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A Minireview of Recent Reported Carboxylesterase Fluorescent Probes: Design and Biological Applications

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As the most important members of serine esterase hydrolase superfamily, mammalian carboxylesterases (CESs) are widely distributed in various tissues like liver, intestine, lung and kidney. Many physiological processes require the hydrolysis of CES with a variety of endogenous and exogenous ester drugs and environmental poisons. So, developing the methods that can detect CESs activity is of great significance. In last decades, huge breakthrough has been made in the development of specific detection technologies including chromatography, quantitative reverse transcription polymerase chain reaction (qRT-PCR), proteomics and western blotting. However, these traditional methods for CESs detection have little effect on the spatiotemporal distribution and in situ activity of CES in living cells and in vivo. Compared with that, fluorogenic methods have been developed for real-time monitoring CESs in biological environments. In this review, we summarized the recent reported CESs probes. The designing strategies as well as their applications are reviewed. We hope this review will inspire the development of novel specific CESs fluorescent probes.

1. Introduction

As a kind of important serine hydrolases in mammals, carboxylesterases (CESs) play significant role in metabolism of various ester and amide molecules, for example, some ester prodrugs (like clopidogrel, irinotecan and oseltamivir) and environmental toxicants (such as pyrethroids).[1] CESs are widely distributed in the body and are mainly expressed in tissues with barrier function, such as lung, intestine, liver, kidney, skin, etc. CESs are concentrated in the endoplasmic reticulum of tissues, depending on the combination of whose c-terminal His-X-Glu -Leu sequence with the KDEL receptors on endoplasmic reticulum membrane.[2] According to the substrate - specific tissue distribution immune characteristics and basal regulation differences, mammalian carboxylesterases are classified into five types, namely, CES1, CES2, CES3, CES4 and CES5. Among them, apple and pear are the main isoenzymes in carboxylesterase and the main subtypes expressed in human body.[3] Moreover, CESs also play vital roles in lipid homeostasis for metabolizing endogenous esters, which means the dysfunction of CESs is closely related to atherosclerosis, cholesterol-induced liver injury and type 2 diabetes mellitus.[4] Especially, human carboxylesterase 1 (hCE1) is a serum marker of liver cancer.[1a,5]

Considering this, developing the rapid, sensitive and precise methods for detecting CESs activity are of great significance.[6] In the last decades, some detection methods have been developed like qRT-PCR, proteomic techniques, and western blotting for quantitative detection of CESs.[7] However, the detection process of these methods has many limitations including time-consuming, fairly complex, need strict requirements and expensive equipment.[8] Moreover, the most significant limitation of these techniques is that the actual function of target enzymes in biological systems cannot be assessed except their mRNA or protein levels.[9] Unlike these, fluorogenic methods have attracted great attention due to many advantages like high sensitivity, fast response time, high selectivity, low-cost as well as easy operation.[10] The detection of carboxylesterase was based on the hydrolysis of ester by enzyme.[11] More importantly, using fluorescent probe can visualize the detection process in vitro and in vivo.[12] Up to now, progress that achieved in CESs detection is not as great as expected.[13] Thus, challenges still remain in the detection of specific CESs.

In this review, we summarized the recent developed probes that can be used for detecting specific CESs as well as specific organelles imaging (Scheme 1). We discussed the designed strategies, detection mechanism, and biological application. The discussions were classified based on specific carboxylesterases fluorescent probes. We hope the designed strategies in this review will inspire the future development of CESs fluorescent probes.

2. Classification and functions of CES_S

Carboxylesterases have many isozymes and are classified into six main groups (CES1–CES6) as well as several subgroups. Although these isozymes share 40–50% similar amino acid sequences, they still display differences in tissue distribution and distinct substrate.[14] Actually, CES1 and CES2 are the major subtypes of CESs in the human body, and CES1 share 48% sequence homology with CES2. Generally, CES1 is mainly distributed in the liver while CES2 mainly expressed in the

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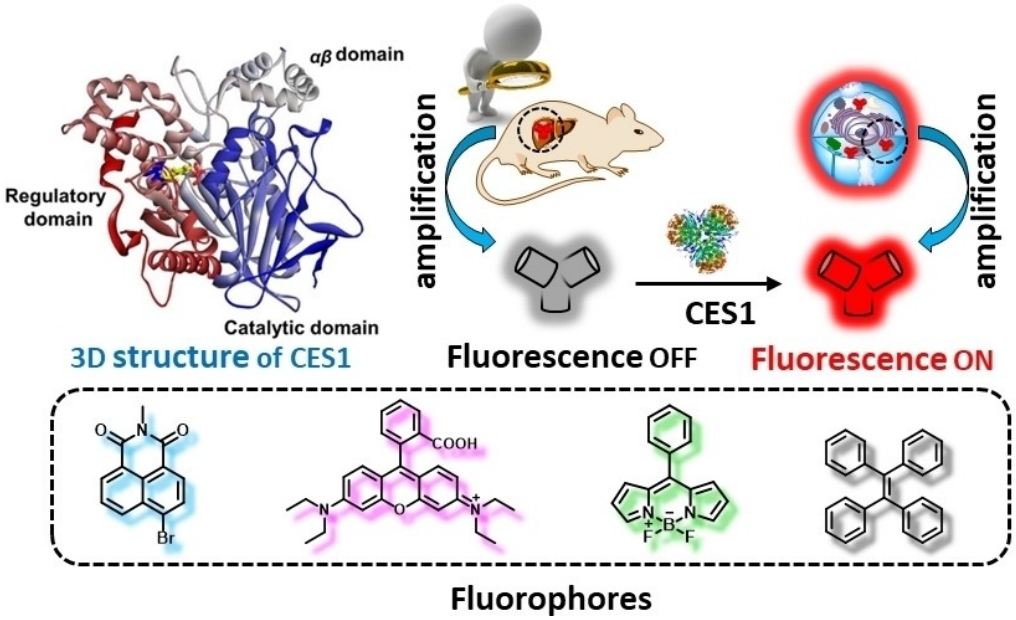

Scheme 1. Summarize carboxylesterases probes.

small intestine. Nevertheless, both CES1 and CES2 are also expressed in the kidney, liver as well as heart.[15] CES1 tends to hydrolyze compounds containing smaller alcohol groups and larger acyl groups, however, CES2 prefers to hydrolyze compounds with larger alcohol groups and smaller acyl groups.[16] They play a vital role in physiological or pathological

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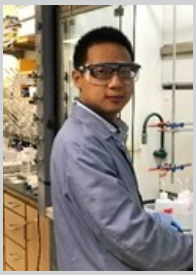

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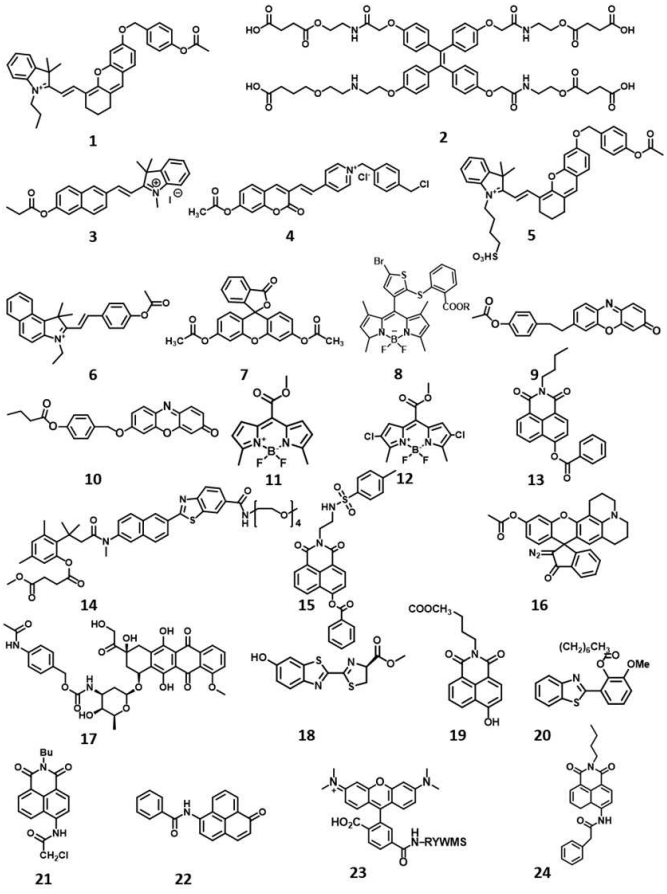
processes.[17] Whereas, only a few fluorescent probes for detection of CESs activity have been reported. The discussion was classified based on specific carboxylesterases fluorescent probes. All the structures of compounds that we summarized are shown in Scheme 2.

2.1. Fluorescent probes for carboxylesterase

In 2017, Li and Yang et al. developed a near-infrared fluorescent turn on probe 1 for detecting carboxylesterase in living HepG-2 cells (Figure 1a).[18] Carboxylate esterase activity in zebrafish treated with carbamate, organophosphorus, pyrethroid and other pesticides was detected by probe 1. The results showed that the three pesticides had inhibitory effect on carboxylesterase. Probe 1 was synthesized based on hemicyanine skeleton using (4-acetoxybenzyl)oxy as a quenching and recognizing group. The detection mechanism relies on enzyme-catalyzed spontaneous hydrolysis of carboxylate bonds and further cleavage of phenyl methyl groups. Probe 1 exhibited excellent analytical performance like near-infrared fluorescence emission at 705 nm, high selectivity and sensitivity, as well as low detection limit of 4.5×10^{-3} U mL^{-1}. More importantly, the results of cell experiments demonstrated that probe 1 has good cell membrane permeability and can be successfully applied to monitor carboxylesterase activity in living HepG-2 cells. Considering the pesticides can effectively inhibit the activity of carboxylesterase, authors applied probe 1 to monitor carboxylesterase in zebrafish pretreated with pesticides. The results suggested that probe 1 is a promising probe in indicating pesticide exposure.



Scheme 2. The structures of all compounds that summarized in this review.

Carboxylesterase-based "on-off" fluorescent probes usually have no fluorescence or weak fluorescence before reacting with CESs. Upon hydrolyzed by CESs, this sort of probes will rapidly show strongly fluorescent. Chen and Li et al. reported a fluorescent "light-up" probe 2 (Figure 1b) for detecting carboxylesterase activity based on tetraphenylethylene derivatives.[19] The carboxylic ester group was introduced and served as detecting group. The detecting mechanism was based on the cleavage of carboxylic ester bonds by carboxylesterase, which lead to the generation of a hydrophobic moiety. After self-assembling into supramolecular microfibers, the fluorescent signals exhibited a "turn-on" phenomenon. Probe 2 has high sensitivity and selectivity towards carboxylesterase. This is much lower than the reported fluorescent probes.

According to the number of photons absorbed, there were two types of fluorescent probes including single-photon and two-photon fluorescent probes. Two-photon absorption refers to the process of transition from the ground state to the excited state with twice the photon energy when the medium molecules absorb two photons at the same time under the excitation of strong light. In addition, two-photon fluorescent probe overcomes the defects of photobleaching and phototoxicity caused by single-photon probe. In 2018, Tang and Jiang et al. designed a two-photon fluorescent probe 3 (Figure 1c) based on the D-π-A structure of hemicyanine and naphthalene derivatives for detecting carboxylesterase.[20] The fluorescence of probe 3 exhibited an obvious light-up process towards carboxylesterase over other biologically-relevant species, enzymes and ROS. Importantly, probe 3 was demonstrated that it can monitor endogenous carboxylesterase in HeLa cells using two-photon imaging under the excitation of 800 nm NIR wavelength. Compared with many other reported carboxylesterase probes, 3 displayed many excellent properties, such as easy preparation, fast response time, high selectivity, high stability and low cytotoxicity, good cell permeability in living cells. They provide a good platform for developing new two-photon specific enzyme fluorescent probes.

An excellent fluorescent probe can collect many beneficial abilities. A dual ratiometric two-photon fluorescent probe was developed by James et al. CESs can produce acidic metabolites after reacting with esters, which will do harm to mitochondria and is essential for the sensing of probe 4. Probe 4 can in situ detect mitochondrial carboxylesterase activity and pH (Figure 1d).[21] When medicated with antipyretic anti-inflammatory drugs, probe 4 can be used to observe the carboxylesterase-mediated acidification of hepatoma cells and hepatic tissues. The skeleton of 4 is an effective platform for exploring two-photon fluorescent probe that can visualize the carboxylesterase-mediated acidification of cells and tissue.

It is urgent to design a better water-soluble fluorescent probe to overcome the defects of high toxic and side effects and fast metabolism in vivo. Li et al. synthesized a hydrosoluble

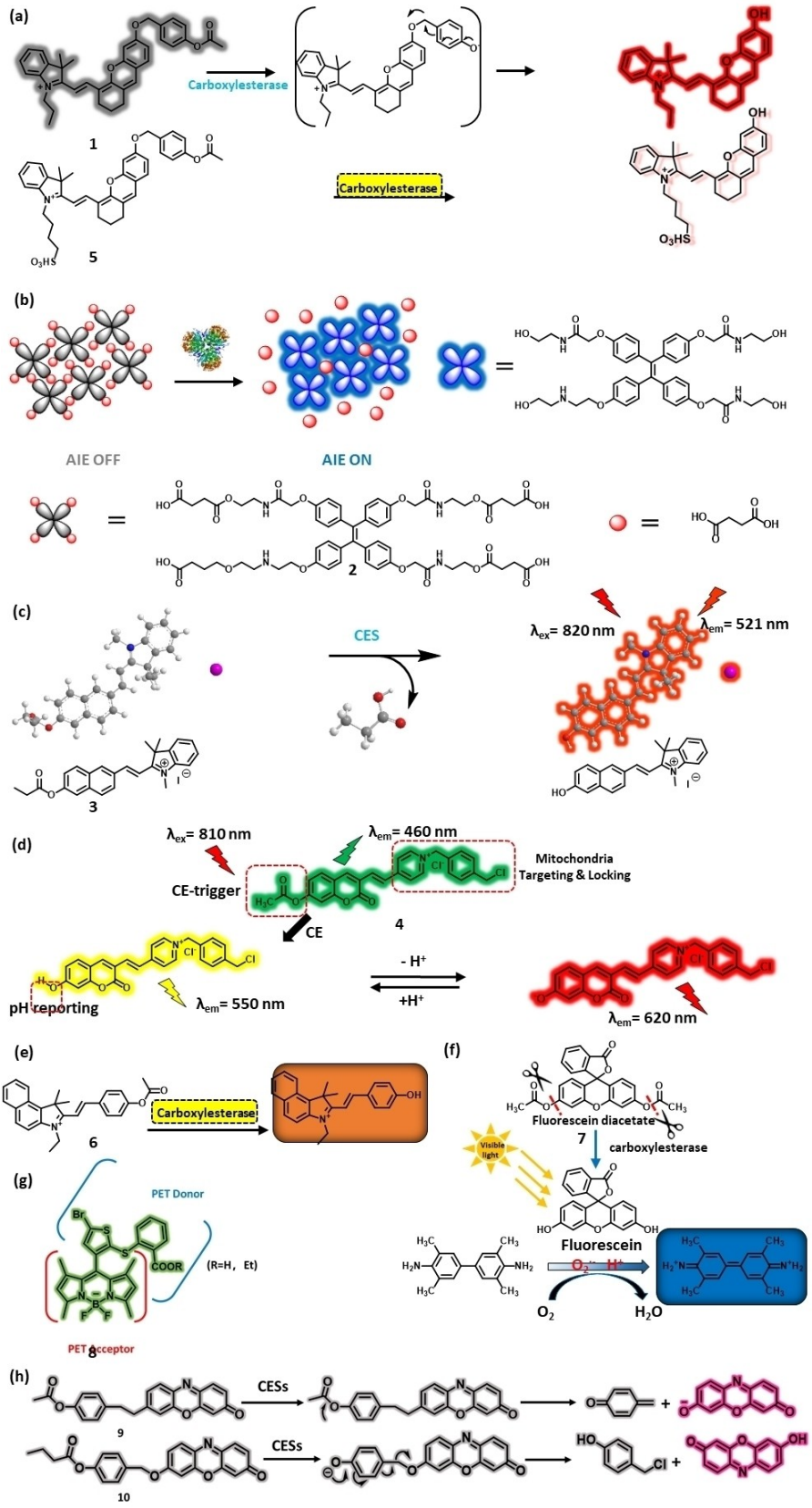


Figure 1. (a) The reactions of 1and 5 with CES; (b) The reactions of aggregation induced fluorescence (AIE) probe 2 with CES; (c) The reactions of 3 with CES; (d) The reactions of 4 with CES; (e) The reactions of6 with CES; (f) The reactions of 7 with CES; (g) The structure of 8; (h) The reactions of 9 and 10 with CES.

near-infrared fluorescent probe 5.[22] This probe is soluble in water and can detect the activity of carboxylesterase (Figure 1a). The fluorescence quenching group used in this probe was (4-acetoxybenzyl)oxy and also served as recognizing group. The responding mechanism of probe 5 towards carboxylesterase was based on the cleavage of carboxylic ester bond through hydrolysis of probe, which was catalyzed by carboxylesterase. This detection process will lead to a fluorescence turn on in near infrared field. More importantly, probe 5 displayed an excellent selectivity toward carboxylesterase in the presence of various substances. Detection limit of probe 5 was calculated as 3.4×10^{-3} UmL^{-1}. Previous probes with short excitation (<500 nm) and emission wavelength were restricted in the performance of cell imaging. After the reaction with carboxylesterase, the probe showed a strong absorption peak at wavelength 670 nm, which meant that it could be better used for in vivo imaging.

In 2019, Zhou's group reported a red emission lysosome-targeted probe 6.[23] It is a fluorescence "off-on" probe with the ability to selectively and sensitively detect carboxylesterase in living cells, sera as well as tissues (Figure 1e). N-methylated benzoindole and 4-(acetyloxy)benzaldehyde were coupled to synthesize probe 6 with hemicyanine skeleton. In probe 6, the acetyl group served as quenching and recognizing unit. 4-(acetyloxy)benzaldehyde was introduced to synthesize probe 6, which led to the property of lysosomal targeting. Due to the rational designing strategy, probe 6 displayed low background interference. The probe was hydrolyzed by carboxylesterase to release hydroxycysteine, which resulted in the fluorescence opening. Further investigation results exhibited that probe 6 can monitor and image carboxylesterase activity in biological samples including serum, live cells as well as tissues. This probe is a promising tool for further biomedical research.

Fluorescein diacetate (FDA) can permeate the cell membrane and store in living cells as luciferin with high amount of leakage in cells. The hydrolysis of FDA is widely accepted as an accurate and simple method for measuring total microbial activity. Zheng et al. constructed a carboxylesterase detecting platform based on fluorescein diacetate (FDA), which exhibit no activity in normal situation (Figure 1f).[24] In the presence of carboxylesterase, however, FDA generated active fluorescein. At the wavelength of 370 nm and 652 nm, there was an obvious linear relationship between absorbance and CES concentration. The detection process can be observed by the naked eye with the colour change from colorless to blue. Probe 7 displayed an excellent analytical performance for the detection of carboxylesterase. It has a good linear relationship in a wide carboxylesterase concentration range from 0.04 to 20 UL^{-1}. The detection limit was calculated as 0.013 UL^{-1}. In summary, this is a promising platform for inspiring researchers designing fluorescein derivatives with specific enzyme detecting ability.

David G. Churchill and his coworkers designed two hydrosoluble ROS-selective fluorescence probes 8 (Figure 1g).[25] The two probes were different from esters on aryl groups, and their substituents were carboxyl and ethyl carboxylate, respectively. These probes were based on BODIPY fluorophore with an aryl sulfide modified by a thiophenyl group at its eighth site. The aryl sulfide was a pendent 2-sulfide-benzoic acid unit which can oxidize with hypochlorous acid to selectively detect ROS in an aqueous solution, furthermore, the thiophenyl group was used to increase the water solubility of probes. Compared with the ethyl carboxylate substituted probe, the carboxyl substituted probe can more rapidly and highly selectively detect hypochlorous acid in aqueous solution. It can poorly imaging cells for its low cell permeability as it was an acid derivative. There was a discovery that the reaction of sulfide with reactive oxygen species changed the function of aryl sulfide group, which influences the detection effect. Inspired by this, we can continuously modify the functional group of aryl sulfide to find the probe with the best detection effect of reactive oxygen species.

Ma, Li and Shi have designed synthesized an off-on probe 9 ($\lambda_{em}=550$ nm) for the detection of carboxylesterase activity with high sensitivity (Figure 1h).[26] The probe was constructed with a resorufin and a p-acetoxyphenylmethyloxy, which played the role of fluorophore and the recognition sites of carboxylesterases relatively. After reacting with carboxylesterases, the carboxylic ester group was hydrolyzed by the enzyme, and the phenylmethyl unit was further dissociated, thus releasing the resorufin and triggering a 321-fold fluorescence. There was something worth mentioning that the detection process could be observed through naked eye, with the color changing from colorless to pink, which brought convenience. The probe 9 was extremely sensitive that its detection limit was calculated as 8.6×10^{5} UmL^{-1}. In this plateau, researchers can modify this probe so that the fluorescence intensity of the probe is linearly related to the activity of carboxylesterase, and then the probe can be used to detect the content of carboxylesterase inhibitors.

Compared with probe designed by Ma, Li and Shi, in 2011, Hakamata et al. also developed a similar fluorescent probe 10 containing with a 4-(hydroxymethyl)phenyl butyrate and a resorufin, which possess the ability of endoplasmic reticulum (Figure 1h).[6a] Since molecule weight of compounds locate at the range of 350–400 can well infiltrate into the cell, and the molecule weight of probe 10 is 389, hence, probe 10 has excellent cell membrane permeability. Upon the probe reacted with carboxylesterase, a series of cleavage reactions leaded to the releasion of resorufin and the appearance of fluorescence. Cell imaging of human fibrosar-coma cell line, HT-1080, and kidney cells of the African green monkey COS-1 indicated that the probes had high endoplasmic reticulum selectivity.

The structures of these two resorufin-based probes were relatively simple, which was the combination of detection groups and fluorophore without any relevant functional modification to improve water solubility and other properties. This kind of probes still have many detection limits to improve.

2.2. Fluorescent probes for carboxylesterase 1

Cui and Ge et al. exploited an isoform-specific fluorescent probe 11 with substrate preference for CES1 based on BODIPY (Figure 2a).[27] Probe 11 and its hydrolytic metabolite have been

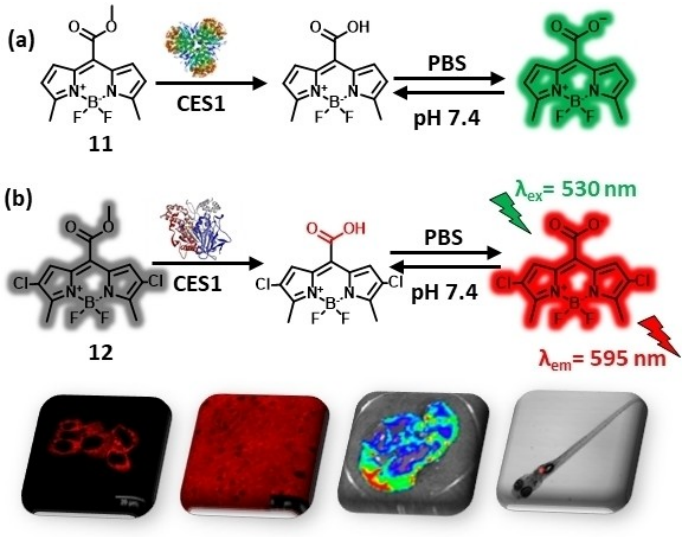


Figure 2. (a) The reactions of 11 with CES1; (b) The reactions of 12 with CES1, and the bioimaging of living cells, tissue slices, organs, as well as zebrafish. Reproduced with permission from Ref. [29]. Copyright 2019, American Chemical Society.

Table 1. The spectroscopic data and bioimaging model.

Probes	λ_{em} (nm)[a]	λ_{ex} (nm)[b]	LOD [c]	Bioimaging model[d]
1	705	670	4.5×10^{-3} U/mL	HepG-2 cells/zebrafish
2	475	365	29 pM	/
3	521	820	/	HeLa/Serum of patients with hepatocellular carcinoma
4	I_{550}/I_{460}	410	/	HepG-2 cells/liver of mice with cardiac perfusion
5	706	670	3.4×10^{-3} U/mL	HeLa
6	575	520	1.2×10^{-4} U/mL	HepG-2 cells/tissues from the liver of 45-day-old Kunming mice
7	/	370/652	0.13×10^{-4} U/mL	/
8(R=H)	506	527	/	/
8(R=Et)	546	573	/	SH-SY5Y human neuroblastoma
9	580	550	8.6×10^{-5} U/mL	HeLa
10	585	571	/	HT1080 cells/COS-1 cells
11	560	505	/	A549 cells/mouse liver slices/zebrafish
12	595	530	/	Caco-2 cells/Zebrafish/HLMs/mouse liver slice/7 week old mouse (Whole-Organ Levels)
13	I_{564}/I_{452}	342	1 μg/mL	HepG2 cells
14	455	373	7.7 nM	RKO cells/HCT 116 cells/SW 837 cells/MCF-7 cell
15	415	350	1 μg/mL	HepG-2 cells
16	600	556	/	HeLa
17	560	488	/	HCC/HepG-2/HT-29/ HeLa/MCF7/NIH3T3
18	/	/	10 μg/mL	HLMs
19	/	/	/	/
20	489	304	/	Zebrafish
21	542/452	354	12 ng/mL	HepG-2/a mouse liver slice
22	I_{605}/I_{562}	548	/	pancreatic cancer cells
23	580	525	/	/
24	470	405	/	Acinetobacter baumannii LMG 994 HAM/Bacillus cereus 994000168 LBK/Brevibacillus parabrevis 090915_03 LBK/Staphylococcus aureus ssp

[a] the emission wavelength of probes; [b] the excitation wavelength of probes; [c] the limit of detection of probes; [d] the bioimaging models of probes.

proved to be low cytotoxic by implementing CCK-8 assay. The result of docking simulations indicated that probe 8 have high specificity toward CES1. In addition, probe 11 was able to monitor the real activities of CES1 in various biological environments in real time, so that probe 11 was regarded as a widely applicable instrument for hypersensitive sensing CES1 activities in complex biological environments. Since probe 11 was synthesized based on BODIPY, thus 11 has optical properties of BODIPY, which generate robust fluorescence signals around 560 nm under excitation at 488 nm.[28] Even through probe 11 can approach the active site of CES2, the distances between the ester bond of probe 11 and the oxyanion hole of CES2 was more than 5.5 Å, which contributed to a strong selectivity to CES1 from CES2. In summary, 11 provided an excellent platform for further investigations on CES1-associated drug discovery.

Cui et al. developed a long-wavelength fluorescent probe 12 which can sensitively monitor carboxylesterase 1 (CES1) utilizing meso-carboxyl-BODIPY as the fluorophore (Figure 2b).[29] BODIPY derivatives have excellent photostabilities, and can modulate photophysical properties easily with proper substitution.[30] This kind of probes had the shortcomings caused by ester derivatives of these polycyclic phenol compounds, on account of ester derivatives preferring to interact with carboxylesterase 2 (CES2). However, molecular docking simulations demonstrated that the small enough spaces between the ester bond of these probes and the oxyanion hole (Gly148 and Gly149) of CES2 resulted in the fact that the interaction between probe 12 and CES2 is unrealizable. Additionally, CES1 could easily hydrolyze probe 12 into acid. In view of this, the change of electronic properties generated a strong fluorescence emission around 595 nm, which occurred at the meso site of fluorophore. Furthermore, the image of CES1 in zebrafish verified the selective localization of probe 12 via receiving strong fluorescence signals from liver region stained by 12. Cui and his coworkers conducted not only fundamental cells imaging, but also the research on the application of probe 12 toward mouse Ces1d (the ortholog of human CES1) at whole-organ levels. Compared with complex and time-consuming traditional methods, probe 12 was regarded as a convenient and efficient tool for monitoring CES1, and provided a precise platform for designing CES1 probe.

2.3. Fluorescent probes for carboxylesterase 2

The most significant advantage of ratiometric fluorescent probes is that the probe concentration has slight effect on the detection. Naphthalimide has rigid plane structure and large conjugated system, and its site 4 has high chemical activity which can easily react with other compounds. Fluorescent probes based on intramolecular charge transfer (ICT) have a striking feature that this type of probes is asymmetrical. Many researchers combined these mechanisms to build new fluorescent probes.

Yang et al. developed a ratiometric fluorescent probe to detect human carboxylesterase 2 (hCES2) based on the ICT mechanism.[31] Probe 13 was synthesized from 4-hydroxy-N-butyl-1,8-naphthalimide via introducing the benzoyl moiety (Figure 3a). The interferences caused by environmental conditions and light sources can be avoided though analyzing the ratio of fluorescence intensities of two maximum emission wavelengths. Due to the electron-withdrawal effect of the benzoyl moiety, florescence response had a remarkable red shift from 416 nm to 564 nm. In view of this, probe 10 can be a ratiometric florescence probe for detecting the bioactivity of hCES2. After adding the CES2, probe would exhibit a colour change from colorless to bright yellow, which allows the probe to perform naked eye detection. Furthermore, there was an unexceptionable liner correlation ($R^2 = 0.9924$) between fluorescence intensities ratio (I_{564} nm/I_{416} nm) and hCES2 concentration in the range of 0–8 μg/mL. The limit of detection was measured to be 1 μg/mL. Moreover, cell imaging experiments exhibited that probe 13 has low cytotoxic and high cell permeability. The exploration can hopefully overcome biomedical challenges as a creative fluorescent probe which can highly and sensitively monitor hCES2.

Kim et al. designed a two-photon ratiometric probe 14 based on succinate ester for monitoring carboxylesterase 2 (CES2) activity (Figure 3b).[32] The detection limit was calculated to be 7.7 nM. Probe 14 exhibited a robust blue fluorescence at the wavelength of 455 nm, which gradually shifted to the wavelength of 540 nm upon adding hCES2 with an obvious color change from blue to yellow. In view of this, the detection process of probe 14 was much more convenient than other forms of detection, such as Western blotting and RT-PCR. Inhibition tests were performed with selective esterase inhibitors in MCF-10 A cells to verify that probe 14 can selectively respond to CES2. Results of CCK-8 suggested that the cytotoxicity of 11 is negligible. Succinate ester functioned as a recognition site. Probe 14 has many outstanding properties including excellent photostability and lightful two-photon microscopy (TPM) imaging capability, and can be applied in quantifying CE2 activity in live samples. Furthermore, according to the accurate data from TPM, the responsiveness to anticancer drugs can be accurately predicted.

Mammalian CESs is a multigene family whose gene products are located in the endoplasmic reticulum of various tissues. Therefore, endoplasmic reticulum targeting detection is of great significance. James and Ma et al. designed an endoplasmic reticulum (ER) targeting ratiometric fluorescent probe 15 which can sensitively detect carboxylesterase 2 (CES2) and has an excellent ER-targeting ability (Figure 3c).[33] P-toluenesulfonamide was introduced to achieve the ER-targeting ability of probe 15. To further confirm the endoplasmic reticulum targeting of the probe, James and his coworkers compared the commercial trackers with the probe 15 and found that probe 15 indeed had excellent endoplasmic reticulum targeting. In spectroscopic experiment, probe 15 exhibited a maximum absorption at 350 nm. Moreover, the increase of fluorescence intensity at 560 nm as well as the decrease fluorescence intensity at 414 nm indicated the hydrolysis of probe 15 with the addition of CES2, and there is an excellent liner correlation ($R^2 = 0.9836$) between fluorescence intensities ratio (I_{560} nm/I_{414} nm) and CES2 concentration in the range of 0–8 μg/mL. An acetaminophen-induced acute liver injury model was established to confirm that the probe 15 can quantitatively and qualitatively detect the decrease of CES2 activity in the condition of ER stress as well, and the CCK-8 assay confirmed that probe 15 will not express cytotoxicity up to 100 μM. The development of ER targeting CES2 ratiometric fluorescent probe can give an inspiration in overcoming diseases connected with ER stress.

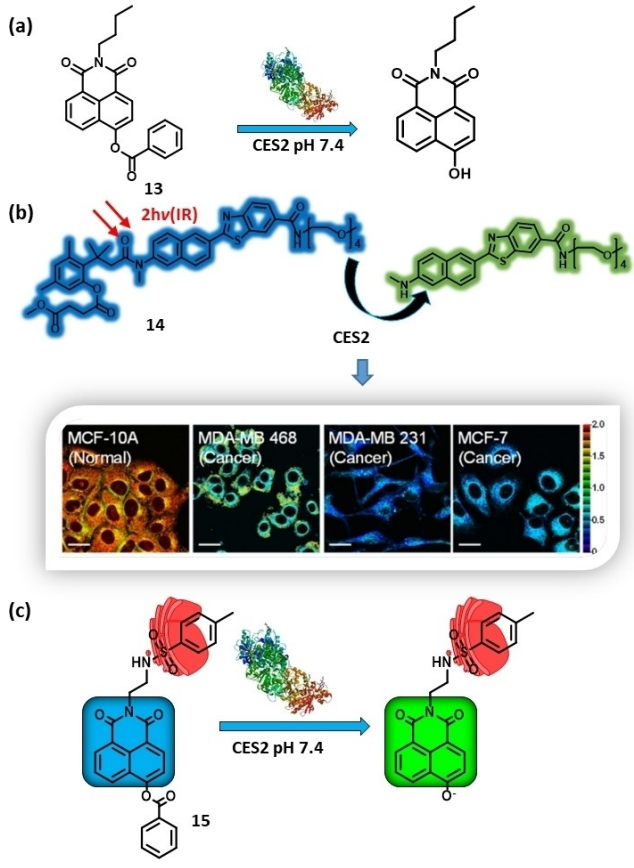


Figure 3. (a) The reactions of 13 with CES2; (b) The reactions of 14 with CES2, and the corresponding living cells imaging; Reproduced with permission from Ref. [32]. Copyright 2018, American Chemical Society. (c) The reactions of 15 with CES2.

2.4. Fluorescent probes for human carboxylesterase

Rivera-Fuentes et al. designed a dual enzyme- and photo-activatable fluorescent probe 16 based on diazoindanone for super-resolution imaging of enzymatic activity in living cells (Figure 4a).[34] The nucleus of the probe lacking electron can transform into a ring-expanded xanthene core that is non-emissive through photoinduced Wolff rearrangement. However, with the pre-activation of carboxylesterases, the core of probe 16 became electron-rich and there was an intense emission through Wolff rearrangement with irradiation. Probe 16 has a photoactivatable fluorophore which was able to combine the sensing mechanism with the photochemi1qcal conversion, which directly leading to the application of 16 in STORM/PALM imaging, compared with the traditional probes. After the reaction of the probe with carboxylesterases, the acetyl group of probe 16 was detached and triggering the fluorescence. The MTT assays demonstrated probe 16 has low cytotoxicity. Probe 16 brought an inspiration for developing small-molecule probes for the detection of other hydrolytic enzymes, reactive signaling molecules, and metal ions at the nanoscale.

Kim et al. demonstrated a physiological pharmic carrier which can convey Doxorubicin (DOX) to hepatocellular carcinoma cells (HCC) based on a small molecule and the response of carboxylesterase (Figure 4b).[35] Traditional methods of selectively delivering drugs were restricted by tumor heterogeneity and endosomal escape, however, the compound 17 solved those difficulties and exhibited excellent performance such as active targeting and easier synthetic access. The main feature of Probe 17 was that it first reacted with carboxylesterase, which produced a fluorescence opening and released the DOX, causing the DOX to act on the tumor sites. The fluorescence opening indicated that the probe bound correctly to the tumor which ensured that the DOX wasn't degraded form enzymes. Compound 17 expressed an increase of fluorescence intensity at 560 nm in physiological environment. In the presence of carboxylesterase, the cleavage of aniline amide bond occurred and released of DOX. The interference experiment displayed that compound 17 has a disturbance rejection behavior which means there is a great high selectivity between carboxylesterase and 17. Furthermore, the compound 17 was confirmed to have anticancer activity in a wide range of 10 μm to 50 μm, and these concentrations are safe for other cell lines. The development gives an inspiration in the delivery of HCC-specific drug and the treatment of related diseases.

2.5. Fluorescent probes for human carboxylesterase 1

Yang et al. innovatively designed a new bioluminescent probe which could be effectively used for detecting the activities of human carboxylesterase 1 (hCES1) based on the natural D-luciferin, whose ester derivatives have highly recognition capability to hCE1 cause of its alcohol groups.[17] The results of researches proved that probe 18 could be promptly hydrolyzed to D-luciferin when in the presence of hCES1, followed by the

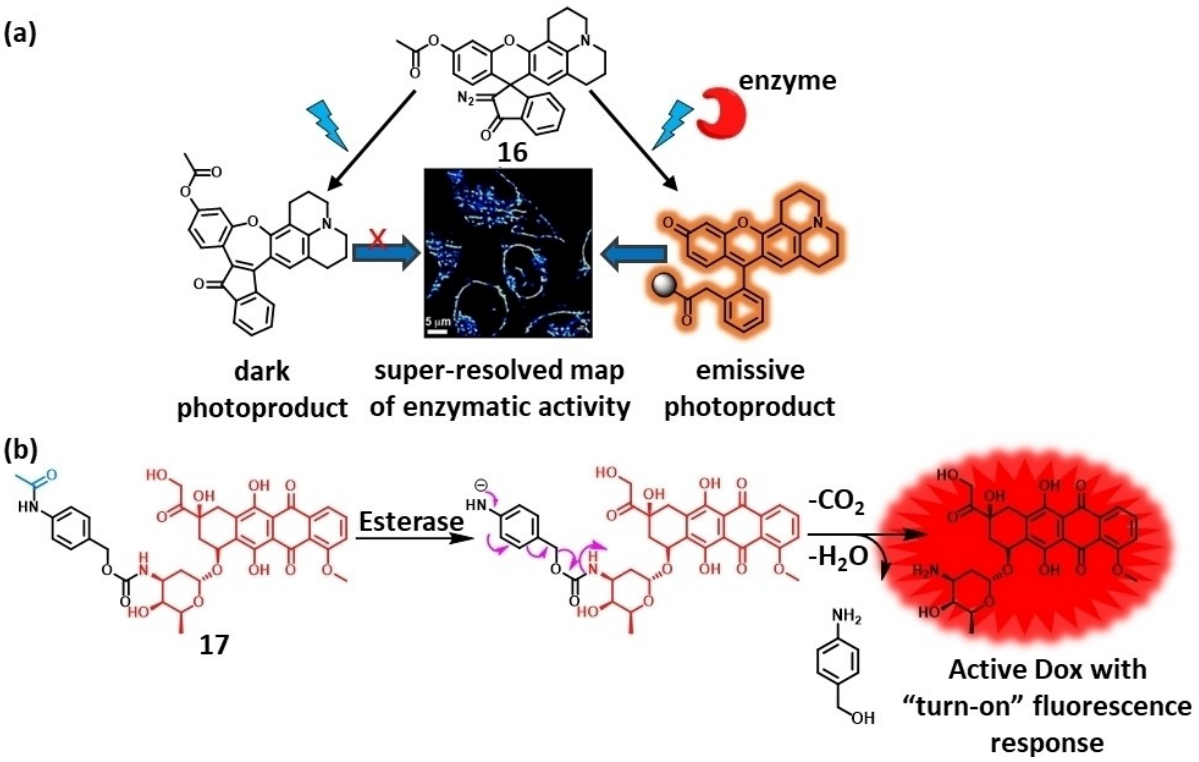


Figure 4. (a) The reactions of 16 with human carboxylesterase; Reproduced with permission from Ref. [34]. Copyright 2017, American Chemical Society; (b) The reactions of 17 with human carboxylesterase.

fluorescence response promoted by firefly luciferase luciferin system (Figure 5a). Additionally, the proper range for hydrolysis is pH 6.0–7.0. Furthermore, the enzymatic kinetic reaction stated the limit detection of probe 18 for hCES1 (0.01 mgmL^{-1}) had a 20-fold sensitivity than that of the reported probe 2-(2-benzoyloxy-3-methoxyphenyl)benzothiazole (BMBT), and the liner relation between luminescence intensity and the increasing hCE1 concentrations ($R^2 = 0.9973$) was splendid. The traditional methods and the fluorescent probes were restricted by detectable biomolecules and wavelength, however, this bioluminescent probe could express the signal without excitation light, which means that is an optimized approach to biology and medicine. Considering these excellent capacities, probe 18 would make contribution to solve academic difficulties about endogenous hCES1 as a new bioluminescent probe.

Yang et al. developed an hCES1 detection probe 19 based on N-(4-Methyl butyrate)-4-hydroxy-1, 8-naphthalimide with a small alcohol group and a large acyl moiety (Figure 5b), which played a key role in specific detection of hCES1.[36] The core design philosophy for monitoring was a highly selective marker reaction. Probe 19 was rapidly hydrolyzed by hCES1 to acid, both of them displayed excellent UV absorption and fluorescent emission properties, indicating that the enzymatic activity of hCE1 can be tested via LC-UV (liquid chromatography with ultraviolet detection) and LC-FD (liquid chromatography with fluorescent detection). Probe 19 can be applied in developing a fast hCES1 quantification method, and measuring the real activities of hCES1 in various biological samples can be realized by this method. In this view, 19 considerably can contribute to the deeper researches on the biological functions of hCES1 in complex biological system. Probe 19 had almost no fluorescence response after reacting with other ester hydrolytic enzymes, such as bovine serum albumin (BSA), human serum albumin (HSA) and so on, which forcefully proved that probe 19 had high selectivity to carboxylesterase. However, Yang et al. didn't exhibit any spectroscopy data and cell imaging results, even though they carried out some relevant experiments.

Wang and his coworkers designed a ratiometric fluorescent probe 20 based on 2-(2-hydroxy-3-methoxyphenyl) benzothiazole (HMBT) for the detection of human carboxylesterase 1 (hCES1) (Figure 5c).[37] Since hCES1 displayed a preference for hydrolyzing ester substrates having smaller alcoholic hydroxyl groups and larger acyl groups. Thus, an octanoyl group was introduced to modulate the excited state intramolecular proton transfer (ESIPT) emission of HMBT. Carboxylesterase 1 can selectively hydrolyze probe 20 with its metabolite HMBT released, which triggered an obvious fluorescence response at the wavelength of 489 nm with a large Stokes shift under the excitation around 304 nm. The result of interference experiment showed that probe 20 has excellent selectivity towards hCES1 over other enzymes and common ions, which means 20 was able to overcome the interference caused by endogenous chemicals. Moreover, 20 can accurately monitor the actual

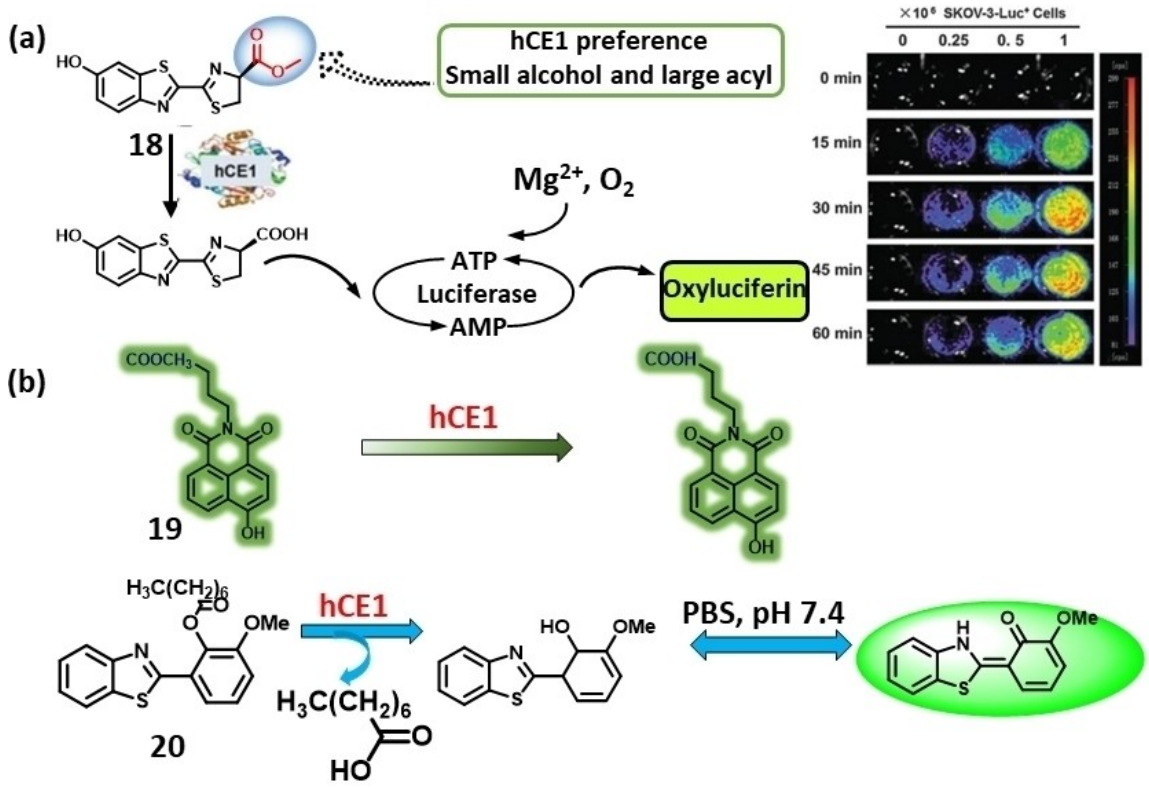


Figure 5. (a) The reactions of 18 with hCES1; Reproduced with permission from Ref. [17]. Copyright 2016, Royal Society of Chemistry. (b) The reactions of 19 with hCES1; (c) The reactions of 20 with hCES1.

activities of hCES1 in complex biological systems. In addition, it provided the possibility for evaluation inhibitory impact of hCES1 inhibitors in human liver microsomes (HLM).

2.6. Fluorescent probes for human carboxylesterase 2

Ge, Cui, and Yang et al. designed a two-photon ratiometric fluorescent probe 21 for the imaging of hCES2 in living samples (Figure 6a).[16] Two-photon excited (TPE) fluorescent probes were superior to single-photon fluorescent probes for bioimaging due to low autofluorescence background, lower photodamage and better spatiotemporal resolution. Probe 21 was derivative of 4-amino-1,8-naphthalimide (NAH), which can be rapidly released by the hydrolysis of probe 21 in the presence of hCE2. Results of the kinetic hydrolysis experiment catalyzed by enzyme indicated that probe 21 followed the michaelis-menten kinetics and showed ideal kinetic behaviors ($K_m <$ 10 μM) ($k_{cat}/K_m >$ 500 μL/min/mg of protein) in various enzyme sources. The detection limit was calculated to be 12 ng/mL. In this view, probe 21 possessed high resolution and sensitivity for the monitoring of real hCE2 activity in living cells and tissue preparations. The probe 21 were imaged not only in living cells but also in fresh slices of mouse liver. Two sets of confocal imaging data showed that probe 21 could measure endogenous CES2 in vivo through two-photon microscopy.

Beharry et al. exploited a small molecule ratiometric fluorescent chemo probe 22 for detecting hCES2 activity based on enzymes catalyze hydrolysis reactions and accompanied by fluorescence changes (Figure 6b).[38] When hCES2 was incubated with bis(p-nitrophenyl) phosphate in the presence of probe 22, a slight fluorescence increase at 605 nm could be observed. The fluorescence response was induced by the hydrolysis of aryl amide bond, which is catalyzed by hCES2. Excitingly, probe 22 has excellent water solubility and no cytotoxicity at active concentrations. Since the pharmacological action of irinotecan in terms of pancreatic cancer depends on the activity of the hCES2, probe 22 can be applied in evaluation of the therapeutic effect of irinotecan for its accurate expression on the activity of hCES2 in patient-derived xenografts. Furthermore, probe 22 can detect the activity of hCES2 in pancreatic cancer cells. Based on these two properties, it can be functioned as an effective tool to efficiently assess the physical status of patients with pancreatic cancer.

Fluorescent probes can be applied in series aspects of detection, including ions, small molecules, proteins, cells as well as pathogens. Indeed, there are currently fewer fluorescent probes for carboxylesterase detection. Carboxylesterase is a hydrolase, therefore, the design of strategies for probes detecting hydrolase and protein can enlighten the development of probes related to carboxylesterase. Tony James designed a series of probes and sensors to detect enzymes and proteins, and his method has important implications for the detection of carboxylesterase (Figure 6c).[39]

In 2020 Tony James reported a fluorogenic peptide sensor array 23 based on graphene oxide for the differential sensing of ebola virus and a ratiometric fluorescent probe 24 for the detection of penicillin G acylase in bacteria. It was worth noting that sensor 23 was a combination of a rhodamine derivative and a targeting peptide, in this plateau, it would make sense to look for carboxyesterase-specific peptide chains or proteins in order to construct a protein-recognized probe, rather than simply esterification.

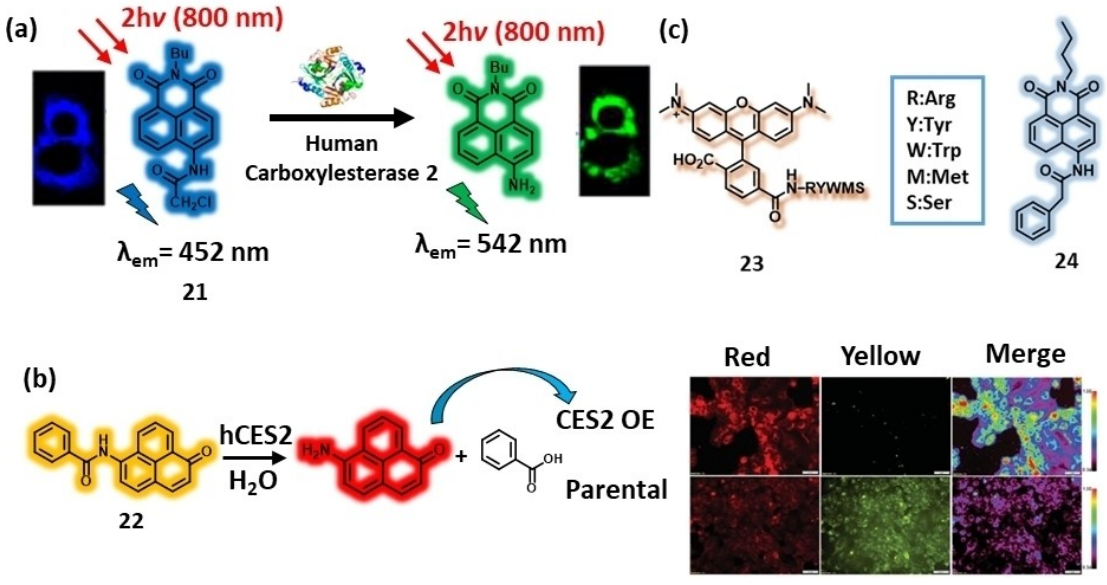


Figure 6. (a) The reactions of 21 with hCES2; Reproduced with permission from Ref. [16]. Copyright 2015, American Chemical Society; (b) The detection mechanism and living cells imaging of probe 22; Reproduced with permission from Ref. [38]. Copyright 2019, Royal Society of Chemistry; (C) The structure of 23 and 24.

3. Summary and Perspective

In brief, CESs are bound up with biotransformation of various drugs, environmental toxicants, and carcinogens containing ester groups, which means the dysfunction of CESs would lead to many diseases. The tracking of the activity of acetate can be used in disease detection, drug metabolism and biodetoxification. Although there have been major breakthroughs in the detection of CESs activity in recent years, there are still many defects in these detection methods, making it impossible to detect easily and efficiently. Thus, carboxylic ester compounds are applied in the design of fluorescence probes for the efficient and convenient detection of CESs activity. The development of fluorogenic method has blossomed in the last decades, because it possesses many excellent properties like speediness, high sensitivity, low background, and real-time visualizing CESs in living cells, tissues or organs. Fluorescent probes can be more suitable for practical applications due to their good tissue penetration and low autofluorescence and biological damage. However, fluorescent probes have some inherent limitations, for example, the samples are easily contaminated by probes and the synthetic probe has biotoxicity and poor biocompatibility, which would limit their wide application in bio-samples. Besides, compared with the large amount of other specific fluorescent probes, only a few studies have focused on the development of probes for detection of CESs activity. We hence, summarized the several recent reported CESs probes. The designed strategies as well as their applications are reviewed. We hope this review will inspire the development of novel specific CESs fluorescent probes.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Carboxylesterases · Fluorescent probe · Living cells imaging · In situ detection · In vivo detection

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