Simultaneous Monitoring of Multi-Enzyme Activity

Subjects: Oncology

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The use of fluorescent imaging probes that monitor the activity of proteases that experience an increase in expression and activity in tumors is well established. These probes can be conjugated to nanoparticles of iron oxide, creating a multimodal probe serving as both a magnetic resonance imaging (MRI) agent and an indicator of local protease activity. Previous works describe probes for cathepsin D (CatD) and metalloproteinase-2 (MMP2) protease activity grafted to cross-linked iron oxide nanoparticles (CLIO). Herein, we have synthesized a triply labeled fluorescent iron oxide nanoparticle molecular imaging (MI) probe, including an AF750 substrate concentration reporter along with probes for cathepsin B (CatB) sand MMP2 protease activity. The reporter provides a baseline signal from which to compare the activity of the two proteases. The activity of the MI probe was verified through incubation with the proteases and tested in vitro using the human HT29 tumor cell line and in vivo using female nude mice injected with HT29 cells. We found the MI probe had the appropriate specificity to the activity of their respective proteases, and the reporter dye did not activate when incubated in the presence of only MMP2 and CatB. Probe fluorescent activity was confirmed in vitro, and reporter signal activation was also noted. The fluorescent activity was also visible in vivo, with injected HT29 cells exhibiting fluorescence, distinguishing them from the rest of the animal. The reporter signal was also observable in vivo, which allowed the signal intensities of the protease probes to be corrected; this is a unique feature of this MI probe design.

Keywords: cathepsin B ; matrix metalloprotease-2 ; biomarker ; near-infrared fluorescent probe ; molecular imaging

1. Introduction

Genomic and proteomic approaches have identified a host of molecular markers associated with disease [1][2][3][4]. A central challenge in contemporary biomedical research is the characterization of these factors in the context of the entire organism. Molecular imaging (MI) techniques hold great promise for mapping molecular activities in living animals, but previously reported probes are thus greatly limited in their ability to measure multiple activities simultaneously. Herein, we report the preparation of a fluorescence-based, in vivo optical imaging probe bearing three fluorescent reporters, two of which are responsive to specific protease activities.

Fluorescence-based imaging probes have been fabricated previously using a high molecular weight graft polymer on which fluorochromes were conjugated to the polymer backbone. The fluorescence from these probes was initially quenched until a particular protease cleaved the polymer backbone. Prior publications report on such probes to monitor CatD protease activity [5], MMP2 [6], and thrombin [7]. Another type imaging probe that has been fabricated previously uses iron oxide nanoparticles as a combined optical imaging and magnetic resonance (MR) agent and, in doing so, becomes multimodal [8][9][10].

A dual-fluorochrome imaging probe using iron oxide nanoparticles was described previously [11], with both enzymatic activity through a fluorescently-labeled cleavable enzyme substrate and, in vivo, via a substrate concentration through a non-cleavable internal standard. The use of these probes initially yielded fluorescence as a function of the intensity of the light used, its depth and the site of interest, and the enzyme activity and delivery of the probe (local substrate concentration) [11][12]. Here, we report on an improvement and extension of our previous dual fluorochrome by creating a triple fluorochrome probe (TFP), containing one fluorochrome to report on the local substrate concentration and two fluorophores to monitor the local activity of two enzymes, CatB and MMP2. Unlike previous synthetic strategies employed to create similar imaging probes, the technique outlined in this report pre-labels the peptide substrates prior to the conjugation of the nanoparticle scaffold. The peptide substrates are then conjugated to the nanoparticle surface, while the reporter fluorochrome (for probe concentration) is attached to the nanoparticle through a proteolytic-resistant linkage. The ratio of fluorescence due to the enzymatic cleavage of each substrate to the fluorescence of the reporter fluorochrome reflects activation by that particular protease and could be used to correct for differences in the size and depth of the target lesions. By using this method, we are able to, simultaneously in vivo, image multiple enzyme activities and multiple molecular parameters.

2. Findings

The probe design and chemistry offer a flexible design for optically activatable nanoparticles that can include different substrates for other enzymes and multiple enzymatic targets. Unlike previous synthetic strategies employed to create similar imaging probes, the technique outlined in this paper pre-labeled the peptide substrates prior to conjugation to the nanoparticle scaffold. By pre-labeling the peptide substrates with a fluorochrome, multiple enzymatic substrates with distinct optical labels can be conjugated to the iron oxide scaffold. However, as more fluorescent labels are added to the imaging probe, more sophisticated techniques such as fluorescence molecular tomography (FMT) [13][14][15] or spectral unmixing techniques [16][17][18] can increase fluorescence sensitivity or further refine and distinguish between similar optical channels, respectively.

The affixing of polyarginyl-containing regions to peptide substrates that are attached to nanoparticles has increased translocation through cell membranes. Internalization can be accomplished through the use of positively charged peptide signals, such as those derived from human immunodeficiency virus (HIV) trans-activator of transcription (Tat) protein, or homeoprotein transcription factor [19][20][21]. CLIO nanoparticles have been conjugated to portions of the tat peptide sequence, and these Tat-CLIO nanoparticles have translocated within cells [19][22][23]. Membrane translocating activity appears to be primarily dependent on the headgroup of arginine [24][25][26], so nanoparticles conjugated to peptides with simply polyarginyl regions may efficiently enter cells, as was seen with flow cytometry data (Figure 5C).

Using a single particle, the TFP multimodal imaging probe would be able to gather through in vivo imaging, in addition to lesion size and depth, new and more types of information simultaneously. By adding an optical channel that monitors the delivery of the probe, the probe can indicate its transport and concentration within the vicinity of the target as the other two fluorochromes monitor the activity of the probe interacting with its molecular target, which, in this case, is a protease. Various physiological factors, including blood flow as well as capillary permeability and volume, can affect probe transport [27][28]. These studies have shown that the TFP activation can provide information independent of the absolute fluorescence of the other two optical channels. Thus, the reporter fluorochrome (AF750) provides an internal standard for determining probe concentration and allows fluorescence from protease activity (Cy5.5 for CatB and AF546 for MMP2) to be corrected for variable levels of probe transport.

The elevated levels of enzymatic activity of CatB and MMP2 are linked to a variety of medical conditions, such as cancer metastasis [29][30][31][32]. The development of enzymatic diagnostic nanoparticles may be realized due to similar particles that are clinically used and accumulated in the liver, spleen, and lymph node macrophages. Designing the probe described in this study may provide a more accurate and global picture of enzymatic activity related to certain diseases given its ability to obtain satisfactory optical images of multi-enzymatic activity in vivo. Future imaging probes of this type might be developed for clinical use.

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