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Substrate-based near-infrared imaging sensors enable fluorescence lifetime contrast via built-in dynamic fluorescence quenching elements

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Abstract

Enzymatic activity sensing in fluorescence lifetime (FLT) mode with “self-quenched” macromolecular near-infrared (NIR) sensors is a highly promising strategy for *in vivo* imaging of proteolysis. However, the mechanisms of FLT changes in such substrate-based NIR sensors have not yet been studied. We synthesized two types of sensors by linking the near-infrared fluorophore IRDye 800CW to macromolecular graft copolymers of methoxy polyethylene glycol and polylysine (MPEG-gPLL) with varying degrees of MPEGylation and studied their fragmentation induced by trypsin, elastase, plasmin and cathepsins (B,S,L,K). We determined that the efficiency of such NIR sensors in FLT mode depends on sensor composition. While MPEG-gPLL with a high degree of MPEGylation showed rapid ($\tau_{1/2}$ =0.1–0.2 min) FLT increase (τ =0.25 ns) upon model proteinase-mediated hydrolysis *in vivo*, lower MPEGylation density resulted in no such FLT increase. Temperature-dependence of fluorescence de-quenching of NIR sensors pointed to a mixed dynamic/static-quenching mode of MPEG-gPLL-linked fluorophores. We further demonstrated that although the bulk of sensor-linked fluorophores were de-quenched due to the elimination of static quenching, proteolysis-mediated deletion of a fraction of short (8–10kD) negatively charged fragments of highly MPEGylated NIR sensor is the most likely event leading to a rapid FLT increase phenomenon in quenched NIR sensors. Therefore, the optimization of “built-

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Conflict of interest disclosure.

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Supporting figures: NMR spectra of sensors I and II, size-exclusion HPLC analysis of sensor fragments, gel electrophoresis and absorbance spectra of sensor I and II and their isolated fragments (PDF)

in” dynamic quenching elements of macromolecular NIR sensors is a potential avenue for improving their response in FLT mode.

Graphical Abstract

Keywords

near-infrared; fluorescence lifetime; macromolecule; pegylated polylysine; enzyme; NIRDye 800CW

Optical imaging continues to hold promise for clinical applications due to its low cost and safety profile, however optimization of the technique at the preclinical level is necessary. Thus far, the majority of preclinical optical imaging strategies have relied on continuous wave (CW) fluorescence excitation and registration^{1, 2}. This strategy has allowed tracking of molecular events of interest (e.g. enzyme activity) by following changes in overall fluorescence intensity of enzyme-reporting “substrate-based” as well as “activity-based” probes (or sensors, reviewed in^{3, 4}). The design of enzyme activity-reporting substrate-based sensors has traditionally relied on the effect of spatial separation of initially quenched fluorophores, such as cyanine dyes, which can be covalently linked directly to the enzyme-cleavable polymer⁵⁻⁷. Alternatively, such macromolecular sensors can harbor enzyme-cleavable pendant peptides, which are terminated with NIR fluorophores or quenchers^{8-10, 11}. These sensors are known to be strongly affected by static quenching including radiative and non-radiative resonance energy transfer, and non-radiative energy transfer either to structurally different acceptors or to molecules of the same kind, which enables exceptional flexibility in designing FLT-based sensors for imaging^{7, 12}. The simplest case of static quenching is a consequence of interaction of the individual cyanine molecules with the formation of intermolecular *H*-aggregates in polar solvents¹²⁻¹⁵. The separation of the fluorophores usually occurs as a consequence of enzyme-mediated sensor breakdown, resulting in the emission of a light photon, as opposed to non-emissive transfer within the originally quenched substrate-based sensors^{16, 17}.

Near-infrared “activatable” substrate-based macromolecular sensors are currently commercially available for imaging of proteolysis *in vivo*^{5, 18, 19}. The backbone of these sensors consists of methoxy polyethylene glycol-graft copolymers of poly-L-lysine (MPEG-gPLL, e.g. Prosense 750 EX, Perkin Elmer) with backbone peptide bonds cleavable by many cysteine- and serine proteinases. These sensors gained widespread use primarily for imaging of animal models of cancer with an aid of fluorescence mediated tomography, FMT². The substrate-based class of imaging sensors has several notable strengths, such as: 1) the preservation of the activity of target enzyme (i.e. the lack of irreversible inhibition such as in the case of “activity-based sensors that act as covalent inhibitors of proteases”⁴); 2) high solubility in water; 3) lack of toxicity at the concentrations far exceeding the doses required for *in vivo* imaging; 4) long half-life of MPEG-gPLL *in vivo* that enable accumulation in tumors due to locally increased tumor vascular permeability and retention^{20, 21}.

However, the dependence on fluorescence intensity increase as a defining feature of substrate-based sensor has several drawbacks, such as the inability to separate concentration from FLT of fluorophores since both the local concentration of sensor and lifetime of the fluorophore signal independently contribute to the measured CW intensity²². As a result, the interpretation of imaging results becomes a complex task. Further, fluorophore-labeled ‘activatable’ substrate-based sensors are usually not completely optically “silent” (i.e., 100% quenching does not occur) contributing background signal that contaminates the signal of interest. Moreover, the most serious challenge for *in vivo* imaging with substrate-based probes stems from the effect of the off-site substrate-based sensor activation occurring by means other than the biological event of interest (e.g. when activated due to the extensive degradation by liver enzymes). This generates a high non-specific “background” signal.

Time- or frequency domain FLT *in vivo* imaging has the potential to overcome such limitations. The power of the technique is based on the unique fluorescence lifetime ‘signature’ of a given fluorophore, which is proportional to quantum yield and independent of fluorophore concentration²³. Other advantages of FLT include the independence of measurements on excitation light intensity, the ability to separate the diffuse excitation signal from true FLT and multiplexing, i.e. simultaneous imaging of several fluorophores, even in the absence of spectral separation of fluorescence signals.

Our group has previously devised a time-domain (TD) *in vivo* imaging approach which uses multi-exponential data fit of fluorescence decay curves that allows fractional analysis of various amounts of long- and short-lifetime components of the same sensor^{24–26}. One of the applications of this approach enabled the detection of protease-activatable sensors *in vivo*. We demonstrated that FLT enables *in vivo* imaging of individual internal organs, which were otherwise not discernible if imaged in the CW mode²⁶. This observation was made during imaging of infarcted hearts in live rodents made possible by eliminating strong background signal originating from the liver, which had a shorter FLT than in the infarct zone²⁶. The mere presence of such short- and long- FLT components in the products of proteolytic cleavage of substrate-based proteinase sensors is somewhat paradoxical since it has been long believed that near-infrared fluorophores in MPEG-gPLL-based sensors are quenched exclusively due to static quenching and that the FLT of a sensor does not change due to exclusively static quenching effects. Here we report the results of the studies aimed at

examining the evidence of potential alternative mechanisms responsible for changes of FLT of such sensors resulting from enzymatic cleavage.

Experimental

Synthesis of imaging sensors

Sensor I. PLL hydrobromide (500 mg) with molecular mass of 46,000–55,200 Da; DP 220–260, Mw/Mn=1.05–1.1 (PL, Sigma P2636, Lot 50K5104) was dissolved at a concentration of 5.1 mg/ml in 0.1 M bicarbonate pH 8.7 prepared in degassed, nitrogen saturated dH₂O (final concentration 25 mM amino groups of PLL), chilled to 4°C and modified by slowly adding dry MPEG5000carboxy-NHS ester (JenKem Technology USA) to achieve final concentration of 16 or 30 mg/ml. Incubation of these solutions at 4°C for 24 h resulted in 21±2% (MPEG5-gPLL carrier of sensor II) and 40±3% MPEGylation (MPEG5-gPLL carrier of sensor I) as determined by TNBS titration²⁷ and verified by ¹H NMR (400 MHz, MPEG5-gPLL solutions were lyophilized and dissolved in D₂O, see Supporting Fig. S-1). The MPEG5-gPLL copolymer I contained 1.25±0.05 mM NH₂ and MPEG5-PLL copolymer II contained 2.20±0.10 mM NH₂ (10 mg MPEG-gPLL/ml). The copolymers were purified using ultrafiltration on UFP-100 columns (exclusion limit 100 kD, GE Healthcare Bio-Sciences, Pittsburgh PA) using 20L of 50 mM NaCl followed by 20 L of dH₂O. The completeness of MPEG5-carbonate removal from MPEG5-gPLL was monitored by using size-exclusion HPLC after acetylating samples with succinimidyl acetate. IRDye 800CW modification of MPEG5-gPLL was accomplished by adding a 1.1 molar excess of IRDye800CW-NHS ester solution (25 mg/ml in DMSO, Li-COR, Lincoln NE) over available amino groups to a 10 mg/ml MPEG-gPLL solution in 0.05 M sodium bicarbonate, pH 8.5. The purification was performed by using two consecutive spins on Bio-Spin P30 mini columns (Bio-Rad, Hercules CA). The purified IRDye 800-labeled sensor I contained 0.20±0.01 mM IRDye 800CW (10 mg MPEG-gPLL/ml, i.e. approximately 12 mol IRDye 800CW/mol MPEG-gPLL). The purified sensor II contained 0.50±0.04 mM IRDye 800CW (10 mg MPEG-gPLL/ml, approximately 14 mol IRDye 800CW/mol MPEG-gPLL).

Kinetics of NIR sensors activation in the presence of purified proteinases

The concentration of IRDye 800CW was normalized by using complete hydrolysis of 1 μM sensor I or II (1 mg/ml trypsin (from bovine pancreas 15,300 BAEE units/mg, Sigma-Aldrich, St. Louis MO) in Na/K-PBS (10 mM KH₂PO₄, 10 mM Na₂HPO₄, 0.1 M NaCl, pH 6.8), 2 h at 37°C and measuring absorbance at 774 nm ($\epsilon=250,000$ [M.cm]⁻¹). To perform measurements of NIR dye fluorescence intensity or lifetime changes as well as absorbance spectrum changes as a result of enzyme-mediated fragmentation the sensors were diluted in Na/K-PBS to concentrations 0.25–2.5 μM and test enzymes were added from stock solutions at the ratio of 1 mol enzyme/2.5–5 moles of sensor. Human recombinant cathepsins B, K, L and S (specific activity >5000 -7,500 mU/mg, BioVision Inc, Milpitas CA), human elastase (purified, > 8 U/mg, Worthington Biochemical Corp, Lakewood NJ) and human active plasmin (Abcam, Cambridge MA) were used without purification. The change in absorbance and FI over time was recorded by using a SpectraMax M5 (Molecular Devices, Sunnyvale CA) plate reader in 96-plates in a volume of 50 μl. The trypsinization reactions were stopped at various time points using tosyl-L-lysyl-chloromethane hydrochloride, TLCK (0.05 mM

final concentration, Sigma-Aldrich). To stop all other fragmentation reactions we used 5x cOmplete™ protease inhibitor (Sigma-Aldrich) added from stock solutions in DMSO. To analyze fragmentation of sensors they were subjected to HPLC using calibrated 10x300 mm Superose 6 Increase (GE Healthcare BioSciences) eluted at 0.5 ml/min in 0.1M ammonium acetate with UV/VIS detection. For electrophoretic (EF) analysis the samples were diluted with 10%SDS/Tris, pH 7.0 (1:1), heated to 95°C for 1 min and loaded on 4–15% TGX™ Protein gradient gels (Bio-Rad) in parallel to IRDye800CW-labeled and purified Precision-Plus protein standards (Bio-Rad); 0.65% agarose gels (run in TAE buffer at 70V) were used to resolve non-denatured fractions of sensors. Upon the completion of EF runs the gels were scanned by using Odyssey NIR Imaging system (Li-COR, Lincoln NE) and/or FLT imaging system described below and the obtained TIFF images were analyzed using ImageJ software. If required, the NIR-fluorescent fragment fraction bands were cut from agarose EF slabs, minced on ice and spun down in a microcentrifuge at 16,000xg for 30 min. Absorbance spectra were measured in supernatants of isolated spun-down fragment bands in micro-cuvettes after 1:1 (vol/vol) dilution. Isolated fragments were also subjected to gradient EF as described above.

Time domain fluorescence lifetime imaging

The primary excitation source was a femtosecond pulse tunable Ti:Sapphire laser (Mai Tai, Newport-Spectra Physics, Mountain View, CA 150-fs pulse width, 80-MHz repetition rate, 750–850-nm tuning range). The detection was performed in free space using a CCD (Picostar HR-12 CAM 2, LaVision GmbH, Goettingen, Germany) with a quantum efficiency of 65%. A time gated image intensifier (Picostar HR-12, LaVision), with a 0.2-ns minimum gate width was used to provide nanosecond time resolution. All FLT imaging done in our study was done using 750-nm excitation and a filter for 800-nm longpass emission detection. The nonlinear fits for the multi-exponential lifetime analysis were numerically implemented using the Matlab function, “fminsearch,” using the Nelder–Mead simplex approach ²⁸.

Time-dependent fluorescence intensity measurements of NIR sensor activation in cell culture

In cell culture experiments, the sensor fragmentation was identified and monitored by measuring near-infrared fluorescence in intracellular vesicles. Cells (human fibroblasts (D551, CRL-110) and human squamous carcinoma cells (A431, CRL-1555, both ATCC) were cultured using 10% FBS in Eagle's MEM or DMEM and used 60% confluent after plating in 24-well plates. Activation of D551 cells with FGF2 was performed by adding 5 ng/ml human recombinant FGF-Basic protein (AA 10–155, ThermoFisher Scientific, Grand Island NY). Sensors were incubated with the cells at a concentration of 0.5 μM for 2–24h and fluorescence intensity changes in intracellular vesicles were measured over time using a Nikon TE2000-U inverted microscope equipped with a 100 W dialluminator and Nikon 800 nm fluorescence filter sets. Images were acquired using a CoolSnapHQ-M CCD (Photometrics, Tucson AZ) and processed using IP Lab Spectrum software (BD Biosciences Bioimaging, Rockville MD). To obtain mean fluorescence readings in individual vesicles, an image segmentation routine was applied by setting region-of-interest (ROI) limits using vesicle area descriptors, which enabled the exclusion of noise (individual pixels), as well as large fluorescent artifacts originating from in-plane but out-of-focus cells. Approximately

100 data points were obtained from a single image and fluorescence intensity values were corrected for background fluorescence that originated from the scattered excitation light. For fragmentation analysis and FL imaging cells were propagated in T-25 cell culture flasks, incubated in the presence of sensor I (0.5 μ M in cell culture medium) and removed from the plastic using enzyme-free Cell Dissociation PBS (Thermo-Fisher).

Results and discussion

Sensors with differential proteolysis-mediated fluorescence signal changes

The backbone copolymer for the sensors used in this study were synthesized by linking MPEG polymer chains to a polylysine backbone for providing protection from rapid removal of the sensor from circulation, and simultaneously affording slower degradation of the backbone by proteinases^{29, 30}. If poly-L-lysine was used for synthesis as a backbone, the resultant copolymer MPEG-gPLL is still readily cleavable by many cysteine- or serine proteases, including cathepsins^{31–33} as well plasmin. Cyanine fluorophore-modified MPEG-gPLL sensors are stable *in vitro* at physiological pH and, regardless of the degree of MPEGylation, the length of MPEG chain or PLL they remain largely non-emissive, i.e. “silent” in the absence of proteases. Hypothetically, proteinase-mediated breakdown of peptide bonds within a macromolecular sensor should result in a spatial separation of a fraction of cyanine fluorophores from the neighboring dyes that initially caused non-emissive “silent” states. Conversely, a high local density of MPEG chains would result in a higher local viscosity, which in turn 1) would give a longer FLT due to slower cyanine dye rotation (i.e. longer correlation times); and 2) interfere with the formation of *H*-aggregates. In addition, it was expected that the sensors with higher MPEGylation would have slower rates of enzymatic cleavage than sensors with lower MPEG content due to “crowding” effect of MPEG chains that slows down enzyme diffusion in the vicinity of the cleavable backbone. We synthesized several classes of substrate-based sensors of which two had similar chemical composition but different density of MPEGylation of the backbone (Table 1). We assumed that the latter would influence the density of NIR fluorophore linking to the backbone⁷. The length of the MPEG protective chain was intentionally kept constant at 5kD, which has a size-exclusion distribution coefficient of a 30 kD globular protein³⁴. Trypsin is traditionally used as a model serine protease capable of cleaving MPEG-gPLL substrate-based sensors. We therefore initially subjected the sensors to trypsinization to investigate changes of fluorescence intensity, lifetime and track the fragmentation.

Under the conditions of pseudo-first order kinetics of proteolysis (i.e. when the MPEG-gPLL concentration exceeded trypsin by approximately 5 fold), we noticed differences in behavior of IRDye 800CW-modified sensors I and II despite the fact that they had approximately the same number of fluorophores linked per MPEG-gPLL carrier (Table 1). As expected, sensor I with the higher density of MPEG-chains showed slower kinetics of fluorescence intensity increase, while sensor II was cleaved much more rapidly under the identical conditions (Fig. 1A). Moreover, trypsinization of sensor I resulted in a change of absorbance spectrum (Fig 1B), with a blue-shifted peak (λ_{max} =705 nm, usually attributed to the presence of IRDye 800 CW *H*-dimers, or aggregates³⁵) decreasing over time, whereas the absorbance peak of dye monomers (λ_{max} =780 nm) was increasing in intensity. In the

case of sensor II, both absorbance peaks were initially increasing over time (at least for 20 min after the addition of the enzyme, Fig. 1C). The differences observed in absorbance spectra could be a result of 800CW fluorophores interacting with each other and/or the backbone of the sensor (i.e. pol-L-lysine backbone) more strongly in sensor II than in sensor I: sensor II-linked 800CW residues showed incomplete disaggregation, possibly due to larger stretches of non-modified protonated N-ε amines of PLL forming an intramolecular polyionic complex. Trypsinolysis resulted in slow but more efficient 800CW dis-aggregation in sensor I fragments as the blue-shifted peak of fluorophore aggregates decreased in intensity.

Further, we noticed that the initial FLT values measured for the fluorophores linked to sensor I and sensor II were different (Fig. 2), while fluorescence intensities were both low. Upon the addition of trypsin the shorter FLT of IRDye 800CW in sensor I rapidly increased to the value similar to that of sensor II after the addition of trypsin. Unlike FI change, it took seconds for the transition of a short FLT sensor I to the products with longer FLT compared to the intensity change: we observed NIR dye FLT increase ($\tau=0.25$ ns) with a half-time of $\tau_{1/2}=0.1-0.2$ min, while the fluorescence intensity increase took minutes ($\tau_{1/2}=10-15$ min). In other words, substantially shorter reaction times were needed to enzymatically “activate” sensor I in FLT mode than in fluorescence intensity mode. It should be noted that FLT values of non-cleaved sensor I showed no fluorophore concentration dependence in non-activated (“silent”) state (0.35 ± 0.05 ns in a concentration range of $0.25-2.5$ μ M) or in activated (fragmented) state (0.53 ± 0.01 ns, 20 min after activation with trypsin). Based on similar fluorescence intensity and lifetime measurements, sensor II was cleaved more rapidly ($\tau_{1/2}=1.5-2$ min), however, the resultant overall FLT increase was an order of a magnitude lower, i.e. less than 0.01 ns. The commercially available NIR imaging sensor (Prosense 750 EX, Perkin Elmer), which is structurally related to the sensors used in this study also showed nearly negligible changes in FLT after the addition of trypsin. At later time points (24 h), and in the presence of higher trypsin amounts (which resulted in high trypsin/sensor molar ratios), the FLT value of sensor I fragments showed a tendency to decrease slowly. This effect appears to correlate well with the effect of macromolecule fragmentation and the removal of rotational barriers for 800CW molecules. Overall, the observed changes in FLT as a result of enzymatic cleavage of sensor I indicated that by switching to FLT imaging from CW mode one could potentially image the presence of proteolytic activity much earlier than with fluorescence intensity imaging using a suitable sensor. Considering the importance of this observation for *in vivo* imaging, we set forth to find an explanation for 1) rapid transition of FLT and 2) the difference between the types of sensors we used for testing resulting in differential FLT changes.

Temperature dependence of FI in intact sensors

We determined that static quenching of IRDye 800CW was clearly present in both sensors I and II. The increase of FI of 800CW upon proteolysis (Fig. 2) and changes of the blue-shifted 710 nm absorbance peaks of dye aggregates (Fig. 1) attested to this in both cases. However, the observed trypsin-mediated increase of IRDye 800CW FLT (Fig. 2) indicated the potential role of an alternative fluorescence quenching mechanism. We suspected that dynamic (collisional) quenching in addition to static quenching could be responsible for the

increase of FLT. We made this assumption based on observation that the initial FLT of IRDye 800CW in sensor I was short but measurable: in the case of 100% static quenching FLT of IRDye 800CW would not differ from the “background lifetime” of the excitation light pulse. Therefore, we performed measurements in an oxygen-free temperature-controlled environment to establish temperature-dependent patterns of IRDye fluorescence intensity changes. Such measurements were expected to provide data that differentiates between dynamic and static modes of NIR fluorophore quenching. Sensor I showed an initial decrease of fluorescence intensity (FI) with the increase of temperatures from ambient (300–310K) to 350K. Further increase of the temperature resulted in FI increase. Sensor II showed only a very small initial “dip” in FI with temperature increase within the same range of temperatures, i.e. FI was nearly independent of temperature below 323K (50°C) but showed a dramatic increase in FI with a further increase of temperature (Fig. 3). A decrease in FI after the initial heating of the sensor I solutions indicates dynamic quenching. Temperature dependence was clearly more pronounced in the case of sensor I as compared to sensor II. However, the pattern of relative FI increase pointed to the combined effects of both dynamic and static quenching of NIR fluorophores because further temperature increase above 350K resulted in the increase of FI (Fig. 3).

Fragmentation analysis

The exposure of both sensors to trypsinization resulted in a time-dependent increase of 1111 as a consequence of backbone cleavage since only the backbone peptide bond hydrolysis by trypsin would result in a spatial separation of NIR fluorophores and the eventual disappearance of static quenching (Fig 1A). The detailed analysis of trypsinization products showed that the treatment of IRDye 800CW-labeled MPEG-gPLL sensors with trypsin for short periods of time (10–15 min) resulted in the formation of multiple fragments. Some of those fragments could be resolved as independently eluting fractions on Superose6 gel-permeation HPLC columns (Fig. S-2). All MPEG-gPLL substrate-based sensors are brush-like copolymers³⁶, and the elution patterns of intact and fragmented MPEG-gPLL resemble much heavier globular polymers: we observed the disappearance of the initial single peak of sensor I after the first 5 min of proteolysis and 4 major peaks of IR Dye 800CW-carrying fragments with hydrodynamic diameters corresponding to globular proteins of 1300, 100, 38.5 and 8 kD were formed. With time, the larger fragments and the 8 kD fragment were further degraded resulting in the formation of 22 kD and 1.6 kD fragments. In the latter fragments were the major fraction containing NIR fluorophore.

Agarose gel electrophoretic analysis of sensor I and II showed that before the addition of trypsin the sensors migrated as large, weakly positively charged macromolecules with very low 800 nm fluorescence intensity (λ_{ex} = 750 nm). Fluorescence lifetime of non-cleaved sensors in the gels could not be measured with sufficient precision due to very low intensity of emitted light, suggesting very efficient quenching. Agarose electrophoresis further showed the formation of slowly migrating positively charged fluorescent fragments at the early time points after the addition of trypsin along with the release of negatively charged fluorescent fragments (Fig. S-3A). The reason for the observed difference in charge (and, consequently, the direction of electrophoretic migration) is due to the high degree of modification of some fragments with negatively charged IRDye 800CW fluorophore each

molecule of which carries four sulfonates. Direct FLT imaging of the gels showed a longer FLT (0.55–0.65 ns) of the positively charged fragments, while the negatively charged sensor I fragments included a fast-migrating component with short FLT (0.35–0.45 ns), as well as at least two slower migrating populations of fragments with FLT values similar to that of the positively charged ones. The trypsinization of sensor II did not result in fast migrating, negatively charged fragments with short FLT. Instead, two populations of negatively charged fragments with longer half-lives were formed. By performing a gradient SDS-polyacrylamide gel electrophoresis, we confirmed that the fragmentation of MPEG-gPLL was non-random (Fig. S-3B). Non-cleaved sensors were entering but not migrating in polyacrylamide gels due to their large mass and hydrodynamic radius. However, their fragments showed well-defined ladder-like patterns that differed slightly for sensor I and sensor II, which were synthesized using PLL backbones with similar masses (compare I.4 and II.4 in Fig. S-3B). The fast-migrating negatively charged fragments were represented by small fragments of sensors both in case of sensor I and II (lanes I.1, I.2 and II.2). Interestingly, while both sensor I and II gave rise to the formation of 8–21 kD fluorescent fragments, only sensor I released small fragments with high content of *H*-aggregates of the fluorophore as evident from absorbance spectra taken after isolating fragments resolved in agarose gels (Fig. S-3C,D). It is possible that such negatively charged small fragments could form intra-molecular polyelectrolyte complexes within the MPEG-gPLL based carrier backbone, which carries residual positive charge due to protonation of free N-ε-amino groups of lysines.

In addition to trypsin in fragmentation assays we used cysteine and serine proteases some of which were previously known to cleave MPEG-gPLL NIRF probes, i.e. cathepsins B, K, L, S, and plasmin^{37, 38}. Since there are similarities in substrate specificities of cathepsin L, S, plasmin and trypsin we anticipated that due to inherently incomplete MPEGylation the exposure of cleavable amino acid stretches on sensors would result in similar fragmentation patterns by these enzymes. Indeed, the observed patterns of enzyme-mediated trypsinization of sensor I and II were nearly identical when cathepsins L and S, plasmin or elastase were used instead of trypsin (Fig. 4A). It should be noted that due to preferred cleavage of sequences that have two small hydrophobic amino acids next to a positively charged one (Val-Val-Arg), cathepsin S was less efficient cleaving enzyme than cathepsin L as judged by fluorescence of sensor fragments that were larger than 100 kD. Elastase also showed less efficient fragmentation. However, the ability of elastase to cleave PEGylated polylysines was unexpected since elastase cleaves proteins rich with hydrophobic amino acids (-Ala-Pro-Val sequence). While cathepsin B has been frequently identified as one of the major contributing factors to MPEG-gPLL activity-based sensor activation *in vivo*^{39, 40}, very little fragmentation of sensor I or II by catalytically active human recombinant cathepsin B was observed, with no low molecular weight fragments released even after a 2h incubation. Cathepsin K which was not expected to catalyze fragmentation of the sensors showed approximately the same level of fragmentation as cathepsin B (Fig. 4). This could be the consequence of cathepsin B specificity to highly positively charged amino acid sequences (e.g. Arg-Arg), and preferred hydrolysis of relatively long stretches of non-polar amino acids (His-Pro-Gly-Gly-Pro-Gln) by cathepsin K. It should be noted that complete fragmentation of sensors in the presence of higher specific activity of trypsin (1:2.5 molar ratio, 8U trypsin/

μ g MPEG-gPLL) showed differences between sensors I and II as only three major fluorescent bands were detected in sensor I digest (approximate average molecular weights of 19, 15, and 8 kD) while sensor II and Prosense 750EX did not show the same level of degradation under the same conditions since larger fragments were still present after 24h (Fig. 4B). This result was consistent with size-exclusion HPLC analysis sensors I and II (Fig. S-2).

To determine whether small fragments were released by proteinases on a time scale comparable to the observed increase of IRDye 800 CW fluorescence lifetime, we conducted a more detailed study of fragmentation kinetics of both sensor types. Electrophoresis revealed a rapid accumulation of small molecular mass fragment (8 kD) within the first minutes of sensor I proteolysis (Fig. 4C). In contrast, in the case of sensor II all small fragments (7.5 kD, 21 kD) were released at much slower rates (Fig. 4D). The combined evidence of this electrophoresis data and spectral analysis of the fast-migrating negatively charged fragment released from sensor I (Fig. S-3) suggests that the only fragment that had overall low fluorescence intensity, short fluorophore lifetime and high relative content of IRDye 800CW dimers was matching with the 8 kD fragment, while all other fragments released from sensor I had higher masses (Fig. S-3C). Furthermore, our comparative analysis of the fractional composition of major degradation products suggests that a strongly negatively charged fragment with low molecular weight was rapidly cleaved off sensor I. Due to its rapid release from the sensor I it is likely that this fragment participated in dynamic quenching of the bulk of non-aggregated IRDye 800CW molecules, since FLT increase upon sensor I fragmentation initially was also very rapid (Fig. 2).

Fragmentation of sensor I in cell culture

Further experiments were performed *in vitro* to compare the extent of sensor I fragmentation in normal human skin fibroblasts and skin cancer cells to determine if differential sensor proteolysis in endolysosomal compartment of normal vs. cancer skin cells could point to a potential source of *in vivo* FLT contrast. The analysis of sensor I activation was performed using control and basic fibroblast (FGF-2) factor-stimulated human D551 fibroblasts and A431 epidermoid carcinoma cells. While non-activated fibroblasts showed very low fluorescence measured in intracellular vesicles after a 24 h incubation with the sensor ($FI=8\pm2$ AU), FGF-2 stimulated fibroblasts showed an increase to $FI=30\pm4$ AU over the same period of time. The average FLT value of IRDye 800CW derived from the imaging maps of suspensions of stimulated cells was also longer: 0.40 ± 0.01 vs. 0.31 ± 0.03 ns. In intact vesicles of A431 cells 800CW fluorescence intensity was 3-times higher than in fibroblasts and FI showed an increase to 0.69 ± 0.01 ns (Fig. 5). The sedimentation of cell suspension and imaging of the tubes containing supernatant and cell precipitates clearly demonstrated the presence of long FLT sensor component, which was cell-bound. In an attempt to identify the fractions of sensor I which were responsible for cell fluorescence and long FLT values, we fragmented the cells and isolated the cytoplasmic and cytoskeletal/nuclear fractions (Fig. 5E). In fibroblasts, the latter was devoid of fluorescent fragments of the imaging sensor, while the cytoplasmic fraction contained a single 14–18 kD fragment with high FI. Unlike fibroblasts, A431 cells contained larger amounts of the fragment of the same size as in fibroblasts in both subcellular fractions. A431 cells also retained a smaller

(8–10 kD), as well as much larger fragments of the sensor. Therefore, sensor incubation with the cells in culture suggested that the retention of sensor I fragments in the cytoplasm of cells resulted in either complete hydrolysis of the sensor with the elimination of very small (<10 kD) fragments in human FGF-2 activated fibroblasts, or an incomplete hydrolysis with the retention without elimination in A431 cells. Both of these scenarios could potentially result in a longer average FLT observed in sedimented cells than averaged FLT of cell suspensions, indicating that intercellular accumulation of sensor fragments is responsible for the long FLT that we previously were able to image in the areas of local inflammation after experimental myocardial infarction ²⁶.

Conclusions

The key findings of this study point to the dependence of FLT of NIR fluorophores on composition and structure of macromolecular proteinase-sensitive carrier molecules to which the fluorophores are linked. The working model of the substrate-based sensor that describes the conclusions derived from our experiment is displayed schematically in Fig. 6. The major structural parameter affecting the fluorescent properties of sensors appears to be the local distribution of fluorophores along the carrier backbone, which is potentially influenced by MPEGylation of the backbone molecule. The extent of MPEGylation appear to influence the distribution of the fluorophores along the backbone carrier molecule (MPEG-gPLL) resulting in aggregated (dimerized) as well as non-aggregated cyanine fluorophore. While the majority of fluorophores are engaged in static self-quenching in both sensors due to formation H-aggregates, the non-aggregated fluorophores can be engaging in intramolecular collisional interactions (Fig 6, Sensor I). If present, the fragments of sensors that act as structural elements responsible for collisional interactions can be quickly released as a result of proteinase-mediated cleavage resulting in a rapid increase of FLT that exceeds the rate of FI increase (see Fig. 6). We anticipate that measurements performed after *in vivo* administration of substrate-based sensors using dedicated imaging equipment will greatly assist in differentiating between signals arising from organs with low levels of proteolytic activity and the organs of the reticuloendothelial system, where the fragmentation of sensors is rapid due to high activity of proteinases and the fragments of sensors are eliminated rather than retained.

Supplementary Material

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Figure 1.

A - kinetics of fluorescence intensity increase as a result of sensor I and II trypsinization at enzyme/sensor molar ratio of 1:5 ([IRDye800CW]= 1 μ M), λ_{ex} =750 nm/ λ_{em} =800; **B** - initial time-dependent changes of sensor I normalized absorbance spectra after the addition of trypsin measured at the indicated time points. Time-dependent decrease of fluorophore H-aggregate peak intensity is shown by an arrow; **C**- initial time-dependent changes of sensor II normalized absorbance spectra after the addition of trypsin measured at the indicated time points. Note that concentrations of sensors and enzyme used for absorbance spectra measurements were 4-times higher than in Fig. 1A.

Figure 2. Time-dependent changes of IRDye 800 CW fluorescence lifetime (FLT, color symbols) and intensity (FI, black/white symbols) of sensor I (open circles) and sensor II (closed circles) after the addition of trypsin at a molar ratio of 1:5 (trypsin/sensor).

Figure 3. Temperature dependence of IRDye 800CW fluorescence intensity ($\lambda_{\text{ex}}=750$ nm/ $\lambda_{\text{em}}=800$) for sensor I (closed squares) and sensor II (open squares). Fluorescence changes are shown as a ratio (F_1/F_0) of fluorescence intensity measured at a given temperature to the initial intensity measured at approximately 300K. Data is shown as mean $\pm$ SEM (n=3).

Figure 4.

Electrophoretic analysis sensor fragmentation. **A-** fragmentation of sensor II in the absence (-) or the presence of cathepsins B, K, L, S, plasmin (P), elastase (E) and trypsin (T) in the presence of approximately 4U of enzyme/ μ g sensor, 2 h at 37°C. **B-** the results of control (-) or experimental (+) complete enzymatic hydrolysis (24 h, 37°C, 8 U trypsin / μ g sensor) of sensor I, II and Prosense 750 EX. The position of 8kD fragment migration is indicated by an arrow; **C-** time-dependent fragmentation of sensor I and **D-** sensor II in the presence of trypsin (1 mol/2.5 mol sensor) shown as a % of total band area for major fragments determined by digital densitometry (ImageJ). The trypsinization reaction was stopped at the time points indicated by adding TLCK and the samples were analyzed by EF. Data was obtained by semi-quantitative image analysis obtained by using near-infrared (800 nm channel) images (Odyssey imaging system (Li-COR) by determining the area of bands. NIR fluorescent images (A,B) were inverted for better displaying.

Figure 5.

The uptake of sensor I in FGF-2 activated human D551 fibroblasts and A431 carcinoma cells. A,B - fluorescent microscopy of intracellular vesicles at 2 (A) and 24 h (B) after the co-incubation of the sensor (0.5 μ M) with FGF-2 activated D551 human fibroblasts. bar- 20 μ m. C- fluorescence lifetime maps of cells (2.10⁶/ml) incubated with sensor I (100 nM) for 24h. D- the intensity and FLT image of a suspension and sedimented A431 cells; E - 4–15% gradient SDS-PAGE of D551 and A431 cell homogenates (S- supernatant (cytoplasmic) and P -precipitate - nuclear+ cytoskeletal fractions). Near-infrared (800 nm channel) images were obtained using Odyssey imaging system (Li-COR) and inverted for display.

Figure 6.

A schematic showing hypothetical mechanisms of sensor proteolysis that lead to differential changes of FLT in the case of sensor I and sensor II. Sensor I (has a short FLT and low FI in its intact form) carries small and potentially mobile elements that create sites exposed to proteinase cleavage (shown by red arrows) that carry pairs of self-quenched fluorophores (*H*-aggregates, shown in blue) that are engaged in dynamic quenching of other nearby fluorophores (shown as dashed blue double arrows). Proteinase cleavage (“fast” phase, solid arrow) rapidly cleaves these fragments off resulting in an increase of FLT due to removal of dynamic quenching. The first phase is followed by the slow second phase (dashed arrow) during which the fragments are subjected to further proteolysis with the resultant increase of FI. Sensor II (having initially a longer FLT and low FI in its intact form) has the most of the fluorophores engaged in static quenching due to the formation of *H*-aggregates, (shown in blue) alongside with non-quenched ones. Proteolysis (“slow” phase) results in formation of smaller fragments carrying non-quenched fluorophores as well as quenched pairs and free fluorophores (which have short FLT and potentially represent a large fraction of products formed at the later stage of proteolysis).

Table 1

Comparative properties of NIRF substrate-based sensors.

Substrate-based sensors	MPEGylation, % modified amino groups (mean $\pm$ SD, n=4)	Calculated mass, kDa	Hydrodynamic diameter, nm (Zav, range by LALLS), mean $\pm$ SD (n=4)	IRDye 800CW, (mol/mol MPEG5-gPLL)*	Proteolysis rate(trypsin), pseudo-first order, k_{eff} 10^{-3} [s] ⁻¹ (mean $\pm$ SD, n=3)
Sensor I (MPEG5-gPLL)	40 $\pm$ 3	565	99 $\pm$ 41	12	0.59 $\pm$ 0.15
Sensor II (MPEG5-gPLL)	21 $\pm$ 2	280	47 $\pm$ 20	14	4.30 $\pm$ 0.71

* Calculated using extinction coefficient of 250,000 [M $\cdot$ cm]⁻¹ at 774 nm