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COMMUNICATION

Iridium complex-based probe for photoluminescence lifetime imaging of human carboxylesterase 2 in living cells

Zihe Yan [a], Jinyu Wang [a], Yanxin Zhang [b], Sichun Zhang [a], Juan Qiao*[b] and Xinrong Zhang*[a]

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A novel photoluminescence lifetime probe (Ir-TB) has been developed for the detection and imaging of hCE2 in living cells. Large lifetime increase by around 300 ns after the enzymatic reaction makes it an ideal tool to distinguish the hCE2-hydrolyzed probes from those non-hydrolyzed ones by PLIM for the first time.

Enzymes are closely related to metabolic processes in the human body and the abnormal levels and activities of metabolic enzymes often cause certain diseases such as various cancers.[1] Therefore, it is in urgent need to realize accurate determination of various important enzymes in living cells. Fluorescent probes have gained much attention to measure enzyme activities both in vitro and in living cells due to their intrinsic sensitive, rapid, non-destructive and high-throughput screening features.[2-5] In particular, NIR probes[6, 7] and two-photon probes[8] have become the focus for bioimaging of important enzymes in living cells. Up to date, however, most fluorescent probes for enzymes can be categorized into two modes, "turn-on" probes and ratiometric probes, both of which are essentially intensity-based probes.

Turn-on probes usually exhibit a fluorescence emission enhancement upon the cleavage of the probe by a specific enzyme, which has the advantages of high sensitivity, high spatial and temporal resolution, and easy design.[9] However, these probes are more susceptible to influence such as probe concentration, instrument precision, and biological environmental factors.[10] Ratiometric probes have received much attention because of its self-calibrating effects.[11] The amount of the analyte can be shown by the ratio of intensities at two distinct wavelengths. But inherent issues such as the interference of unwanted autofluorescence in biological samples still remain troubles.[12]

Photoluminescence lifetime imaging (PLIM) is a powerful strategy to enhance the signal-to-noise ratio in cellular imaging and sensing.[13, 14] Compared with intensity-based probes, the long lifetime probes can make it possible to eliminate the undesirable short-lifetime background autofluorescence in the complicated biological environment. A series of PLIM probes for anions (fluoride)[15] and small molecules (hydrogen sulfide)[16] have been established with great advantages. Recently, our group has developed a PLIM probe to image the newly synthesized proteins.[17] However, none of such PLIM probes for the detection of metabolic enzymes have been reported yet.

Carboxylesterases (CEs) are key members of metabolic enzymes.[18, 19] As a carboxylesterase isoform mainly distributed in the intestine and liver, human carboxylesterase 2 (hCE2) plays a key role in the metabolic activation of various anticancer prodrugs.[20-23] Herein, we developed a PLIM method to detect and image hCE2 in living cells by designing a photoluminescence lifetime probe based on an Iridium complex, Iridium (III) bis(1-phenylisoquinoline-N,C2')-N,N'-(2-(4-([2,2'-bipyridin]-4-yl)-1H-1,2,3-triazol-1-yl)benzoate) hexafluorophosphate, namely Ir-TB. With 1-phenylisoquinoline (piq) as its main ligand, the probe realizes deep red to near-infrared emission which makes it possible to avoid the interference of background autofluorescence. The ancillary ligand 2-(4-([2,2'-bipyridin]-4-yl)-1H-1,2,3-triazol-1-yl) benzoate is constructed by using 2,2'-bipyridin moiety as great contributions to better solubility and easier loading into cells, and the recognition group carboxylic ester bond on the ancillary ligand allows the probe to be selectively cleaved by the hCE2. Upon reaction with hCE2, Ir-TB shows a significant lifetime change from 338 ns to 625 ns. With such a huge lifetime increase, we realized the sensitive detection of hCE2 in living cells by PLIM for the first time.

[a] Department of Chemistry, Beijing Key laboratory of Microanalytical Methods and Instrumentation, Tsinghua University, Beijing 100084, China. E-mail: xrzhang@mail.tsinghua.edu.cn.
[b] Department of Chemistry, Key Lab of Organic Optoelectronics and Molecular Engineering of Ministry of Education, Tsinghua University, Beijing 100084, China. E-mail: qjuan@mail.tsinghua.edu.cn. † Footnotes relating to the title and/or authors should appear here.
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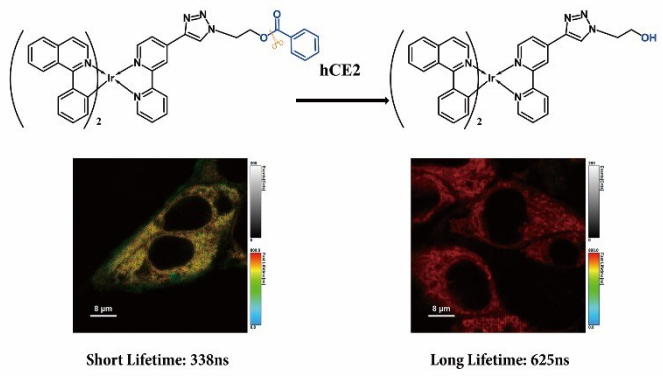

Scheme 1 The structure of Ir-TB and its photoluminescence lifetime response towards hCE2

The photophysical properties of Ir-TB itself and its responses towards the addition of hCE2 were investigated in PBS buffer (10 mM) under physiological conditions (pH=7.4, 37 °C). As shown in Fig. 1a, both absorption spectra with/without hCE2 displayed intense absorption bands below 400 nm from the intra-ligand π-π* transitions, moderate bands from 400 to 700 nm belonging to mixed transitions of ^{1}MLCT (metal-to-ligand charge transfer) and ^{3}MLCT transitions. Reaction of Ir-TB with hCE2 didn't cause noticeable changes to the absorption spectra and the maximum absorption in the visible region remained at 445 nm. Using 445 nm as the excitation wavelength, Ir-TB itself showed the maximum emission at 639 nm. In order to determine the reaction time, time course of the reaction was studied. With the procession of the incubation time, the photoluminescence lifetime increased within 20 minutes and reached the plateau at this time point (Fig. S1). Upon the addition of different concentrations of hCE2 (1 μg /mL-10 μg /mL) in the presence of Ir-TB, the fluorescence intensity was slightly stronger as the concentration increases, and the maximum emission wavelength appears to be blue-shifted for about 5 nm. The little variation is hardly employed for analysis perporse. (Fig. 1b).

However, significant differences can be seen in the photoluminescence lifetime decay curves as depicted in Fig. 1c. The black curve representing Ir-TB alone without any hCE2 showed a sharp drop trend, and it quickly decayed completely. On the other hand, other curves representing the additions of hCE2 decayed more slowly as the hCE2 concentration increased. Double exponential function was used here to fit the measured decay curves and then the corresponding lifetime values were analyzed. The average lifetime of the 10 μM Ir-TB solution (10 mM PBS buffer) was 338 ns, while the reaction solution with the addition of 10 μg/mL hCE2 increased to 625 ns. Under physiological conditions (pH=7.4, 37 °C), the photoluminescence lifetime response of Ir-TB towards hCE2 at various concentrations was further studied. The measured decay curves (Fig. 1c) were fitted by the common double exponential function. As depicted in Fig. 1d, the lifetime enhancement is proportional to the hCE2 concentration in the range of 0-10 μg/mL and exhibited a good linearity (y=341.48x+27.946, R^2=0.9938). This linear relationship between the photoluminescence lifetime and the hCE2 concentration would provide a reliable and effective way to carry out rapid and quantitative analysis.

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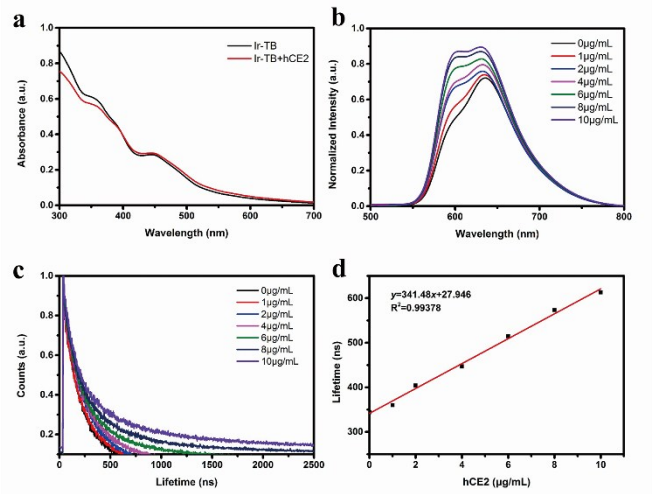

Fig. 1 (a) The absorbance spectra of Ir-TB before and after the addition of hCE2. (b) The emission spectra of Ir-TB before and after the addition of different concentrations of hCE2. (c) The lifetime decay curves of Ir-TB reacted with different concentrations of hCE2. (d) The fitted curve of lifetime to different concentrations of hCE2.

Besides, we studied the kinetic behaviours of Ir-TB hydrolysis by hCE2 as shown in Table S1 and Fig. S2, the Michaelis Constant (K_M) and maximum reaction rate (V_m) values implied that Ir-TB has good affinity and reactivity towards hCE2. To investigate if it is the hydrolysis reaction that leads to the lifetime change, we carried out ESI-MS experiments. Ir-TB alone was stable in PBS (10 mM, pH=7.4) without any metabolite detected for 20 minutes incubation time (Fig. S3a). In the experiment group, hCE2 and Ir-TB were incubated together under the same conditions for the same time, Ir-TB could be hydrolyzed and the only metabolite Ir-TO could be generated. As depicted in Fig. S3b, a mass peak at m/z 868.27, characteristic of Ir-TO is detected in the reaction solution. Therefore, it is confirmed that such a significant increase of photoluminescence lifetime was due to the hydrolysis of Ir-TB by hCE2 and the production of the only metabolite Ir-TO.

To explain the reason for the change in the lifetime, the electronic structures of Iridium complexes before and after hydrolysis were studied theoretically by using density functional theory (DFT) and time-dependent DFT calculation methods (Fig. S4). As shown in Table S2, the metal-to-ligand charge transfer (MLCT) percentage for the triplet states of Ir-TO after hydrolysis (40.37%) was slightly lower than that of Ir-TB before reaction (40.41%), which would decelerate radiative rate. In addition, the shortened substitute of the Iridium complex after hydrolysis (Fig. S4) would help to decrease non-radiative decay pathways arising from intramolecular large motion of vibrational, torsional and librational modes.[24] Therefore, the prolonged decay lifetime of the probe upon hCE2 hydrolysis could be anticipated. This lifetime change will help to distinguish the reacted probes from the unreacted ones from a more effective perspective.

To validate that Ir-TB is a reliable hCE2 substrate, stability and selectivity experiments were carried out under various conditions. Firstly, photostability of Ir-TB was investigated as it is of great importance for fluorescent probes both in vitro and in vivo. It can be shown that the photoluminescence intensity of Ir-TB undergoes only 4.5% decline following 30 rounds of detection under a UV lamp illumination, indicating stable photoluminescence intensity of the probe (Fig. S5). It is known that pH is a vital factor on the photophysical properties of many fluorescent probes. A suitable pH range was then tested. The results showed that the photoluminescence lifetime was quite stable in a wide pH range of 3–11 (Fig. S6a). Besides, viscosity is also an important factor that may influence the photoluminescence lifetime.[25] Different viscosities are regulated by different proportions of glycerol added. It was found that the lifetime remained stable in the range from 0% to 35% of glycerol (Fig. S6b). The above results suggest that there is minimum interference of pH and viscosity for the detection of hCE2 using Ir-TB.

The selectivity of Ir-TB for hCE2 was examined over a variety of enzyme species and proteins. There were only subtle changes in photoluminescence lifetime upon the addition of hCE1, AChE, BChE, BSA, HSA, compared with the control group. Only when treated with hCE2 was the distinct lifetime difference observed (Fig. 2a). To further confirm the lifetime change was the consequence of the cleavage reaction by hCE2, we conducted inhibitor assays. As shown in Fig. 2b, both BNPP (an effective inhibitor for hCEs) and LPA (a common inhibitor for hCE2) have a significant inhibitory effect on the activity of hCE2, while EDTA and HA, inhibitors of other enzymes, show little influence on the activities of hCE2. We also tested the photoluminescence lifetime response of Ir-TB towards various species of amino acids and bio-molecules (Fig. 2c), as well as different kinds of metal ions (Fig. 2d), where the results demonstrated that these small molecules and metal ions had little influence on the lifetime change of Ir-TB. Therefore, the above results clearly indicate that Ir-TB can be used as an effective tool for the selective detection of hCE2.

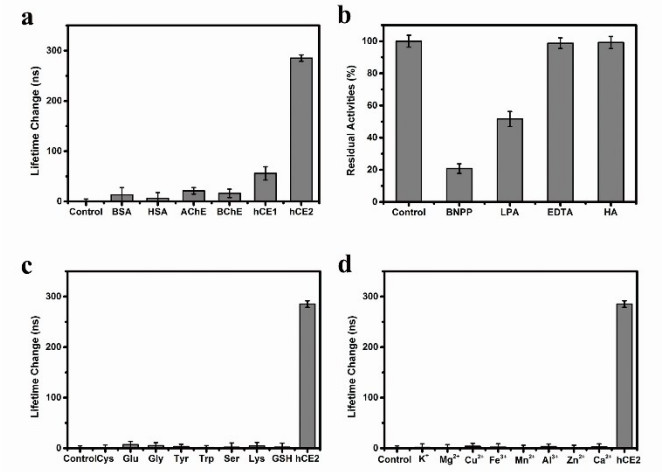

Fig. 2 (a) The photoluminescence lifetime response of Ir-TB (10 μM) towards various species of enzymes. (b) Inhibitory effects of various specific inhibitors of human esterases on Ir-TB (10 μM). (c) The photoluminescence lifetime response of Ir-TB (10 μM) towards various species of amino acids and bio-molecules. (d) The photoluminescence lifetime response of Ir-TB (10 μM) towards various species of metal ions.

Based on the in vitro photoluminescence lifetime studies, we are inspired that Ir-TB could be used for the imaging and detection of hCE2 activities in living cells. We first performed cytotoxicity experiments before conducting live cell imaging experiments. The probe Ir-TB showed very low cytotoxicity because even under a concentration of up to 5 μM, there is not significant influence on the cell viability and the results showed that more than 83% cells were still alive after 6 h treatment of the probe Ir-TB (Fig. S7). All these ensured good biocompatibility of Ir-TB. Next, both photoluminescence imaging and lifetime-imaging methods were employed in living cells to evaluate reacted probes and the unreacted probes due to inhibitors of hCE2. HepG2 cells, a human hepatocyte cell line, in which there are relatively higher hCE2 expression, were used for the cell imaging studies. After culturing the cells to a suitable density, the old DMEM medium was replaced and the cells were incubated with fresh medium containing 2 μM Ir-TB for 30 minutes. For the control group, 200 μM BNPP was pre-treated to the cells for 1 hour before incubation of Ir-TB, as we have proved in the in vitro experiments that BNPP is an effective inhibitor to hCE2. One blank group was also set in which there is nothing adding to the cells. Afterwards, the three groups of cells were directly subjected to photoluminescence imaging and lifetime imaging without any further washing steps. When the cells were incubated with Ir-TB, the photoluminescence intensity in the cells increased gradually and became stable and quite clear after 16 minutes (Fig. S8), indicating good cell permeability of Ir-TB. In addition, photostability experiments showed that the probe can maintain stable photoluminescence properties after circular imaging of 20 seconds interval for a long time of up to 30minutes, thus ensuring stable and reliable results (Fig. S9).

As shown in Fig. 3a, there was no signal in the blank group (no Ir-TB added), indicating that long-lifetime and NIR-emissive Ir-TB could eliminate the interference of cell autofluorescence. In the group of cells to which Ir-TB was added, Ir-TB was digested by hCE2 in the cells to produce Ir-TO, thus indicating a longer lifetime which was shown red (Fig. 3b). While in the group in which the inhibitor BNPP was added in advance, the activity of hCE2 in the cells was inhibited, so hCE2 could not function and digest the Ir-TB, thus indicating a shorter lifetime which was shown green (Fig. 3c). The lifetime distribution histogram of these two groups was depicted in Fig. S8. In addition, we could see that there is also a small part of reddish areas in the group with inhibitors, which means due to the complexity of the biological system, a small quantity of hCE2 is not inhibited and still exerted its own effect to cut off Ir-TB and showed longer lifetime.

This is where our method stands out. As depicted in Fig.3e and Fig. 3f, it's almost impossible to distinguish the reacted probes from those unreacted ones in the photoluminescence images, as they have similar intensity and emission wavelength. In the PLIM images, however, reacted and unreacted probes showed totally different lifetime which could be demonstrated by the pseudo colors. In this way, we can intuitively distinguish the hydrolyzed and non-hydrolyzed probes from the lifetime

images so as to determine the activity of hCE2 to be tested in this group of experiments. Therefore, Ir-TB has proven to be a promising PLIM-probe to realize rapid and efficient imaging and detection of hCE2 in living cells.

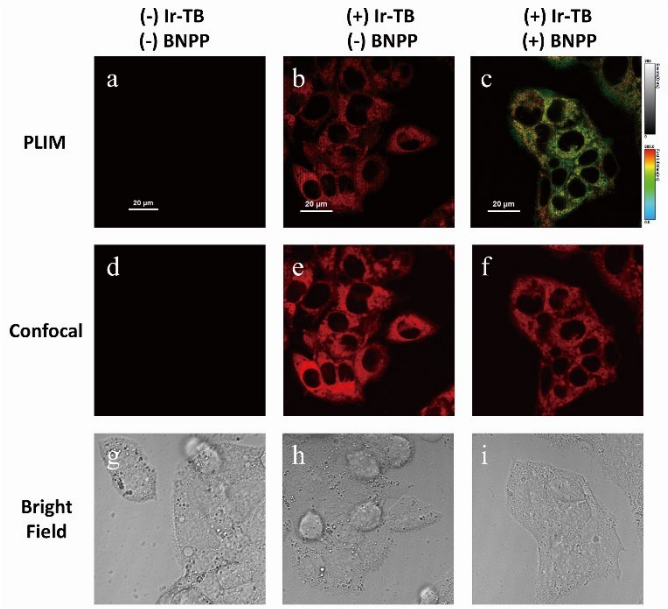

Fig. 3 Confocal images and photoluminescence lifetime images of HepG2 cells. Photoluminescence lifetime images (a-c), confocal images (d-f), and bright field images (g-i). The PLIM images were collected from the emission through a 561 nm long-pass filter upon excitation at 441 nm.

In conclusion, the lifetime-based Iridium complex probe Ir-TB for real-time detection and imaging of hCE2 in living cells by PLIM has been developed. This probe displays near-infrared emission and high selectivity, as well as good linearity towards different concentrations of hCE2. Besides, insensitivity to pH and viscosity, easy loading into cells, high photostability and low cytotoxicity all make it a relatively ideal photoluminescence probe for live cell imaging. Compared to traditional intensity-based sensing and imaging methods, lifetime-based detections are not affected by the interference of background autofluorescence at all, and with a significant lifetime change, it's easy to distinguish the hCE2-hydrolyzed probes from those non-hydrolyzed ones by PLIM without washing steps for the first time. All these findings suggest that Ir-TB holds great potential for further applications in biomedical research of other metabolic enzymes and other corresponding studies. And such photoluminescence lifetime method will help to develop more efficient probes and solve more important biomedical issues in the future.

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Conflicts of interest

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There are no conflicts to declare

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