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A tyrosinase-triggered oxidative reaction-based "Turn-on" fluorescent probe for imaging in living melanoma cells

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ABSTRACT

Melanoma, with poor prognosis and highly metastatic spread, is the most deadly skin cancer, but the monitoring and diagnosis of melanoma is still a challenging. The rate-limiting enzyme tyrosinase is crucial for controlling melanin production, and is a well-known biomarker closely associated with the level of malignancy. However, effective probes for detecting tyrosinase in living cells are currently lacking. This paper describes the design, synthesis and characterization of F2, a high sensitive type of "turn-on" fluorescent probe for imaging living melanoma cells. F2 could be activated by tyrosinase-catalyzed oxidation followed by hydrolysis of a urea linkage. The results demonstrate that F2 displays cyan fluorescence when activated by tyrosinase, and has sufficient sensitivity and selectivity to detect tyrosinase in aqueous solution and in living cells. F2 has potential for the noninvasive real-time diagnosis and tracking of melanoma.

1. Introduction

Exploring biological processes and monitoring the stage of disease progression is important for physicians, patients and biologists. As a noninvasive technology, fluorescence imaging is integral to detecting diseases and physiological processes, especially skin cancer, which is one of the most common types of cancer [1–3]. Among skin cancers, melanoma is the most deadly, with very poor prognosis and highly metastatic spread. However, the fluorescence imaging of melanoma is still a challenging and there is a lack of highly sensitive Tyrosinase(TYR)-based probes [4–7]. Thus, there is a crucial and urgent demand for noninvasive monitoring and diagnosis of melanoma. TYR is an oxidase that converts monophenols or catechols to the corresponding *o*-quinone and is the rate-limiting enzyme for biosynthesis of melanin [8–10]. TYR is a potential biomarker for melanoma due to its overexpression in melanoma cells and its close association with the level of malignancy. TYR is also related with various other human diseases, such as vitiligo, oculocutaneous albinism and age spots [11,12]. TYR-based molecular imaging, in which fluorescent signal could be activated by the catalytic activity of the enzyme, is potentially a valuable tool for visually monitoring TYR-related diseases.

In the last decade, the detection of the activity of TYR mostly relied on colorimetric detection, but the methods were limited by the poor sensitivity [13–16]. In addition, several methods have been developed for the detection of TYR *in vitro*, such as electrochemistry and the fluorescence method [17–31]. Although the sensitivity has been improved by the electrochemical method, it cannot be easily and conveniently applied to living cells. The fluorescence method, which possesses the advantages of simplicity and high sensitivity and selectivity, has been widely used in living cells imaging [32–34]. So far, some fluorescent probes, such as nanoparticles [20,22,23,30], conjugated polymers [24] and small

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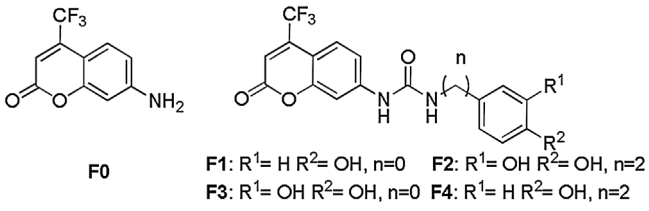

Scheme 1. Design of compounds F1–F4.

molecular organic dyes [25–27,29], have been reported for TYR detection in aqueous solution. However, most of these probes work through a "turn-off" response, which has high background fluorescence and low sensitivity. Although there are a few fluorescent molecules based on "turn-on" response mechanism, but they showed a low sensitive to TYR, due to the steric interference reason in the design of these molecules. Thus, high sensitive fluorescent molecules based on "turn-on" response mechanism are urgently needed to improve the signal-to-noise ratio for imaging living cells.

Inspired by the TYR-catalyzed bio-oxidation of monophenols or catechols, and the subsequent hydrolysis of the corresponding urea compounds, we have designed and synthesized a series of compounds, F1–F4, which respond to TYR for imaging living melanoma cells (Scheme 1) [35–38]. These probes can be divided into three parts: the phenol or *o*-dihydroxybenzene moiety which acts as the substrate of TYR; 7-amino-4-(trifluoromethyl)-coumarin (F0) which serves as the fluorophore; The urea linkage couples the two components together. F0 was selected as the fluorophore because of its small size and lower steric hindrance to TYR catalysis [39]. The probes show almost no fluorescence in aqueous media, but intense cyan fluorescence is observed when the compounds are selectively activated by TYR. The proposed reaction mechanism of the compounds in the presence of TYR is illustrated in Scheme 2. First, the probe is oxidized to an *o*-quinone intermediate, then the fluorophore F0 is released from the intermediate with the cleavage of the N—CO bond in the urea linkage.

2. Result and discussion

The long, narrow tyrosinase catalytic pocket makes it difficult for the substrate to reach the tyrosinase reactive site (Fig. S1). This poses a challenge for the design of sensitive and enzyme-responsive fluorescent probe. Thus, beforesynthesizing the designed probes, we conducted molecular docking calculations to predict whether these probes match with the catalytic site of TYR. As shown in Fig. S2, compounds F1–F4 all can dock into the catalytic pocket of TYR. The docking energy of F1, F2, F3 and F4 is −6.15, −8.16, −5.83 and −8.04 kcal/mol, respectively. The difference between the binding energies should be mainly caused by the difference in steric hindrance. Therefore, F2 and F4, with a long distance between the phenol group (substrate) and the fluorophore have a lower docking energy and easier binding than F1 and F3 (Scheme 1). Thus, the molecular docking calculation results indicated that F2 and F4 are the best potential substrates of TYR. In order to verify the molecular docking results, compounds F0–F4 were synthesized with a simple and high-yield method (Scheme S1). The chemical structures were fully characterized by ^{1}H NMR, ^{13}C NMR and ESI-HRMS (Schemes S13–30).

The optical properties of compounds F0–F4 were firstly examined in 20 mM potassium phosphate buffer (PPBS, pH = 6.5). As shown in Fig. S3, the maximum absorption peaks of F1–F4 have 19–20 nm blueshifted compared with F0, which is the result of intramolecular charge transfer effect (ICT). As expected, these compounds were almost non-fluorescent before triggered by TYR. The fluorescence quantum efficiencies of compounds F1–F4 were also lower than that of F0 (Table S1), which may be caused by the acceptor-excited photoinduced electron transfer (a-PeT) mechanism [40].

Next, the fluorescence properties of F1–F4 were investigated in response to TYR (50 U/mL) at 37 °C. As shown in Figs. S4 and S5 , the fluorescence intensities of the probes gradually increased upon incubation with TYR. The fluorescence intensities of F2 and F4 increased ~60-fold and ~15-fold respectively after 3 h. However, the fluorescence intensity of F1 increased only ~8-fold, and F3 showed almost no change. These results demonstrated that F2 and F4 are more effectively activated by TYR than F1 and F3, in accordance with the results of the molecular docking calculations. Therefore, F2 and F4 were selected for further study in medium and living cells.

It is vital that a molecular probe shows excellent sensitivity and selectivity for practical applications. Thus, the sensitivity of F2 and F4 toward TYR was evaluated. Fig. 1A and B show the fluorescence intensities of F2 and F4 after incubated with various concentrations of TYR at 37 °C for 3 h. The results indicated that the fluorescence intensities increase as the TYR concentration increased and reach a plateau when the concentration of TYR is greater than 5 U/mL. In addition, strong linear relationships ($R^2 > 0.99$) were obtained in certain ranges of TYR concentrations (Fig. 1C and D), which indicated that F2 and F4 can be used to quantitatively determine the concentration of TYR. Moreover, the reaction rate of F2 was obviously higher than F4. Taken together, these results indicate that F2 and F4 can be turned on by TYR with great sensitivity. Also, it is noting that the sensitivity of F2 and F4 are better than the reported turn-on probes (Table S2).

To further confirm that F2 and F4 were catalytically oxidized by TYR, the reaction products were subjected to HPLC (Fig. S6). As shown in Fig. S6B and S6C , the peak from F2 at 9.94 min decreased markedly after F2 was incubated with TYR for 4 h at 37 °C. Meanwhile, a new peak at 7.53 min was observed, which belongs to F0 (Fig. S6A). A similar result was also obtained from F4 (Fig. S6E). Then, we also analyzed the enzyme kinetic parameters of the probes and found that the K_m of F2 and F4 was 14 μM and 7 μM respectively (Fig. S7). These data clarified that the fluorescence enhancement of F2 and F4 is the result of the release of F0 from F2 and F4 after oxidation by TYR.

Considering the complexity of the intracellular environment, including temperature, biomacromolecule activity and physiological factors, we next investigated the selectivity of F2 and F4 activation. Probes F2 and F4 were treated with various potential interfering species, including inorganic salts ($CaCl_2$ and $MgCl_2$), amino acids (cysteine, glutamic acid and arginine), reduced glutathione, BSA, glucose, H_2O_2, Vitamin C, Human immunoglobulin G, Glucose Oxidase. The fluorescence intensities were measured after these probes were incubated with these interfering species. As shown in Fig. 2, the fluorescence intensities have very little changes following treatment with the interfering species, whereas the signals from the TYR-treated probes dramatically increased. In fact, the signal from F4 was slightly increased compared to F2 in the presence of the interfering species, but the effect was very weak. In conclusion, the data indicated that the probes F2 and F4 could be selectively activated by TYR but not by other biologically relevant species.

The effect of pH and temperature on the stability of these probes was also evaluated. The fluorescence intensities of F0, F2 and F4 in solutions of different pH are shown in Fig. S8. It was observed that the fluorescence intensities of F0 and F2 stayed almost constant in the pH range from 4.0 to 9.0. The fluorescence intensities of F4 decreased under alkaline conditions, due to ionization of the phenol group, but were stable in neutral and acidic environments. These results suggested that these probes