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COMMUNICATION

A novel fibroblast activation protein-targeted near-infrared fluorescent off-on probe for cancer cells detection, *in vitro* and *in vivo* imaging†

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A new hemicyanine-based near-infrared fluorescent probe which designs as fibroblast activation protein-targeted and shows high selectivity and sensitivity to cancer cells detection, in vitro and in vivo imaging. This probe is successfully applied to fluorescence detection of living cells (with a detection limit of 1500 cells/mL). It is believed that many new functions or distributions of FAP could be discovered by this new probe later.

Introduction

Fibroblast activation protein (FAP), also known as seprase, belongs to the clan SC proteases and is a member of the S9B prolyl oligopeptidase subfamily.[1] It plays many important roles in wound healing and tissue remodelling, liver disease, inflammatory disease, malignancy (including proliferation process of cancer cells).[2-5] Moreover, many studies have shown that FAP can be a new target in cancer therapeutics.[6-8]

In spite of the important progress made in recent years in studying FAP, there are still some problems to be solved such as its precise distribution in living organisms and mechanisms in many physiological processes. Obviously, it would be helpful to clarify these issues by the development of excellent fluorescent probes due to the fact that fluorescence spectroscopy shows not only high sensitivity but also unrivaled spatiotemporal resolution ability.[9-15] So far, some fluorescent FAP probes have been obtained.[3,16-18] Among them, there exists only one near-infrared fluorescence probe (NIR probe),[16] but polypeptide was used as the recognition group in this probe, which can lead to its complicated synthetic route. Furthermore, no existing FAP probe was used to not detect the FAP-expressed living cells but image. Thus, a NIR fluorescent probe with a simple FAP recognition group is developed herein to detect FAP-expressed living cells and image.

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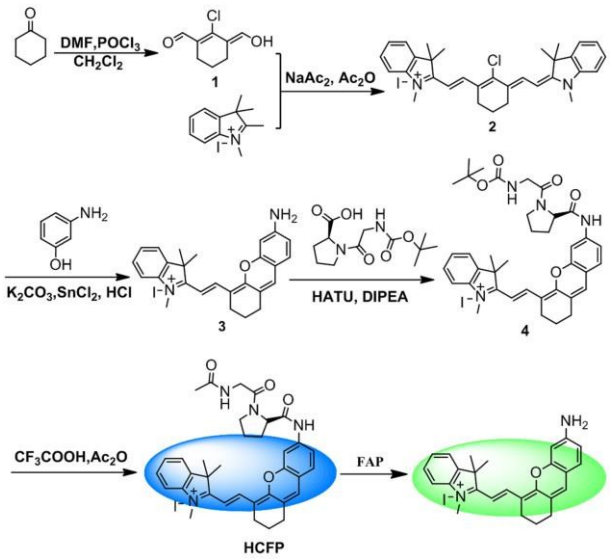
Results and discussion

Design and Synthesis

As shown in Scheme 1, the NIR FAP probe (**HCFP**) can be prepared by coupling the hemicyanine fluorophore with a dipeptide derivative (a sample recognition group for FAP) through an amido bond.[19-21] Detailed synthetic protocols and structure characterizations are provided in the Electronic supplementary information (ESI†) (Figs. S1-S4).



Scheme 1 Synthesis of probe and its reaction with FAP.

Photophysical properties of HCFP and cells detection

Fig. 1 shows the spectroscopic properties of **HCFP**. **HCFP** itself

exhibits an absorption peak at 596 nm in PBS. After reaction with MCF-7 cells (Because FAP is expressed in many cancer cells,[22-24] we choose MCF-7 cells as a model), the mixed liquid absorption peak is around 670 nm and the colour changes from blue to cyan (Fig. 1A). During this reaction, **HCFP** was degraded by FAP, and then the fluorophore is released, so the reaction system shows cyan (the colour of fluorophore), this result can be further verified by the mass spectral analysis (*m/z* = 383.2 $[M]^+$; Fig. S5) and inhibitor experiments (Fig. S6). Fig. 1B displays the fluorescence spectra of probe before and after the reaction with different cell concentrations. It can be seen that with the increase of cell concentrations, the fluorescence at 710 nm enhances accordingly. Under optimum reaction conditions (Figs. S7-S8, in order to image in cells and mice, we choose 37 °C for the following measurements), **HCFP** exhibits a good linear fluorescence response to FAP in the concentration range of 1×10^4-6×10^5cells/mL (see also inset of Fig. 1B), with an equation of $\Delta F = 1.095 \times C$ (10^4cells/mL) +1.88 (R = 0.995). The detection limit (k = 3)[25-26] of **HCFP** for cells was determined to be 1500 cells/mL. Furthermore, **HCFP** shows good selectivity and biocompatibility (Figs. S9-S10).

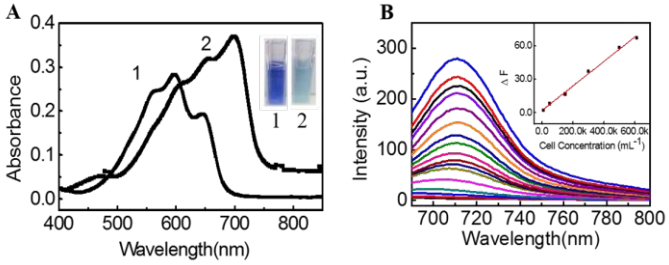


Fig.1 (A) Absorption spectra of **HCFP** (7.5 μM) before (curve 1) and after (curve 2) reaction with MCF-7 cells (2×10^6/mL) (The inset shows the color change before and after reaction). (B) Fluorescence response of **HCFP** (7.5 μM) to FAP at different concentrations in 10 mM phosphate buffer (pH 7.4) (The inset shows linear relationship between ΔF and the FAP concentration (1×10^4-6×10^5cells/mL). ΔF is the fluorescence intensity difference after and before reaction. $\lambda_{ex}/_{em}$ = 670/710 nm. (All solutions contain 0.75% DMSO as co-solvent)

Targeted validation of HCFP

Although in interference experiments we exclude many substances that may affect probe fluorescence, the effects of other substances on the probe must be ruled out because the inner cell is complex. Targeted validation of **HCFP** was performed using fluorescence confocal imaging, flow cytometry, cell transfection, and Western blotting. First. Fig. 2A and 2B show that HCFP was optimized for cell confocal imaging conditions (time and concentration), the most optimum incubation conditions are determined to be 5 μM of **HCFP** and 30 min. The small RNA interference experiments were used to specifically knock down the expression of FAP in MCF-7 cells.Control group cells, after incubation with 5 μM **HCFP** for 30 min, the cells show bright brilliant fluorescence (C1). However, the siRNA transfected MCF-7 cells show a decrease of fluorescence (C2), which means that the down-regulation of FAP, indicating the high selectivity of **HCFP** living cells. This result is further confirmed by flow cytometer results (Fig. 2D) and western blot (Fig. 2E). At the same time, the introduction of inhibitor into cells leads to a decrease of fluorescence in cells (Fig. S11), which further confirm that the fluorescence of reaction system arise from the interaction of probe and FAP.

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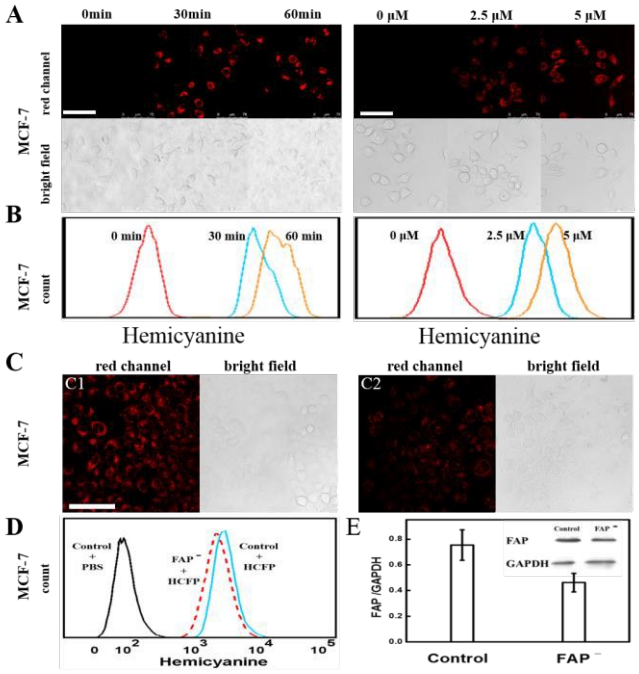

Fig.2 HCFP for Confocal fluorescence images, Flow cytometry and WB of FAP in MCF-7 cells. (A) The effects of **HCFP** (5 μM) incubation time and concentrations on the fluorescence of cells. Scale bar 75 μm.. (B) Time and concentration correspond to the flow pattern. (C1) MCF-7 cells incubated with 5 μM **HCFP** for 30 min. (C2) siRNA transfected MCF-7 cells incubated with 5 μM **HCFP** for 30 min. (D) The flow cytometer results of above (C) cells. (E) The western blot results of above cells. (All solutions contain 0.5% DMSO as co-solvent)

In vivo imaging

Finally, we investigated the applicability of the probe for visualizing FAP *in vivo*.

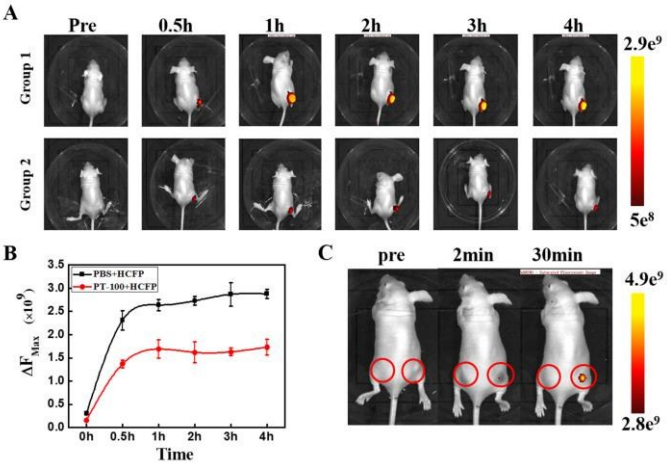

Fig. 3 Representative fluorescent images of MCF-7 tumor-bearing BALB/c mice. (A) Groups 1 were subjected to an intratumoral injection with **HCFP** (50 μL of 50 μM) for 0, 30, 60, 120, 180, 240 min, respectively. Groups 2 were subjected to an intratumoral injection with PT-100 (50 μL of 100 μM) firstly and then intratumoral injected with **HCFP** (50 μL of 50 μM). (B) Maximum fluorescence value(A) with time chart. (C) Fluorescence images of

injection **HCFP** (50 μL of 50 μM) in normal(left) and cancer tissues(right). (All solutions contain 5% DMSO as co-solvent)

As shown in Fig. 3A and 3B, the fluorescence from group 1 is bright all the time (from 30 min to 4 h) in MCF-7 tumor-bearing mice, reaches a platform in 2 h. However, the fluorescence from group 2 (pre-injection of PT-100: FAP inhibitor) is weaker than group 1. In addition, we also injected probes (50 μL of 50 μM) into different tissues of the same mice (normal tissue to the left and transplanted tumor tissue to the right). Results in Fig. 3C. shows that no fluorescence was detected on the left side of the mice and a strong fluorescence on the right side after **HCFP** was injected for 30 min. These results indicate that the probe can be used to targeted FAP and selective imaging in vivo.

Conclusions

In summary, we have successfully synthesized a new hemicyanine-based near-infrared fluorescent probe for FAP and this probe is successfully applied to fluorescence detection of living cells (with a detection limit of 1500 cells/mL). The probe shows good selectivity and near-infrared excitation and emission make the probe as a useful tool to image *in vivo*. It is believed that many new functions or distributions of FAP can be discovered by this new probe.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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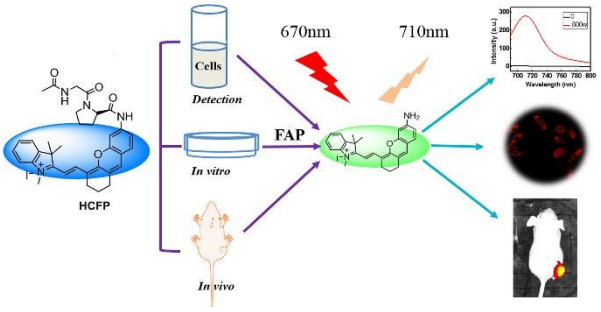

Design, synthesis a novel fibroblast activation protein "Off-ON" near-infrared fluorescent probe for cells detection, *in vitro* and *in vivo* imaging