental demands by adjusting the grafted substrate. Furthermore, it escapes the processes of protein denaturation and renaturation and provides more accurate information on proteinase activity. The AuNPs-based method can be characterized with obvious advantages in aspects of: (1) one-step operation and short assay time (60 min), (2) high sensitivity and specificity for MMP activity, (3) use of a common spectrophotometer or the naked eye, and (4) a high correlation with the traditional zymography method. In addition, the optical AuNPs-based method has a potential for further application in the efficient screening of class MMP inhibitors in vitro and the ability to obtain significantly faster diagnostic times and lower doses than with substrate zymography. Thus, the optical AuNPs-based method may serve as a useful tool for evaluating activities of various proteases and for protease inhibitor screening.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.11.002.

References

Alby, C., Ben Abdesselam, O., Foglietti, M.J., Beaudeux, J.L., 2002. Clin. Chim. Acta 325, 183–186.
Almholt, K., Juncker-Jensen, A., Laerum, O.D., Danø, K., Johnsen, M., Lund, L.R., Rømer, J., 2008. Mol. Cancer Ther. 7, 2758–2767.
Ando, H., Twining, S.S., Yue, B.Y., Zhou, X., Fini, M.E., Kaiya, T., Higginbotham, E.J., Sugar, J., 1993. Invest. Ophthalmol. Vis. Sci. 34, 3541–3548.
Augé, F., Hornebeck, W., Laronze, J.Y., 2004. Crit. Rev. Oncol. Hematol. 49, 277–282.
Bonomi, R., Cazzolaro, A., Sansone, A., Scrimin, P., Prins, L.J., 2011. Angew. Chem. Int. Ed. Engl. 50, 2307–2312.
Chang, J.Y., 1983. FEBS Lett. 164, 307–313.
Crabbe, T., Ioannou, C., Docherty, A.J., 1993. Eur. J. Biochem. 218, 431–438.
Chen, C.L., Huang, S.K., Lin, J.L., Lai, L.P., Lai, S.C., Liu, C.W., Chen, W.C., Wen, C.H., Lin, C.S., 2008. J. Mol. Cell. Cardiol. 45, 742–753.
Chen, S.H., Chuang, Y.C., Lu, Y.C., Lin, H.C., Yang, Y.L., Lin, C.S., 2009a. Nanotechnology 20, 215501.

Chen, S.H., Lin, K.I., Tang, C.Y., Peng, S.L., Chuang, Y.C., Lin, Y.R., Wang, J.P., Lin, C.S., 2009b. IEEE Trans. Nanobiosci. 8, 120–131.
Chuang, Y.C., Li, J.C., Chen, S.H., Liu, T.Y., Kuo, C.H., Huang, W.T., Lin, C.S., 2010. Biomaterials 31, 6087–6095.
Dormán, G., Cseh, S., Hajdú, I., Barna, L., Kónya, D., Kupai, K., Kovács, L., Ferdinandy, P., 2010. Drugs 70, 949–964.
Du, B., Li, Z., Cheng, Y., 2008. Talanta 75, 959–964.
Faust, A., Waschkau, B., Waldeck, J., Höltke, C., Breyholz, H.J., Wagner, S., Kopka, K., Heindel, W., Schäfers, M., Bremer, C., 2008. Bioconjug. Chem. 19, 1001–1008.
Fisher, J.F., Mobashery, S., 2006. Cancer Metastasis Rev. 25, 115–136.
Gerlach, R.F., Demacq, C., Jung, K., Tanus-Santos, J.E., 2007. Clin. Biochem. 40, 119–123.
Griffin, J., Singh, A.K., Senapati, D., Lee, E., Gaylor, K., Jones-Boone, J., Ray, P.C., 2009. Small 5, 839–845.
Gupta, S., Andresen, H., Ghadiali, J.E., Stevens, M.M., 2010. Small 6, 1509–1513.
Guo, Y., Wang, Z., Qu, W., Shao, H., Jiang, X., 2011. Biosens. Bioelectron. 26, 4064–4069.
Haas, T.L., Davis, S.J., Madri, J.A., 1998. J. Biol. Chem. 273, 3604–3610.
Hanauer, M., Pierrat, S., Zins, I., Lotz, A., Sönnichsen, C., 2007. Nano Lett. 7, 2881–2885.
Itoh, Y., Binner, S., Nagase, H., 1995. Biochem. J. 308, 645–651.
Jiang, Y., Zhao, H., Lin, Y., Zhu, N., Ma, Y., Mao, L., 2010. Angew. Chem. Int. Ed. Engl. 49, 4800–4804.
Jung, K., Nowak, L., Lein, M., Henke, W., Schnorr, D., Loening, S.A., 1996. Clin. Chem. 42, 2043–2045.
Jung, K., Lein, M., Laube, C., Lichtinghagen, R., 2001. Clin. Chim. Acta 314, 241–244.
Kai, H., Ikeda, H., Yasukawa, H., Kai, M., Seki, Y., Kuwahara, F., Ueno, T., Sugi, K., Imaizumi, T., 1998. J. Am. Coll. Cardiol. 32, 368–372.
Kanesaka, T., Mori, M., Hattori, T., Oki, T., Kuwabara, S., 2006. J. Neurol. Neurosurg. Psychiatry 77, 185–188.
Kang, J.H., Asami, Y., Murata, M., Kitazaki, H., Sadanaga, N., Tokunaga, E., Shiotani, S., Okada, S., Maehara, Y., Niidome, T., Hashizume, M., Mori, T., Katayama, Y., 2010. Biosens. Bioelectron. 25, 1869–1874.
Kim, J.H., Chung, B.H., 2011. Biosens. Bioelectron. 26, 2805–2809.
Kim, Y.P., Oh, Y.H., Oh, E., Ko, S., Han, M.K., Kim, H.S., 2008. Anal. Chem. 80, 4634–4641.
Kleiner, D.E., Stetler-Stevenson, W.G., 1994. Anal. Biochem. 218, 325–329.
Kupai, K., Szucs, G., Cseh, S., Hajdu, I., Csonka, C., Csont, T., Ferdinandy, P., 2010. J. Pharmacol. Toxicol. Methods 61, 205–209.
Lantz, M.S., Ciborowski, P., 1994. Methods Enzymol. 235, 563–594.
Leber, T.M., Balkwill, F.R., 1997. Anal. Biochem. 249, 24–28.
Lee, S., Cha, E.J., Park, K., Lee, S.Y., Hong, J.K., Sun, I.C., Kim, S.Y., Choi, K., Kwon, I.C., Kim, K., Ahn, C.H., 2008. Angew. Chem. Int. Ed. Engl. 47, 2804–2807.
Li, F., Feng, Y., Zhao, C., Tang, B., 2011. Biosens. Bioelectron. 26, 4628–4631.
Li, X., Wang, J., Sun, L., Wang, Z., 2010. Chem. Commun. (Camb.) 46, 988–990.
Lin, Y.H., Lin, L.Y., Wu, Y.W., Chien, K.L., Lee, C.M., Hsu, R.B., Liao, L.C., Ho, Y.L., Chen, M.F., 2009. Clin. Chim. Acta 409, 96–99.
Löffek, S., Schilling, O., Franzke, C.W., 2011. Eur. Respir. J. 38, 191–208.
Lu, W., Arumugam, S.R., Senapati, D., Singh, A.K., Arbneshi, T., Khan, S.A., Yu, H., Ray, P.C., 2010. ACS Nano 4, 1739–1749.
Mannello, F., 2003. Clin. Chem. 49, 339–340.
Mannello, F., Jung, K., Tonti, G.A., Canestrari, F., 2008. Clin. Biochem. 41, 1466–1473.
Mao, X., Ma, Y., Zhang, A., Zhang, L., Zeng, L., Liu, G., 2009. Anal. Chem. 81, 1660–1668.
Schulz, R., 2007. Annu. Rev. Pharmacol. Toxicol. 47, 211–242.
Takahashi, M., Naito, K., Abe, M., Sawada, T., Nagano, A., 2004. Arthritis Res. Ther. 6, R208–R212.
Travis, J., Salvesen, G.S., 1983. Annu. Rev. Biochem. 52, 655–709.
Vlachou, M.A., Glynou, K.M., Ioannou, P.C., Christopoulos, T.K., Vartholomatos, G., 2010. Biosens. Bioelectron. 6, 228–234.
Yamamoto, A., Yano, S., Shiraga, M., Ogawa, H., Goto, H., Miki, T., Zhang, H., Sone, S., 2003. Int. J. Cancer 103, 822–828.
Yi, D.K., Sun, I.C., Ryu, J.H., Koo, H., Park, C.W., Youn, I.C., Choi, K., Kwon, I.C., Kim, K., Ahn, C.H., 2010. Bioconjug. Chem. 21, 2173–2177.
Zucker, S., Hymowitz, M., Conner, C., Zarrabi, H.M., Hurewitz, A.N., Matrisian, L., Boyd, D., Nicolson, G., Montana, S., 1999. Ann. N. Y. Acad. Sci. 878, 212–227.
Zhang, Z., Yang, J., Lu, J., Lin, J., Zeng, S., Luo, Q., 2008. J. Biomed. Opt. 13, 011006.