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Monitoring Matrix Metalloproteases Based on the Selective Interaction between Ir(III) Solvent Complex with Histidine-rich Polypeptide

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A luminescent biosensor has been developed for matrix metalloproteinase 9 (MMP-9) assay based on a selective interaction between Ir(III) solvent complex with histidine-rich peptide, which avoids the complicated double labeling of substrate polypeptides commonly-used in the FRET MMPs detections and provides a promising strategy for MMPs detection in clinical application.

Matrix metalloproteases (MMPs) belong to a zinc-dependent endopeptidase enzyme family, playing important roles in pivotal physiological functions, such as, angiogenesis, inflammation, reproduction, growth and development.[1] In the microenvironment of malignancies, MMPs not only participate in degrading the basement membrane, which offers space for tumor mass expansion and tumor cell evasion, resulting in metastasis formation, but also are involved in the neovascularization and angiogenesis in tumors.[2] In the MMP family, the gelatinase MMP-9 and MMP-2 have been found to be overexpressed in cervical, breast, colon and other types of tumors.[3] Especially, it can directly influence tumor progression and thus promote metastasis formation. Therefore, MMPs become the valuable biomarkers in tumor evaluation.

Traditionally, gelatinase zymography and immunoassay have been widely employed to assay MMPs. However, gelatinase zymography requires tedious sample preparation and signal readout procedures, which is time-consuming, while immunoassay is in the need of antibody which is vulnerable to the certain detection conditions. Hence, surface plasmon resonance (SPR),[4] electrochemical[5] and Förster resonance energy transfer (FRET) assays[6] have been developed to monitor MMPs. Among these strategies, FRET-based methods are considered as a promising strategy since fluorescence-based strategies usually demonstrate high sensitivity, simplicity and point-of-care advantages.[7] For instance, gao's group designed a novel dual-ratiometric fluorescent probe for the analysis of MMP-9 activity. In the same time, imaging of MMP-9 in cells can also be achieved.[8] Tang's group constructed a multicolor fluorescence nanoprobe for MMPs detection. MMPs can specifically cleave polypeptides at their recognition sites, so the MMP-cleavable peptides were usually used as the detection substrates.[9] Therefore, most of the reported fluorescent methods are based on the FRET interactions between fluorophores with quenchers, both of which were grafted in the polypeptides. When the doubly labeled polypeptides were cleaved by MMPs, an enhanced fluorescent signal was obtained.[9] Although FRET methods are effective strategies for MMPs assay, the double labeling of the peptides is usually complicated and expensive. In addition, the labeled fluorophores or quenchers may influence the interaction between peptides with MMPs.

Cyclometalated Ir(III) complexes have become a topical area of interest in inorganic photochemistry and phosphorescent materials for luminescent chemo-sensing or bio-sensing due to their long lifetime, high quantum yields, adjustable photospectra and easy preparation.[10] Luminescent Ir(III) solvent (Solv) complexes are formulated as $[Ir(C^N)_2(Solv)_2]^+$ [C^N = 2-phenylpyridine, 2-phenylquinoline or other similar structures; Solv = H_2O, dimethyl sulfoxide ($(CH_3)_2SO$), acetonitrile (CH_3CN)] or methanol (CH_3OH)], which possess the ability to stain certain proteins through the selective reaction with histidine (His) in proteins.[11] Ir(III) solvent complex demonstrates very weak emission in aqueous solution. By contrast, it exhibited an enhanced emission in the presence of His through generating a new strong luminescent complex $[Ir(C^N)_2(His)]^+$. Further experiments showed that Ir(III) solvent complex only recognizes His among the 20 kinds of natural amino acids (AAs). In view of this interesting phenomenon, Ir(III) solvent complexes can be used as signal transducers in MMPs detection to avoid the complicated double labeling of the peptide probe which was widely used by the previous FRET-based strategy.

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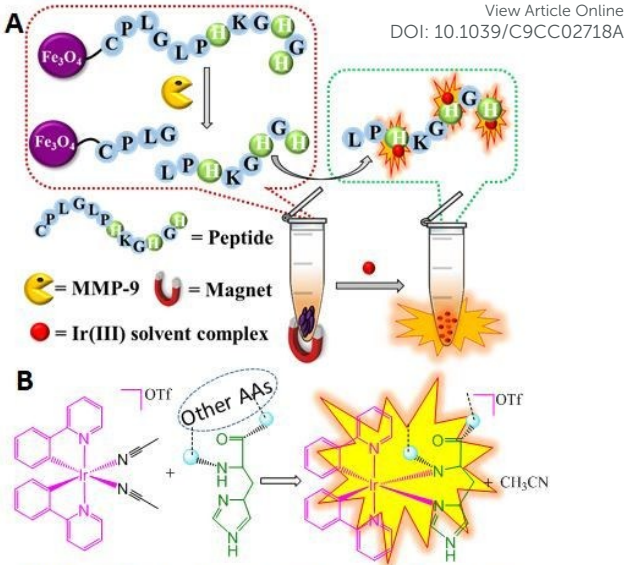
Magnetic nanoparticles, have been applied as attractive materials for load, delivery, diagnosis and treatment in medical or biological fields due to their unique and superior physical and chemical properties.[12] Spearheaded by the exquisite synthetic technology, a series of magnetic nanoparticles with controllable shape, good stability and monodispersity have been successfully fabricated.[13] Through the modification of biological molecules, such as DNA, AA and polypeptides on the surface of magnetic beads (MBs) by biological or chemical methods, they could possess better biocompatibility and easy entrance into cells and tissues for disease diagnosis and treatment.[14] Therefore, MBs have been widely used as a versatile biological immobilization carrier in bio-sensing field.

Inspired by these concepts, a sensitive luminescent MMPs biosensor was developed on the basis of peptide-linked MBs and the selective reaction between Ir(III) solvent complex with His. The detection process is very simple and no complicated double labeling of pepetides are needed. MMP-9 was used as a proof of concept to verify the proposed strategy.

As depicted in scheme 1A, the His-rich peptides containing the MMP-9 recognition site (between G and L) were covalently grafted to the surface of the MBs.[15] In the presence of MMP-9, the part of His-rich peptide was cleaved off from the MBs. After magnetic separation, Ir(III) Solv-**1** was added in the supernatant fraction. Subsequently, enhanced luminescence appeared due to the selective reaction between Ir(III) Solv-**1** with the His in substrate peptides, which produced a highly emissive Ir(III) complex (named as Ir(III) His-**P**) as described in Scheme 1B. In the absence of MMP-9, the part of His-rich peptides could not be cut off, and the subsequent magnetic separation would take away the His-rich peptides from the supernatant. After the addition of Ir(III) Solv-**1**, no luminescent Ir(III) His-**P** was produced, resulting in no luminescence enhancement. To the best of our knowledge, luminescent cyclometalated Ir(III) solvent complex-based strategy for the assay of MMP-9 has not yet been reported.

To confirm the workability of the presented strategy, the luminescent responses of cyclometalated Ir(III) Solv-**1** was investigated under different conditions. Fig. 1A exhibited that no obvious luminescent signal appeared when just Ir(III) Solv-**1** was added into the aqueous solution (curve a). Then, the polypeptide-modified MBs were added to the buffer solution, and Ir(III) Solv-**1** was mixed into the supernatant after the magnetic separation. As expected, there is no luminescence signal because no polypeptides were left in the supernatant after the magnetic separation (curve b). In contrast, when MMP-9 was added to the above reaction system, the supernatant showed a strong luminescence upon the addition of Ir(III) Solv-**1,** which indicated that a large number of His-rich polypeptide residues cleaved by MMP-9 remained in the supernatant after magnetic separation and the Ir(III) Solv-**1** quickly bound to His in the polypeptide residues, resulting in a significant increase in luminescence signal (curve c). In the same time, in order to verify that the newly produced Ir(III) His-**P** can be used as a luminescent signal in this detection, a His-rich peptide titration experiment was performed by using peptide **2** (Table S1). Fig. 1B showed that the luminescence



Scheme 1. (A) Schematic illustration of MMP-9 assay based on the selective reaction between Ir(III) solvent complex with histidine-rich peptide. (B) The chemical reaction between Ir(III) Solv-**1** with His contained by peptide. Where, Ir(III) Solv-**1** = $Ir[(2\text{-phenylpyridine})_2(CH_3CN)_2]CF_3SO_3$, Ir(III) His-**P** = $Ir[(2\text{-phenylpyridine})_2(His, \text{which contained by peptide})]CF_3SO_3$, AAs = amino acids.

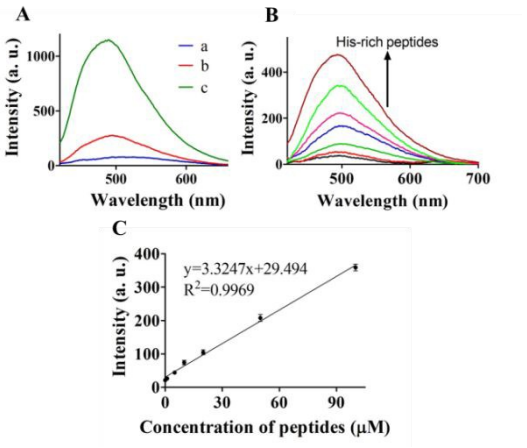


Fig. 1 (A) The luminescence responses of Ir(III) Sov-**1** at different conditions under 350 nm excitation. (a) Ir(III) Solv-**1** in the buffered solution, (b) supernatant + Ir(III) Solv-**1** in the absence of MMP-9, (c) supernatant + Ir(III) Solv-**1** in the presence of MMP-9. (B) The luminescence responses of Ir(III) Solv-**1** towards increased His-rich peptide **2** (0, 5, 10, 20, 40, 60 and 100 μM) under 350 nm excitation. (C) The linear relation between the intensity of Ir(III) Solv-**1** at λ = 490 nm with the concentrations of His-rich peptides.

intensity linearly ascended as the increase of His-rich peptides. These results clearly verified the detection mechanism depicted in Scheme 1, indicating that the cyclometalated Ir(III) solvent complex and magnetic separation-based label-free platform can sensitively assay MMP-9.

The absorbance, excitation and emission spectra of Ir(III) Solv-**1** in the presence/absence of His were presented in Fig. S1 which demonstrated the clear luminescence changes,

indicating that the reaction product between Ir(III) Solv-**1** with His can be used as an efficient luminescent sensor. The preparation route of His-rich polypeptide modified MBs was depicted in Fig. S2. To confirm that the His-rich polypeptide was attached to MBs, UV spectra of the MBs, His-rich polypeptide and His-rich polypeptide modified MBs were tested by spectrophotometer. As shown in Fig. S3, the absorption spectrum of His-rich polypeptide modified MBs exhibited the characteristic absorption band of polypeptide at 260 nm, which indicated that polypeptide had been successfully grafted to MBs through the chemical reaction between the mercapto group of polypeptide with maleimide-modified MBs. Zeta potential was also used to verify this attachment. Fig. S4 demonstrated that the peptide had a negative potential of −14.7 mV, while the maleimide-modified MBs possessed a positively charged zeta-potential of 19.3 mV. After the MBs' surface was grafted with peptides, the potential was switched to 9.9 mV, confirming the successful grafting of the peptide onto the surface of MBs.

In this work, several experimental factors that may influence the performance of this assay were investigated through single-factor testing. Due to the best reaction sites for Ir(III) Solv-**1**, peptide **2** in the three peptides (Table S1) presented the highest luminescence signal (Fig. S5). As shown in Fig. S6, The optimal pH for the detection was 7.5, which is favorable for the application of this platform in biological system. In order to achieve the best detection performance, the concentration of Ir(III) Solv-**1** and the time for the cleavage reaction between MMP-9 with the His-rich peptide were optimized as 3.0 μM (Fig. S7) and 60 min (Fig. S8), respectively.

After optimization of the detection factors, the luminescent responses of the detection system towards different concentrations of MMP-9 (0 to 5 nM) were studied. Fig. 2A demonstrated that the luminescence intensities gradually grew with the increased MMP-9 concentrations. The luminescence intensity presented a linear relation y = 1.2616x + 65.843 (R^2 = 0.9935) with MMP-9 concentrations from 5 pM to 0.25 nM. The limit of detection (LOD) in this assay for MMP-9 was estimated to be 2.1 pM (S/N=3) according to the linear relation (Fig. 2B), which was superior to those reported fluorescent MMPs detections.[7, 16] Therefore, the developed detection platform can be successfully used in luminescent detection of MMP-9. Table S2 listed the various MMPs detection strategies reported in recent years.

The specificity of the detection platform for MMP-9 assay was demonstrated through investigating luminescence intensity of the detection system to four kinds of interfering proteins including prostate specific antigen (PSA), fibronectin (FN), insulin and thrombin (TB). The results showed that only MMP-9 and the mixture of MMP-9/other proteins can produce a significantly enhanced illuminating signal (Fig. S9). In contrast, no significant luminescence enhancement was observed upon the addition of PSA, FN, insulin and TB. In the substrate peptide, there is a K unit, which could be cleaved on the C-terminal by trypsin. As expected, this cleavage also led to the luminescence enhancement of the detection platform because of the generation of the two His in residual peptides (Fig. S10).

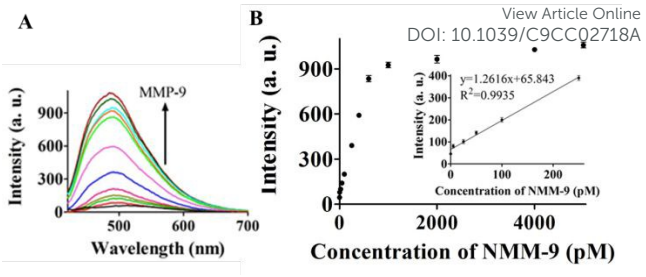


Fig. 2 (A) The luminescence responses of Ir(III) His-**P** to the increased concentrations of MMP-9 (0, 5, 25, 50, 100, 250, 400, 600, 1000, 2000, 4000, 5000 pM) under 350 nm excitation. (B) The relationship between luminescent intensity of Ir(III) His-**P** at λ = 490 nm with the concentrations of NMM-9. The plot showed the linear relation between luminescence intensity with the concentrations of NMM-9.

Worthy to note, this could be completely avoided through the design and optimization of the substrate peptide. Taken together, these results showed that the excellent specificity and anti-interference of the developed strategy for MMP-9 detection could be easily achieved through the adjustment of the substrate peptide.

In order to verify the applicability of the detection platform, the performance of the developed assay for MMP-9 detection in the presence of different amount of MCF-7 lysate was investigated. MCF-7 is a human breast cancer cells, which is positively expressed MMP-9. The result showed that the reaction system presented a linear growth in luminescence intensity with the increased density of MCF-7 cells (Fig. 3A and B). The detection platform can detect as low as 5000 MCF-7 cells per mL. Then, the detection platform was also used to screen the inhibitor of MMP-9. 1,10-phenanthroline (Phen) was reported as a broad-spectrum inhibitor of MMPs.[17] As shown in Fig. S11, The luminescence intensity of the detection system was gradually decreased as the concentrations of Phen increased from 0 to 10 μM, indicating that Phen inhibited the activity of MMP-9 and caused the non-generation of cleaved His-rich peptides. The IC_{50} of Phen was estimated as 5 μM towards 1 nM of MMP-9 (Fig. S12). Hence, the proposed

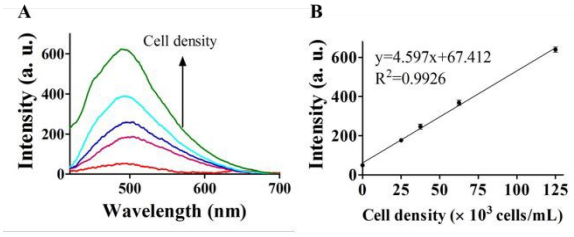


Fig. 3 (A) The luminescence responses of Ir(III) His-**P** to the increased numbers of cell (0, 25, 37.5, 62.5, 125 × 10^3 cells/mL) under 350 nm excitation. (B) The relationship between luminescent intensity of Ir(III) His-**P** at λ = 490 nm with the increased density of MCF-7 cells. The plot showed the linear relation between luminescence intensity with the density of MCF-7 cells.

detection platform can be used to screen the inhibitor of MMPs.

In summary, the cyclometalated Ir(III) Solv-**1** was for the first time served as a luminescent signal transducer in MMP-9 detection, which avoided the complicated double labeling of MMP-9 specific polypeptides just through a simple ligand exchange reaction between Ir(III) solvent complex with the His in the designed polypeptides. The polypeptides were linked on the surface of MBs through the covalent reaction between maleimide attached by the MBs and the mercapto in the polypeptides. MMP-9 can specifically cleave the polypeptides at the recognition site. After magnetic separation, Ir(III) Solv-**1** were added to the supernatant and then specifically bound to the His, resulting in an enhanced luminescence. Such a "signal-on" strategy enables the detection system to sensitively detect MMP-9 in cancer cell lysates and screen MMP-9 inhibitors. Thus, the proposed strategy can be used as an effective strategy to achieve MMPs detection and expect to be applied clinically.

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Conflicts of interest

There are no conflicts to declare.

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