博士論文（要約）

Multicolour Activatable Protease Probes for Fluorescence and Photoacoustic Cancer Imaging

（多色 Activatable 型プロテアーゼプローブ群の開発とがん蛍光・光音響イメージングへの応用）

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論文の内容の要旨

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Cancer imaging is now a vital part of cancer treatment as early detection and diagnosis can greatly influence patient's prognosis, and a variety of cancer imaging modalities are used for detecting different types of tumours. For intraoperative imaging, organic small molecule-based activatable fluorescent probes have been developed to achieve rapid and sensitive detection of cancer that are otherwise often missed. These include gGlu-HMRG, a fluorescent probe for γ-glutamyltransferase (GGT) based on Hydroxymethyl Rhodamine Green (HMRG) scaffold, which has achieved rapid *in vivo* tumour detection with high tumour-to-background ratio. However, not all tumours can be characterised with single enzymatic activity, and to simultaneously visualize altered activities of multiple enzymes in cancer sites, other scaffolds with distinct spectroscopic properties from those of HMRG were desired. The introductory chapter discusses the current situation of various imaging modalities in clinical settings, in particular, the modalities utilising light. The advantages of using activatable probes and multicolour imaging are also discussed.

There were no reported rhodamines bearing a hydroxymethyl group that could function as a scaffold for protease probes and have spectroscopic properties that could be differentiated from

HMRG. Thus, in Chapter 2, synthetic schemes and a novel rational design strategy to develop asymmetrically modified spirocyclising rhodamines bearing a hydroxymethyl group are introduced. Asymmetrical rhodamine derivatives were designed by extending alkyl chains from one of the unsubstituted amines of the xanthene moiety of HMRG. As a result, the maximum wavelengths of absorption and fluorescence were successfully shifted to longer wavelengths than that of HMRG. However, their equilibrium constants of intramolecular spirocyclization pK_{cycl} (the pH at which the absorbance or fluorescence of the compound decreases to a half of the maximum value as a result of spirocyclization) were larger than that of HMRG, resulting in the elevation of background fluorescence at the physiological pH of 7.4. Thus, further optimisations were attempted to the equilibrium constant by introducing electron-withdrawing halogens at the 2 position of the xanthene moiety. This resulted in successful shifting of pK_{cycl} without affecting the red-shifted wavelengths.

Some of the resulting novel rhodamine derivatives had the suitable spectroscopic properties to be the scaffolds for fluorescent probes for protease activities. Their fluorescence was distinguishable from that of HMRG, and they preferentially took the intramolecular spirocyclised form before the protease reaction along with reduced absorption in the visible region. Upon reaction with the targeted proteases, they were converted to rhodamine derivatives that preferentially took the open form to show strong fluorescence.

As a proof of concept, a red-shifted GGT probe, gGlu-HMJCR, was developed based on a novel asymmetrical rhodamine, HMJCR. gGlu-HMJCR was confirmed to activate and fluoresce in yellow/orange region of visible light upon reaction with GGT by *in vitro* and cell spheroid assays. Following this, gGlu-HMJCR and HMRG-based probes, with green fluorescence, were applied simultaneously for cancer imaging *in vivo* with mouse models of peritoneal metastases. By applying a cocktail of probes, tumours were labelled depending on their protease activity profiles and emitted

fluorescence in either one or both colours. This is the first example known to date of multicolour *in vivo* detection and discrimination of cancer according to the protease activity profile. This imaging enabled:

1. Simultaneous imaging of a wide range of tumours that cannot be characterised with a single protease activity.
2. *In vivo* distinction of tumours with different protease activity profiles, opening up a possibility for early and personalised interventions after surgery.
3. Multicolour imaging with both probes emitting fluorescence in the visible region that are strong enough to be detected by the naked human eye, suggesting good translatability in clinical settings such as intraoperative imaging.

The extension of the rational design strategy to silicon-rhodamine (Si-rhodamine) derivatives is discussed in Chapter 3. Si-rhodamines are derivatives of rhodamine with the oxygen atom at the 10 position replaced with a silicon atom, emitting fluorescence of over 600 nm. A Si-rhodamine was also reported to have a blue-shift in absorption spectra following amidation of an amine on the xanthene moiety, which could contribute to reducing background signals in *in vivo* imaging, but was not fully exploited at the time.

Since there was only one previous report of a Si-rhodamine bearing a hydroxymethyl group, other asymmetrical derivatives were synthesised to examine pK_{cycl} and wavelengths. From these data, a novel asymmetrical Si-rhodamine, HMJSiR, was designed and synthesised according to the rational design strategy developed in Chapter 2, and was shown to have suitable spectroscopic properties to be used as a scaffold for protease probes.

A GGT probe, gGlu-HMJSiR, was developed based on HMJSiR, with fluorescence in the

near infrared region and large activation ratio of over 700 fold utilising both intramolecular spirocyclisation and blue-shift in absorption by amidation. Its functions were confirmed by *in vitro*, *in cellulo* and *in vivo* fluorescence imaging.

In Chapter 4, the novel protease probes were applied to photoacoustic imaging in cells and in clinical specimens. Photoacoustic imaging is an emerging imaging modality with the capability to image deeper tissues that are unreachable by fluorescence. gGlu-HMDiMeR and gGlu-HMJSiR were both shown to function as GGT-activatable photoacoustic protease probes in cells. Since both GGT probes still retain their function as fluorescent probes, dual modality imaging using florescence and photoacoustics was possible.

In the imaging of clinical specimens, clear contrasts between cancerous legions and normal tissues were observed in both fluorescence and photoacoustic imaging. Even though there is still room for further investigation by immunostaining, the results of pathological analyses, fluorescence imaging and photoacoustic imaging were generally in good agreement.

Further research on contrast agents, hardware and image processing are required for photoacoustic imaging to provide convincing results. Nevertheless, these probes showed promising characteristics as examples of photoacoustic probes for endogenous protease activities.