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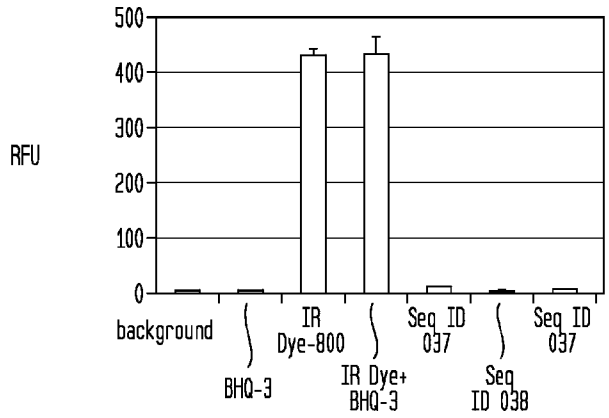
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(54) Title: ENZYME-CLEAVABLE DYE-CONTAINING FLUOR-QUENCHER CONSTRUCTS

FIG. 3



(57) Abstract: This invention relates to novel compounds that contain a fluor and a quencher linked by an enzyme cleavable substrate. The constructs contain fluor/quencher moieties that are optically silent until cleaved by enzymes, in a preferred embodiment by matrix metalloproteases (MMPs). After cleavage, the constructs become fluorescent - the resulting fluorescent moieties provide imageable emissions that can be used for in vitro evaluation of enzyme activity or for in vivo detection of disease. In a preferred embodiment the fluor is detectable in the hear infrared (NIR). Unlike imaging methods that provide information on morphology, these constructs provide biochemical information about the disease.

Enzyme-Cleavable Dye-Containing Fluor-Quencher Constructs

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S.S.N 61/078,014, filed July 3, 2008, all of which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] Novel constructs for in vitro assays and for in vivo fluorescence imaging, using a construct of the form Fluor-enzyme cleavable substrate-Quencher. In a preferred embodiment , the fluor emits fluorescent emissions in the Near Infrared and the quencher is a moiety that quenches the fluorescence of the fluor by contact quenching such as, for example, IR Dye800CW-(enzyme cleavable substrate)-BHQ-3. In another preferred embodiment, the enzyme cleavable substrate is cleaved selectively by one or more MMPs and is not substantially cleaved in vivo by enzymes present in normal tissue or blood, such as Neprilysin.

BACKGROUND OF THE INVENTION

[0003] Arthritis is a highly debilitating disease that can take many forms, including osteoarthritis, rheumatoid arthritis, septic arthritis, gout and pseudogout, juvenile idiopathic arthritis, Still's disease and ankylosing spondylitis. The most common form of arthritis, osteoarthritis (degenerative joint disease) is a result of trauma to the joint, infection of the joint, or age. Rheumatoid arthritis and psoriatic arthritis are believed to be autoimmune diseases in which the body attacks itself. Septic arthritis is caused by an infection of the joint. Gouty arthritis and pseudogout are respectively caused by deposition of uric acid or calcium pyrophosphate crystals in the joint, causing inflammation.

[0004] Diagnosis and staging of the disease can be challenging, as many of the symptoms are nonspecific. Diagnostic and monitoring methods can include physical examination, scoring systems to determine the degree of symptoms such as pain and inflammation, laboratory tests, and imaging studies.

[0005] The laboratory tests used are non-specific, and most cannot provide a definitive diagnosis – in practice, a variety of tests are required. For example, Rheumatoid Factor is only present in about 70 to 80 percent of adults who have rheumatoid arthritis and may be found in patients that do not have the disease. Erythrocyte Sedimentation Rate (an indicator of the presence of nonspecific inflammation) only suggests that inflammation is

present. C-Reactive Protein (CRP) is elevated when any acute inflammation or infection is present. Anti-cyclic Citrullinated Peptide Antibody (anti-CCP, a blood marker that can be elevated in subjects with rheumatoid arthritis) is seen in many subjects that do not yet show clinical signs of the disease. Positive tests for Antinuclear Antibodies (ANA) are suggestive of a variety of autoimmune diseases, including lupus, scleroderma, Sjogren's syndrome, and rheumatoid arthritis, but only 30 to 50 percent of rheumatoid arthritis patients express this marker.

[0006] Complete Blood Count provides information on elevation or depression of the WBC (white blood cell count), RBC (red blood cell count), hemoglobin, hematocrit, several red blood cell indices and a platelet count. Elevated white blood cell counts suggest the possibility of an active infection, but patients taking corticosteroids may have an elevated WBC due to the medication. Chronic inflammation can cause a low red blood cell count. Low hemoglobin and hematocrit may be indicative of anemia associated with chronic diseases or possible bleeding caused by medications. The platelet count is often high in rheumatoid arthritis patients, while some potent arthritis medications can cause platelets to be low.

[0007] Human Leukocyte Antigens (HLA Tissue Typing) are proteins on the surface of cells. Specific HLA proteins are genetic markers for some of the rheumatic diseases, including ankylosing spondylitis and rheumatoid arthritis; however, such markers only indicate the potential for disease. High levels of uric acid in the blood can indicate the potential that crystals have been deposited in the joints and tissues, causing painful gout attacks. For certain types of systemic rheumatic diseases, biopsies of certain organs can provide important diagnostic information. Also, joint fluid analysis can provide a doctor with many details about the health of a person's joint.

[0008] In practice, several laboratory tests are required to confirm the diagnosis of, for example, rheumatoid arthritis, and many patients begin treatment without a firm diagnosis.

[0009] Imaging methods that have been used for the detection and staging of arthritis so far include X-ray imaging, MRI and ultrasound imaging. Biopsies are also used, but are highly invasive.

[00010] X-Ray imaging has the limitation that by the time erosions of the bone can be seen, the disease has already progressed to the point of irreversible damage. X-Ray provides no information about the causative agents for the observed destruction of bone and cartilage. MRI, although it is able to detect inflammatory changes such as edema, synovitis, and bone

loss, is expensive and time-consuming to carry out. Again, MRI provides no biochemical information regarding the nature of the agent(s) responsible for bone and cartilage destruction. Ultrasound has also been used for the diagnosis and staging of arthritis, but again, provides only morphological information. When diagnosing or treating a disease such as arthritis, it is important to identify morphological changes caused by the disease, but a determination of the biochemical changes that are occurring, especially the presence of the enzymes that ultimately cause tissue damage, would be highly advantageous.

[00011] Biopsy can sometimes provide definitive diagnosis and the resulting tissue can be used to determine the presence of disease markers such bone-degrading enzymes, which are overexpressed in the disease state. However, this technique is highly invasive and is not suitable for following changes in disease over time.

[00012] In the treatment of debilitating diseases such as arthritis, it is useful to know if the disease is responding to the drugs that are being administered. For example, initial treatments for arthritis typically rely on drugs that are inexpensive, but if the disease fails to respond, drugs that are significantly more expensive can be prescribed. As the disease is very heterogeneous, it is not possible, a priori, to determine which patient will respond to which drug. Response to treatment can be slow, and it is difficult to determine if the patient is going to be responsive or non-responsive without a prolonged wait to determine if a drug is effective. During this time, damage can occur in the joints of non-responsive patients.

[00013] It is known that the proteolytic enzymes known as Matrix metalloproteases (MMPs), for example MMPs such as MMP-2, MMP-9 and MMP-13, are increased in arthritic conditions such as rheumatoid arthritis (RA), where they are expressed, for example, by synovial cells, chondrocytes, and macrophages in synovial fluid, cartilage and invasive synovial pannus. Active MMPs cleave selective cut sites in cartilage and bone and can also cause proteolysis of tissue. MMPs have been reported to be responsible for the degradation of bone, tendons and cartilage in arthritis. Levels of MMP-1, 3, 9, and 13 have been reported to be strongly stimulated by inflammatory cytokines such as TNF-α and IL-1, for example. Expression of MMP-2 and MMP-9 correlates with bone erosions (but with wide variation). Patients who are responding to therapy are known to show declines in joint total MMPs.

[00014] MMPs are also known to be upregulated in various types of cancer and in artherosclerosis. The MMPs play an important role in tissue remodeling which occurs in angiogenesis, cirrhosis, metastasis and various inflammatory processes.

[00015] Some MMPs can be detected in the serum using laboratory assays, but such tests do not provide information about their expression levels in a particular involved joint. At present, MMP levels in joints of patients afflicted by arthritis are most readily detected using biopsy, a highly invasive procedure (vide supra).

[00016] The situation is confounded by the fact that although many in vitro assays for MMPs exist, they are typically unable to distinguish between the Proenzyme form of the MMP (ProMMPs), which are proteolytically inactive, and active MMPs, which are capable of enzymatic cleavage. MMPs may also be present in tissue or blood as complexes with TIMPs (Tissue inhibitor of matrix metalloproteinase) or as α-macroglobulin complexes. These latter forms of MMP are also inactive. As these in vitro assays for MMPs cannot distinguish between overexpression of Pro, active and TIMP forms of the MMP, the existing assays only provide information on total MMP levels. Furthermore, only active forms of MMPs cause proteolytic damage.

[00017] MMP expression also correlates with carcinoma survival time. For example, in one study, patients with matrilysin-positive carcinoma had a significantly shorter overall survival time than did those with matrilysin-negative carcinoma (H Yamamoto, J Clin Oncol. 2001 Feb 15;19(4):1118-27). K. Sakata et al (Int J Oncol. 2000;17(4):673-81) found that MMP-2, MT1-MMP, TIMP-2, and MMP-9 and down-regulation of TIMP-1 may contribute to the development or enhanced growth capacity of ovarian tumors. Over-expression of MMP-2 has been correlated with poor prognosis in cervical cancer (B Davidson et al, Gynecol Oncol. 1999 Jun;73(3):372-82. Other cancers where MMPs have been implicated include breast, colorectal, endometrial, lung, pancreatic, thyroid and other cancers.

[00018] Increased expression of MMPs has also been demonstrated in the setting of myocardial ischemia, reperfusion injury, and during the progression to congestive heart failure. MMPs are also believed to be major contributors to the progression of atherosclerotic lesions. They are also suspected of contributing to the progression of chronic obstructive pulmonary disease (COPD) and multiple sclerosis (MS), and are implicated in periodontal disease as well.

[00019] Several therapeutic strategies based on blocking these enzymes using various matrix metalloproteinase inhibitors have been attempted.

[00020] The use of Near Infrared ("NIR") fluors has been exploited for optical imaging. When excited with the proper wavelength of light, fluorescent dyes absorb light,

which places the dye in an excited state. The dye then returns to the ground state from the excited state by emitting light (fluorescence) of a different (longer) wavelength than that of the light used to excite the compound. This emission can be detected using fluorescence detectors. Detection methods include reflectance imaging and fluorescence molecular tomography ("FMT"), among others.

[00021] It has been reported that certain NIR emitting fluors such as Indocyanine Green and analogs can be used to detect arthritis and tumors. The fluor is injected, and the fluorescence emitted in parts of the body (e.g. the tumor or arthritic joint) is detected as a function of time. Several fluorescent agents that contain a targeting group have also been reported. It has been found in studies with such constructs that the kinetics of clearance of the fluorescent compound from diseased tissue is modified relative to that of normal tissue, so by watching clearance curves, the presence of such diseased tissue can be detected. This is due either to changes in pharmacokinetics of untargeted agents between diseased and normal tissue, or due to specific targeting, for fluorescent constructs that contain a targeting group such as a receptor binding moiety.

[00022] However, these constructs, which have a structure that is different than those described in the instant invention, are always fluorescent. A fluorescence signal is also present in normal tissue, potentially reducing the ability to detect the disease. In addition, studies with such fluors do not provide information about the biochemistry (in particular the presence of proteases) in the affected tissue.

[00023] In an attempt to solve this problem, some compounds have been developed having the general formula Fluor-Enzyme Cleavable Substrate-Quencher that are optically silent due to the phenomenon known as FRET, or Fluorescence Resonant Energy Transfer, also known as Förster type energy transfer. In this quenching method, when the fluorophore is excited by light of a proper wavelength, a photon from the energetically excited fluorophore (the "donor") serves to raise the energy state of an electron in another molecule, (the "acceptor") to higher vibrational levels of the excited singlet state. In the process, the energy level of the donor fluorophore returns to the ground state, without emitting fluorescence. The acceptor can be another fluorophore or a non-fluorescent molecule. If the acceptor is a fluorophore, the transferred energy can be emitted as fluorescence. If the acceptor is a non-fluorescent molecule (or quencher), no fluorescence is observed. In such constructs, fluorescence is "quenched" until the linker between the fluor and quencher is broken.

[00024] For example, Weissleder et al (US2006/0275775) have described intra-molecularly-quenched near infrared fluorescent probes comprising a polymeric backbone and a plurality of NIR fluorochromes that are covalently linked to the backbone in such a way that the fluors are quenched by one another until they are "activated" by enzymatic cleavage. The constructs do not have the same composition as those described herein.

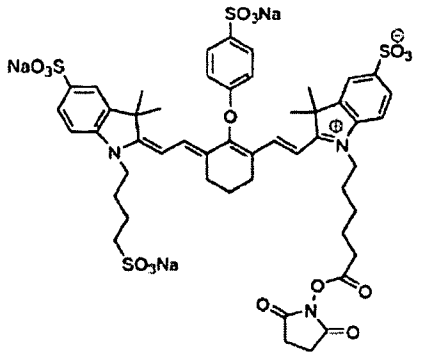
[00025] Several such constructs that are quenched by FRET have also been prepared using visible fluors for use in *in vitro* assays, for example, DNA probes that use FRET detect many types of reactions involving DNA, RNA, proteins, and inorganic substances (see e.g. Vladimir V. Didenko, DNA Probes Using Fluorescence Resonance Energy Transfer (FRET): Designs and Applications, Biotechniques, 2001 November ; 31(5): 1106–1121). The specific limited distance at which energy transfer is effective forms a basis for utilization of FRET as a "molecular ruler", measuring 0.5–10-nm distances within biomolecules with high precision (Selection of Fluorophore and Quencher Pairs for Fluorescent Nucleic Acid Hybridization Probes, Salvatore A. E. Marras, Methods in Molecular Biology: Fluorescent Energy Transfer Nucleic Acid Probes: Designs and Protocols. Edited by: V.V. Didenko). Since such fluor-enzyme cleavable linker-quencher constructs do not emit fluorescence in the NIR, they cannot be used for in vivo applications.

[00026] In order for FRET to occur, several constraints must be satisfied.

- The mechanism is highly dependent on the dipole orientations of the molecules – if they do not orient properly, no FRET quenching occurs.
- The degree of FRET quenching is strongly affected by the distance between the donor and the acceptor molecule. Typical effective distances between the donor and acceptor molecules are in the 10 to 100 Å range.
- Another significant requirement is that the donor fluorescent dye's emission spectrum must overlap the absorption spectrum of the acceptor or quencher, as shown in Figure 1.

[00027] However, for many dyes that emit fluorescence in the Near IR, suitable quenchers to provide the proper spectral overlap between the emission wavelength of the fluor and the absorbance wavelength of the quencher needed for FRET do not exist. A case in point is the NIR fluor known as IRDye 800CW, whose structure (as its N-hydroxysuccinimidyl ester) is shown below.

Structure of IRDye800 CW



[00028] The emission maximum for IRDye800CW has been reported to be above 800 nm.

[00029] Table 1 below summarizes the absorbance maxima of the commercially available Quenchers:

Table 1: Absorbance Maxima of Commercially Available Quenchers

Quencher	Absorbance Maximum (nm)	Quencher	Absorbance Maximum (nm)
Deep Dark Quencher	430	Black Hole Quencher 2	580
Dabcyl and Dabsyl	475	Deep Dark Quencher II	630
Epoche Eclipse	530	Blackberry Quencher 650	650
Iowa Black FQ	532	Iowa Black RQ	645
Black Hole Quencher 1	534	QSY-21	660
QSY-7	571	Black Hole Quencher 3	670

[00030] Other quenchers such as Black Hole Quencher 0 and QXL520 are also known to those skilled in the art. Their absorption maxima are suitable for quenching fluors that fluoresce in the visible, not in the NIR. None of the fluors in the table above has the proper spectral overlap for FRET to occur with a NIR fluor such as IRDye800CW, as there is very poor overlap between the absorbance spectrum of these quenchers and the emission spectrum of IRDye800CW or other NIR fluors. The quencher that has an absorption band that is closest

to that of the wavelength of the fluorescent emission maximum of IRDye800 (Black Hole Quencher 3) is not satisfactory for FRET.

[00031] In another attempt to solve this problem, some mechanisms have been developed whereby a fluor and quencher can interact to cause quenching of fluorescence, namely static (or contact) quenching, wherein interaction of a fluorophore with quencher gives rise to a stable, non-fluorescent complex. Unlike the dynamic quenching observed with FRET, static quenching occurs when the donor and acceptor molecules are in the ground state. The donor and acceptor molecules bind together to form a ground state complex, an intramolecular dimer with its own unique properties. Typically such a construct has a unique absorption spectrum that is diagnostic for this mechanism. Such contact quenching can be a dominant energy transfer mechanism for some reporter-quencher probes. A special case of static quenching is self-quenching, where the fluorophore and the quencher are identical.

[00032] However, for contact quenching to occur, the fluor and quencher should be close to one another, as shown schematically in Fig. 2, and described in Salvatore A. E. Marras, Selection of Fluorophore and Quencher Pairs for Fluorescent Nucleic Acid Hybridization Probes, Methods in Molecular Biology: Fluorescent Energy Transfer Nucleic Acid Probes: Designs and Protocols. Edited by: V.V. Didenko. In, for example, probes that can hybridize, bringing fluor and quencher close to one another, contact quenching is observed. However, if the fluor and quencher are separated, contact quenching is not observed, and the compounds fluoresce. This rule has been used to determine nanometer distances in biomolecules.

[00033] In light of the limitations of present diagnostic tools and methods, a compound capable of being used in a method to detect active MMPs in the body, especially MMPs that are overexpressed in diseased states is needed. It would also be highly useful to have a fluorescent compound that could detect the presence or absence of MMP proteolytic activity and that could be used to determine the efficacy of matrix metalloproteinase inhibitors.

[00034] For example, if relative MMP (protease) levels in joints could be detected by administration of an imaging agent that gave rise to an imaging signal that was generated selectively in the presence of active MMPs, then such a compound would be useful to determine disease severity or response to therapy. Such a construct would provide a simple way to detect the enzymes that give rise to damage in joints. When using such a construct, patients with active disease (high levels of MMPs), or patients who are not responding to drugs for the treatment of arthritis would have higher signal in their joints than responsive

patients. Sequential monitoring would show response to therapy faster and more accurately than it can be assessed by pain scores or other current imaging modalities.

SUMMARY OF THE INVENTION

[00035] The present invention relates, *inter alia*, to constructs (also referred to herein as compounds) which comprise a fluorescent moiety that is linked to a quencher by an enzymatically cleavable substrate where the linkage prevents the compound from emitting fluorescence (also referred to herein as being "optically silent" or "non-fluorescent") until the linkage is cleaved. In certain embodiments of the present invention, the linkage can be an enzyme-cleavable substrate. Upon cleavage by an enzyme, the fluorescent moiety and the quencher become separated, allowing the generation of a fluorescent signal if illuminated with light. In embodiments described below the light is in the Near Infrared range.

[00036] Light in the Near Infrared (NIR) is known to have the ability to pass through living tissues with significantly less scattering and absorption than does visible light. Fluorescent emission in the NIR can penetrate tissue of joints such as those in the hand and be detected externally using, for example, a CCD camera or other fluorescence detection equipment. Therefore, for in vivo applications, the use of compounds that have the potential to fluoresce in the NIR is preferred.

[00037] In one aspect of the present invention, the compounds of embodiments of the present invention have been shown to be cleaved selectively by enzymes such as active MMPs, generating, post cleavage, a fluorescent signal that can be used for imaging. These constructs comprise a fluorescent moiety that is linked to a quencher by an enzymatically cleavable substrate; the substrate prevents the compound from emitting fluorescence due to the enforced proximity of the fluor and quencher moieties. Upon cleavage of the enzyme-cleavable substrate by the MMP, the fluorescent moiety and the quencher become separated and migrate away from each other in solution as they are no longer tethered by the substrate. The resulting distance between the fluor and the quencher exceeds the maximum distance allowable for the quenching of the fluor by the quencher. Thus the fluorescence emission due to the fluor is no longer quenched and the emitted light can be detected when the system is illuminated with light in the Near Infrared. The presence and magnitude of a fluorescence signal in an affected tissue such as a joint of a patient with rheumatoid arthritis, following administration of Fluor-enzyme cleavable substrate-Quencher constructs of the present invention, can provide valuable diagnostic information about the nature and levels of the enzymes present at the imaged site.

BRIEF DESCRIPTION OF THE DRAWINGS

[00038] FIG. 1 depicts a donor fluorescent dye emission spectrum overlapping the absorption spectrum of an acceptor or quencher.

[00039] FIG. 2 is a diagram of contact quenching.

[00040] FIG. 3 is a bar graph showing quenching of Fluor-Enzyme cleavable substrate quencher constructs.

[00041] FIG. 4 depicts in vitro cleavage curves of IRDye800CW-enzyme cleavable substrate-BHQ3 constructs by MMP-2, 9 and 13.

[00042] FIG. 5 is a schematic of the synthesis of the SEQ ID NO. 053.

DETAILED DESCRIPTION OF THE INVENTION

[00043] Applicants have now unexpectedly and surprisingly found, contrary to what was expected based on FRET or contact quenching, that good quenching (that is quenching suitable for diagnostic use) occurs in constructs where a Near IR fluor such as IRDye 800CW and quencher such as Black Hole Quencher 3 are linked to an interposed enzyme-cleavable substrate that serves to separate them from one another by several amino acid residues. In such constructs, significant quenching via either FRET or contact quenching is not expected to occur.

[00044] The present invention is directed, *inter alia*, to constructs which comprise a fluorescent moiety that is linked to a quencher by an enzymatically cleavable substrate where the linkage substantially prevents the compound from emitting fluorescence (also referred to herein as being "optically silent" or "non-fluorescent") until the linkage is cleaved. The linkage can be an enzyme-cleavable substrate. Upon cleavage by an enzyme, the fluorescent moiety and the quencher become separated, allowing the generation of a fluorescent signal if illuminated with light in the Near Infrared.

[00045] Embodiments of the invention are directed to novel fluor/quencher constructs, particularly those containing the fluor known as IRDye800CW and analogs thereof and the quencher known as Black Hole Quencher-3 (BHQ-3) and analogs thereof. The fluor and quencher are linked to one another by an enzyme cleavable substrate and are substantially optically silent (non-fluorescent) until such cleavage occurs.

[00046] Further embodiments of the invention are also directed to the use of these compounds for imaging, comprising the administration of said constructs to a mammalian

species, followed by the imaging of the fluorescence that is generated. There are applications for in vitro assays as well.

[00047] In a preferred embodiment, the fluor and quencher of the constructs are linked by an enzyme cleavable substrate that can be selectively cleaved by enzymes known as Matrix Metalloproteinases.

[00048] In an especially preferred embodiment, the enzyme cleavable substrate, which links the fluor and the quencher, can be selectively cleaved by one or more MMPs but cannot be substantially cleaved in vivo by the enzymes present in normal tissue or blood, such as the enzyme known as Neprilysin.

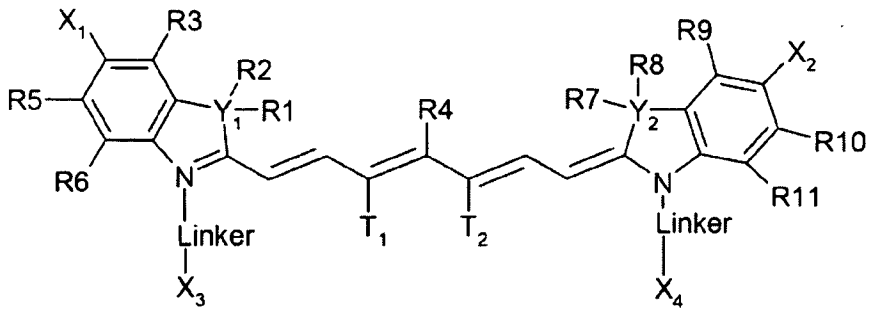
[00049] The enzyme cleavable substrate may be a peptide, a polypeptide, a monomer, a dimer, a multimer, a peptidomimetic, a non-peptide, an antibody fragment, an antibody (humanized or non-humanized), a protein, a hormone, a growth factor, a cytokine or a drug. Peptides, monomers, dimers and multimers may optionally contain one or more unnatural amino acids and or D-amino acids. In a preferred embodiment, the enzyme cleavable substrate is a peptide, and in an especially preferred embodiment, the peptide is a substrate for one or more MMP. One or more of the residues in the enzyme cleavable substrate may optionally be substituted with solubilizing charged or uncharged substituents such as SO_3^-, or COO^-, or with polymers (e.g PEG, polyglycine, polyproline, polyhydroxymethylacrylate, polylysinealkyl amines or N-acylated polylysines, polyaspartic acid, polyglutaminc acid or quanidines) to modify its pharmacokinetics.

[00050] In one embodiment the fluorescent dye in the fluor-enzyme cleavable substrate-quencher construct is a dye that is capable of emitting fluorescence with an emission maximum wavelength from about 700 to about 900 nm. Suitable dyes include, for example tetrapyrrole, tetraazapyrrole, xanthine, phenoxazine, phenothiazine, and especially polymethine dyes such as cyanine dyes. This includes those having indocarbocyanine, indodicarbocyanine and indotricarbocyanine skeletons, such as those described previously (Licha, K (2002) Contrast Agents for Optical Imaging in Topics in Current Chemistry – Contrast Agents II, (Editor: W. Krause), Vol 222, Springer Heidelberg, pp. 1-31 and references therein) and derivatives of the NIR emitting Alexa Fluor dyes, cyanine dyes such as Cy5.5 and Cy7.0 and the like. Several commercially available candidate fluorochromes that can be used to prepare the compounds of the present invention are known including IR Dye® 800CW, IRDye® 680, and IRDye® 700DX (LI-COR Biosciences), Cy5.5 and Cy7 (GE Life Sciences), and Alexa® Fluor 750 (Invitrogen) and HiLytePlus™750 available from AnaSpec.

[00051] Cy5.5 has excitation/emission maxima at 675 nm/694 nm, making it a borderline candidate labeling agent for in vivo applications. In contrast, a recently developed fluorochrome, IRDye 800CW, has its excitation/emission maxima at 785 nm/810 nm, precisely centered in the region known to give optimal signal to background for optical imaging in living systems.

[00052] Constructs wherein the fluor is a derivative of Alexa Fluor 750, HiLytePlus750, or IRDye 800CW are preferred, with IRDye800CW being particularly preferred.

[00053] In a preferred embodiment, the fluor is a construct of Formula I:



wherein

R1, R2, R5, R6, R7, R8, R9, R10 and R11 are each independently H, Me, substituted or unsubstituted alkyl, halo, carboxy, amino, sulfonate, R12-COOH, R12OR13, R12SR13, or R12COOR13, wherein R12 is a bond or alkylene and R13 is a substituted or unsubstituted alkyl,

R4 is –O-Aryl, NH-Aryl, substituted or unsubstituted Aryl, substituted or unsubstituted C-1-C10 alkyl, halo, S-Aryl or SO_2-Aryl, where the aryl ring can, in all cases, be substituted or unsubstituted. Alternatively, it may be a linker (e.g. a substituted or unsubstituted alkyl, substituted or unsubstituted Aryl, PEG, alkyl amine or quanidine) that is either covalently bonded (e.g. via an amine, thiol or acid functionality) to an enzymatically cleavable substrate or contains a reactive moiety suitable for coupling to an enzyme cleavable substrate.

T1 and T2 are each H, or are joined together to form a substituted or unsubstituted 5- or 6-membered ring.

X1, X2, X3 and X4 independently represent SO_3H, COOH or physiologically acceptable salts thereof, or polyethylene glycol, or one or more of X1-X4 may be a linker (e.g. a substituted or unsubstituted alkyl, substituted or unsubstituted Aryl,

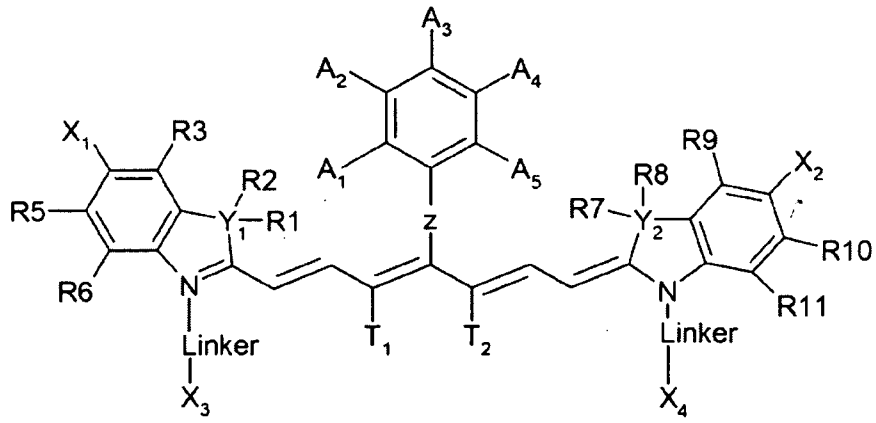
PEG, alkyl amine or quanidine) that is either covalently bonded (e.g. via an amine, thiol or acid functionality) to an enzymatically cleavable substrate or contains a reactive moiety suitable for coupling to an enzyme cleavable substrate,

or X1 and R3 and/or X2 and R9 can be cyclized together to form a 5- or 6 membered saturated or unsaturated ring that is optionally derivatized with H, substituted or unsubstituted C1-C6 alkyl, polyethylene glycol, substituted or unsubstituted aryl, halogen, a cyano, carboxy, and/or a sulfo group(s).

Y1 and Y2 are independently C, N (in which case R2 and/or R8 is absent), or O, S, Se, sulfide, sulfone or sulfoxide (in which case both R1 and R2 and/or R7 and R8 are absent).

[00054] The Linker is a substituted or unsubstituted C1-C10 alkyl chain, a polyethylene glycol derivative, or a substituted or unsubstituted aryl group.

[00055] Also preferred are compounds containing a fluor of the structure shown below (Formula II),



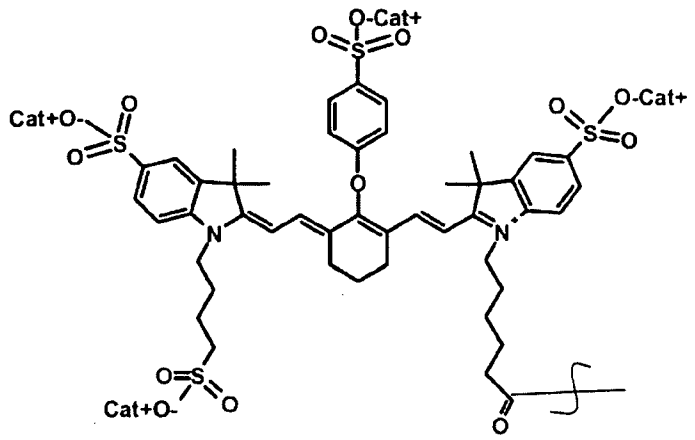
wherein

Z is –O-, -S-, -S(-O)-, -SO_2- -NH, HN-NH- or N-Alkyl, or absent and

wherein A1-A5 are each independently H, alkyl, halo, carboxy, alkylcarboxy, amino, alkylamino, amino alkyl, or SO_3^-Cat+,

wherein Cat^+ is a H^+ or a cation, and the remaining residues are defined as described above for Formula I.

[00056] Also preferred are Fluor-Enzyme cleavable substrate-Quencher compounds containing a fluor of the structure shown below:



[00057] where Cat+ is a cation. Cations include, but are not limited to H^+, Na^+, K^+, NH_4^+, meglumine, or a protonated organic base. The cation is preferably one that is physiologically acceptable.

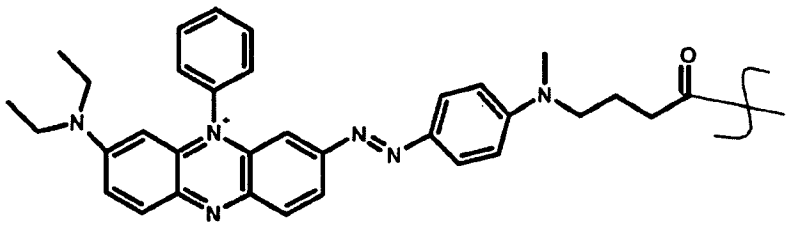
[00058] Also preferred is the fluorophore known as HyLitePlus750, a proprietary NIR fluorophore whose structure is unknown. It is commercially available from AnaSpec.

[00059] Other fluors suitable for use in embodiments of the present invention include the various fluors that emit in the NIR region that have been developed in recent years, including derivatives of the various cyanine compounds reported in EP 1 480 683 B1 (Kawakami et al, Near Infrared Fluorescent Contrast Agent and Method for Fluorescence Imaging), Indocyanine Green (as described by Li, X. et al, SPIE 2005, SPIE Vol. 2389 p. 789, Tumor Localization Using Fluorescence of Indocyanine Green (ICG) In Rat Models), Indocyanine Green tetrasulfonate (WO2004/065491 A1, Licha et al), IRDye 700DX (as described in Phthalocyanine dye as an extremely photostable and highly fluorescent near-infrared labeling reagent, Xinzhan Peng et al. Proc. of SPIE Vol. 6097 60970E, (2006) in Optical Molecular Probes for Biomedical Applications, ed. S. Achilefu, D. J. Bornhop, R. Raghavachari), IRDye800CW and analogs reported by Li-Cor (Lugade et al, US6,995,274), HiLytePlus™750, and those compounds described in US7297325 (Achilefu et al, Hydrophilic light absorbing compositions for determination of physiological function), for example, all of which are hereby incorporated by reference.

[00060] **Quenchers:** The quenchers on the Fluor-Enzyme cleavable substrate-Quencher constructs are electron deficient compounds that comprise diazo-aromatic or nitro-aromatic rings. Quenchers useful in embodiments of the present invention include Deep Dark Quenchers, Dabcyl, Eclipse, Iowa Black FQ and RQ, Blackberry Quencher 650, QSY-

7, QSY-21, and Black Hole Quenchers 0, 1, 2, and 3. It is to be appreciated that other quenchers known to those skilled in the art can also be used.

[00061] The quencher shown below (Black Hole Quencher 3) is particularly preferred.



[00062] An enzyme cleavable substrate is a key component of the Fluor-Enzyme cleavable substrate-Quencher constructs of the invention. This substrate may be branched or unbranched, and may be a substrate for any proteolytic enzyme, although compounds comprising a sequence that is selectively cleaved by one or more active MMP(s) are preferred.

[00063] Referring now to Fig. 3, applicants have surprisingly discovered that in certain IR Dye800 CW-enzyme cleavable substrate-BHQ-3 constructs, good quenching is observed. This is shown schematically FIG. 3. BHQ-3 alone does not give rise to fluorescence, as shown by the low RFU value observed (RFU = relative fluorescence units). IRDye 800CW, or mixtures of IRDye 800CW and BHQ-3 do not exhibit fluorescence quenching, instead they give rise to fluorescence when illuminated by NIR light. However, for the three representative IRDye800 CW-enzyme cleavable substrate-BHQ-3 constructs shown (Seq ID 037, 038 and 039, provided at the same concentration at that of IRDye800CW or BHQ-3 alone), good quenching of fluorescence is found.

[00064] In vitro studies with these and other Fluor-Enzyme cleavable substrate-Quencher constructs of the invention, showed that the constructs are cleaved by active MMPs, such as MMP-1, -2, -3, -7, -8, -9, -10, and -13, generating significant NIR fluorescence. These studies are described in detail in the Experimental section, and the relative rate of cleavage of the constructs by various enzymes are shown in Table 3 (below).

[00065] Such constructs have the potential to be useful for the diagnosis and staging of arthritis and other diseases where active MMPs are implicated. In addition to playing an important role in arthritis, the family of human matrix metalloproteinases (MMPs) and their specific inhibitors play important roles in tumor progression and the metastatic process –

MMPs are known to facilitate extracellular matrix degradation as well as degradation of bone and cartilage.

[00066] The enzyme cleavable substrates need not be substrates for active MMPs. It should be understood that based on the teachings herein, several IR Dye800CW-enzyme cleavable substrate-BHQ-3 constructs can be constructed, having sensitivity to and selectivity for the wide range of proteolytic enzymes that exist in nature.

[00067] Specific examples of such constructs that are cleaved by MMPs are those shown in Table 2 below having Seq ID 037, 038, 039, 040, 041, 042, 043, 044, 046, 047, 048, 051, 052, 053, 054, and 055.

Table 2. Characterization Data of Intermediate Peptides and Fluorogenic Substrate Peptides

Seq. ID	Peptide Sequence	M. Wt	HPLC Data (System, t_R, min)	Mass Spectral Data (Mode: ion)
Seq001	Fmoc-Lys(ivDde)-Pro-Leu-Gly-Leu-Lys-Ala-Arg-NH_2	1310	A, 7.29	Pos. ion: [M+H]: 1310.7
Seq002	Fmoc-Gly-Pro-Leu-Gly-Leu-Lys-Ala-Arg-NH_2	1032	A, 6.24	Pos. ion: [M+H]: 1032.6; [M+H]/2: 517.0
Seq003	Fmoc-Pro-Leu-Gly-Leu-Lys-Ala-Arg-NH_2	975	B, 6.29	Pos. ion: [M+H]:975.2; [M+H]/2: 487.5
Seq004	Fmoc-Lys(Boc)-Pro-Leu-Gly-Leu-Lys-Ala-Arg(Pmc)-OH	1471	C, 7.78	Pos. ion: [M+H]:1471.2; [M+H]/2: 735.8
Seq005	Fmoc-Abu4-Pro-Gln-Gly-Leu-Lys-Ala-Arg-NH_2	1075	D, 4.69	Pos. ion: [M+H]: 1075.4
Seq006	Fmoc-Pro-Cha-Gly-Smc-His-Ala-Lys-NH_2	1000	A, 5.87	Pos. ion: [2M+H]: 2001.8; [M+H]:1000.4
Seq007	Fmoc-Pro-Leu-Ala-Leu-Lys-Ala-Arg-OH	990	C, 6.69	Pos. ion: [M+H]: 990.4
Seq008	Fmoc-Pro-Cha-Gly-Nva-His-Ala-Lys-NH_2	982	A, 5.94	Pos. ion: [M+H]: 982.4
Seq009	Fmoc-Abu4-Pro-Cha-Abu4-Smc-His-Ala-Lys-Ala-Lys(Boc)-NH_2	1415	C, 6.92	Pos. ion: [M+H]:1415.6; [M+H]/2: 707.0
Seq010	Fmoc-Abu4-Pro-Cha-Abu2-Smc-His-Ala-Lys-Ala-Lys(Boc)-NH_2	1415	B, 6.80	Pos. ion: [M+H]:1415.2; [M+H]/2: 707.0
Seq011	Fmoc-Pro-Leu-Gly-Met-Lys-Ser-Arg-OH	1010	D, 5.66	Pos. ion: [M+H]:1010.4
Seq012	Fmoc-Pro-Leu-Ala-Tyr-Lys-Ala-Arg-OH	1040	C, 5.75	Pos. ion: [M+H]:1040.8
Seq013	Fmoc-Pro-Leu-Ala-Tyr-Trp-Met-Arg-Lys-Ala-Arg-NH_2	1512	D, 6.21	Pos. ion: [M+H]:1513.2; [M+H]/2: 756.5
Seq014	Fmoc-Pro-Cha-Smc-His-Ala-Lys-NH_2	943	A, 4.41	Pos. ion: [M+H]: 944.4; [M+H]/2: 472.3

Seq015	Fmoc-Abu4-Pro-Gln-Gly-Leu-Ala-Lys-NH_2	919	C, 5.34	Pos. ion: [M+H]: 919.4
Seq016	Fmoc-Abu4-Pro-Gln(Trt)-Gly-Leu-Ala-Lys-Ala-Lys(Boc)-NH_2	1389	A, 5.38	Pos. ion: [M+H]:1390.6
Seq017	Fmoc-Lys(Boc)-Pro-Leu-Gly-Leu-Lys-Ala-Arg(Pmc)-NH_2	1470	B, 7.85	Pos. ion: [M+H]:1470.6
Seq018	Fmoc-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys-Ala-Arg-NH_2	1225	D, 5.61	Pos. ion: [M+H]:1225.8
Seq019	H-Lys(ivDde)-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	1619	E, 6.72	Pos. ion: [M+H]: 1619.8; [M+H]/2: 808.6; [M+3H]/3: 540.4
Seq020	H-Gly-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	1340	E, 6.11	Pos. ion: [M+H]: 1339.8; [M+H]/2: 670.0; [M+3H]/3: 447.2
Seq021	H-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	1283	F, 6.23	Pos. ion: [M+H]: 1282.7; [M+H]/2: 641.5
Seq022	H-Lys(Boc)-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg(Pmc)-OH	1779	H, 7.05	Pos. ion: [M+H]: 1779.5
Seq023	H-Abu4-Pro-Gln-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	1383	H, 1.70	Pos. ion: [M+H]: 1382.6; [M+H]/2: 691.4; [M+3H]/3: 461.5
Seq024	H-Pro-Cha-Gly-Smc-His-Ala-Lys(BHQ3)-NH_2	1308	G, 8.29	Pos. ion: [M+H]: 1308.5; [M+H]/2: 655.9
Seq025	H-Pro-Leu-Ala-Leu-Lys(BHQ3)-Ala-Arg-OH	1297	G, 2.82	Pos. ion: [M+H]: 1298.7; [M+H]/2: 649.0
Seq026	H-Pro-Cha-Gly-Nva-His-Ala-Lys(BHQ3)-NH_2	1290	H, 3.98	Pos. ion: [M+H]: 1291.2 [M+H]/2: 645.5
Seq027	H-Abu4-Pro-Cha-Abu4-Smc-His-Ala-Lys(BHQ3)-Ala-Lys-NH_2	1718	E, 5.11	Pos. ion: [M+H]: 1718.8; [M+H]/2: 860.0
Seq028	H-Abu4-Pro-Cha-Abu2-Smc-His-Ala-Lys(BHQ3)-Ala-Lys-NH_2	1718	H, 4.90	Pos. ion: [M+H]: 1718.6; [M+H]/2: 860.0
Seq029	H-Pro-Leu-Gly-Met-Lys(BHQ3)-Ser-Arg-OH	1318	I, 5.20	Pos. ion: [M+H]: 1318.6; [M+H]/2: 659.8

Seq030	H-Pro-Leu-Ala-Tyr-Lys(BHQ3)-Ala-Arg-OH	1348	E, 5.29	Pos. ion: [M+H]: 1348.8 [M+H]/2: 674.6
Seq031	H-Pro-Leu-Ala-Tyr-Trp-Met-Arg-Lys(BHQ3)-Ala-Arg-NH_2	1820	G, 7.28	Pos. ion: [M+H]: 1820.8; [M+H]/2: 911.2
Seq032	H-Pro-Cha-Smc-His-Ala-Lys(BHQ3)-NH_2	1251	J, 4.26	Pos. ion: [M+H]: 1251.8; [M+H]/2: 626.4
Seq033	H-Abu4-Pro-Gln-Gly-Leu-Ala-Lys(BHQ3)-NH_2	1227	H, 4.10	Pos. ion: [M+H]: 1228.2; [M+H]/2: 614.8
Seq034	H-Abu4-Pro-Gln(Trt)-Gly-Leu-Ala-Lys(BHQ3)-Ala-Lys(Boc)-NH_2	1698	I, 4.15	Pos. ion: [M+H]: 1697.8; [M+H]/2: 848.6
Seq035	H-Lys(Boc)-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg(Pmc)-NH_2	1778	J, 9.16	Pos. ion: [M+H]: 1778.8; [M+H]/2: 888.8
Seq036	H-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys(BHQ3)-Ala-Arg-NH_2	1534	J, 4.08	Pos. ion: [M+H]: 1534.8; [M+H]/2: 767.5
Seq037	IRDye800CW-Lys(ivDde)-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2603	H, 6.60	Pos. ion: [M+H]: 2602.2; [M+Na]/2: 1312.2; [M+H]/2: 1301.2; [M+3H]/3: 867.8
Seq038	IRDye800CW-Gly-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2323	F, 6.21	Pos. ion: [M+H]: 2324.0; [M+2H]/2: 1162.6; [M+3H]/3: 775.4
Seq039	IRDye800CW-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2268	G, 6.27	Pos. ion: [M+H]: 2268.8; [M+2H]/2: 1134.0; [M+3H]/3: 756.4
Seq040	IRDye800CW-Lys-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-OH	2397	F, 2.29	Pos. ion: [M+H]/2: 1199.0; [M+3H]/3: 799.6
Seq041	IRDye800CW-Abu4-Pro-Gln-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2368	J, 5.77	Neg. ion: [M-H] = 2365.4; 2M/3: 1576.2; [M-2H]/2: 1182.0
Seq042	IRDye800CW-Pro-Cha-Gly-Smc-His-Ala-	2293	I, 9.00	Neg. ion: [M-H]: 2292.6; [2M-3H]/3: 1526.6; [M-2H]/2: 1146.4; [M-3H]/3: 764.8

	Lys(BHQ3)-NH_2			
Seq043	IRDye800CW-Pro-Leu-Ala-Leu-Lys(BHQ3)-Ala-Arg-OH	2283	E, 9.29	Neg. ion: [M-H]: 2281.6; [2M-3H]/3: 1521.6; [M-2H]/2: 1141.4; [M-3H]/3: 761.2
Seq044	IRDye800CW-Pro-Cha-Gly-Nva-His-Ala-Lys(BHQ3)-NH_2	2276	H, 10.24	Neg. ion: [M-H]: 2272.6; [M-2H]/2: 1137.2; [M-3H]/3: 758.6
Seq045	IRDye800CW-Abu4-Pro-Cha-Abu4-Smc-His-Ala-Lys(BHQ3)-Ala-Lys-NH_2	2605	F, 9.28	Neg. ion: [M-2H]/2: 1302.6; [M-3H]/3: 868.6; [M-4H]/4: 651.8
Seq046	IRDye800CW-Abu4-Pro-Cha-Abu2-Smc-His-Ala-Lys(BHQ3)-Ala-Lys-NH_2	2605	J, 9.24	Neg. ion: [M-2H]/2: 1302.6; [M-3H]/3: 868.8; [M-4H]/4: 651.6
Seq047	IRDye800CW-Pro-Leu-Gly-Met-Lys(BHQ3)-Ser-Arg-OH	2303	E, 4.09	Pos. ion: [M+H]: 2302.4; [M+2H]/2: 1151.4; [M+3H]/3: 768.3;
Seq048	IRDye800CW-Pro-Leu-Ala-Tyr-Lys(BHQ3)-Ala-Arg-OH	2333	F, 9.23	Neg. ion: [M-H]: 2329.5; [2M-3H]/3: 1553.1; [M-2H]/2: 1164.3
Seq049	IRDye800CW-Pro-Leu-Ala-Tyr-Trp-Met-Arg-Lys(BHQ3)-Ala-Arg-NH_2	2628	H, 4.98	Pos. ion: [M+2H]/2: 1313.6; [M+3H]/3: 876.6; [M+H+Na]/2: 1324.8.
Seq040	IRDye800CW-Pro-Cha-Smc-His-Ala-Lys(BHQ3)-NH_2	2236	J, 4.89	Pos. ion: [M+2H]/2: 1117.8; [M+3H]/3: 745.5; [3M+4H/4]: 1676.2;
Seq051	IRDye800CW-Abu4-Pro-Gln-Gly-Leu-Ala-Lys(BHQ3)-NH_2	2212	I, 4.19	Pos. ion: [M+2H]/2: 1106.0; [M+3H]/3: 737.5; [2M+Na/3]: 1482.2; [3M+Na/4]: 1664.3.
Seq052	IRDye800CW-Abu4-Pro-Gln-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2339	H, 4.18	Neg. ion: [M-H]: 2336.8; [M-2H]/2: 1168.0; [2M-3H]/3: 1557.8
Seq053	IRDye800CW-Lys-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2396	J, 7.58	Pos. ion: [M+H]: 2395.0; [3M+4H]/4: 1796.8; [2M+3H]/3: 1597.2; [M+3Na-H]/2: 1234.4; [M+2H]/2: 1197.8
Seq054	HyLitePlus-Lys-Pro-Leu-Gly-Leu-Lys(BHQ3)-Ala-Arg-NH_2	2304	J, 6.63	Pos. ion: [M+H]: 2305.2; [3M+4H]/4: 1729; [2M+3H]/3: 1536.8; [M+2H]/2: 1153.0
Seq055	IRDye800CW-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys(BHQ3)-Ala-Arg-NH_2	2518	J, 12.53	Pos. ion: [M+Na+H]/2: 1270.0; [M+2H]/2: 1259.2; [M+3H]/3: 839.8

System A. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water (0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 20% B, linear gradient 20-45% B over 15 min; Flow rate; 3 ml/min; Detection: UV @ 230 and 254 nm.

System B. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water (0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 20% B, linear gradient 20-65% B over 15 min; Flow rate; 3 ml/min; Detection: UV @ 230 and 254 nm.

System C. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water (0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 25% B, linear gradient 25-65% B over 15 min; Flow rate; 3 ml/min; Detection: UV @ 230 and 254 nm.

System D. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water (0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 30% B, linear gradient 30-45% B over 15 min; Flow rate; 3 ml/min; Detection: UV @ 230 and 254 nm.

System E. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water (0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 25% B, linear gradient 25-45% B over 20 min; Flow rate; 3 ml/min; Detection: UV @ 701 and 790 nm.

System F. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water(0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 20% B, linear gradient 20-65% B over 25 min; Flow rate; 3 ml/min; Detection: UV @ 701 and 790 nm.

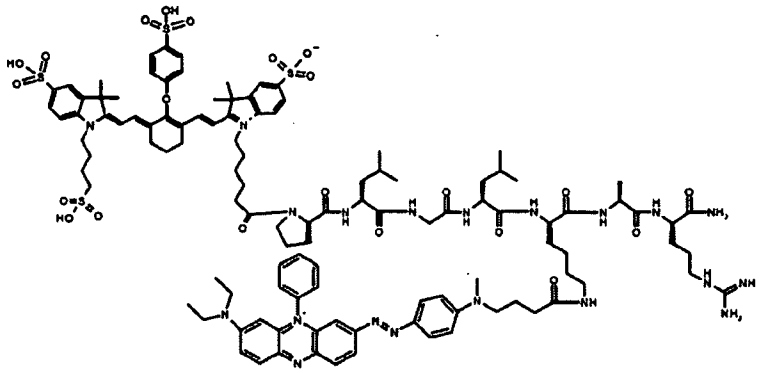
System G. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water(0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 15% B, linear gradient 15-45% B over 20 min; Flow rate; 3 ml/min; Detection: UV @ 701 and 790 nm.

System H. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water(0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 20% B, linear gradient 20-60% B over 20 min; Flow rate; 3 ml/min; Detection: UV @ 701 and 790 nm.

System I. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water(0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 30% B, linear gradient 30-50% B over 20 min; Flow rate; 3 ml/min; Detection: UV @ 701 and 790 nm.

System J. Column: Waters XTerra MS-C18 4.6 x 50 mm; Particle size: 5 microns; Eluents: A: Water(0.1% TFA), B: Acetonitrile (0.1% TFA); Elution: Initial condition: 25% B, linear gradient 25-55% B over 30 min; Flow rate; 3 ml/min; Detection: UV @ 701 and 790 nm.

[00068] Especially preferred are the compounds having Seq ID 037 and 039. The structure of Seq ID 039 is shown below.



IRDye 800CW-P-L-G-L-K(BHQ3)-A-R-NH_2

[00069] However, it should be appreciated that the surprising results found with these compounds can apply to Fluor-Enzyme cleavable substrate-Quencher constructs where the enzyme cleavable substrate is designed to be a substrate for proteolytic enzymes other than MMPs.

[00070] Several proteolytic enzymes are known, including those that are described in the Handbook of Proteolytic Enzymes, 2nd Edition, Vol 1 and 2, A, J. Barrett et al. The enzyme-cleavable constructs, properly designed could serve as selective substrates for a large variety of enzymes including, for example,

- Aspartic peptidases such as the Pepsins, Cathepsins, Presenilins, Renin and the like
- Cysteine Peptidases such as the Cathepsins, Ubiquitin-specific proteases, Caspases, Dipeptidyl peptidase and the like,
- Metallopeptidases such as aminopeptidases, Angiotensin-converting enzyme, Neprilysin, Endothelin-converting enzyme, Matrix Metalloproteases including Collagenase-1, -3, and -4, Gelatinase A and B, Stromelysin 1, 2 and 3, Matrilysin, Membrane-type matrix metalloproteinases such as Membrane-type matrix metalloproteinase 1-6, the ADAM metalloproteinases, ADAMSTS metalloproteases, TNF-α converting enzyme, the Carboxypeptidases, aminopeptidases such as Leucyl and Methionyl aminopeptidases,
- Lipases such as Phospholipase A 1,A2, B, C and D,
- Serine and Threonine peptidases such as trypsin, chymotrypsin, leukocyte elastase, kallikreins, Complement factors, Plasminogen activators, Plasmin,

Hyaluronon-binding protease, Oligopeptidases, Serine carboxypeptidase D and the like.

[00071] These proteases are examples and are not intended to be limiting.

[00072] Preferred Fluor-Enzyme cleavable substrate-Quencher constructs are those that are selectively cleaved by proteolytic enzymes that are overexpressed in a disease state. In one embodiment these substrates are not substantially cleaved by enzymes present in normal tissues.

[00073] The Fluor-Enzyme cleavable substrate-Quencher constructs may be provided as freeze-dried solids that are reconstituted with a physiologically acceptable solution prior to administration, or may be provided in a physiologically acceptable aqueous or non-aqueous solution, in the presence of such buffers, stabilizers, and solubilizers as are necessary to prepare a stable solution of said constructs. The agents may also be formulated as micelles, liposomes and the like. Prior to administration, the solid or solution may stored frozen or at room temperature, depending on the stability requirements of the compound.

[00074] For imaging studies, the part of the body to be studied is positioned under the detector, and a slow or fast bolus of the compounds of the invention of sufficient quantity to provide a diagnostic image is administered by intravenous, intraperitoneal, subcutaneous or intramuscular injection. The agent may alternatively be administered to the surface of an organ or disease site, e.g. the lumen of an artery, esophagus, colon etc. Scanning can be initiated within 1-2 minutes or may be delayed, depending upon the pharmacokinetics of the test compound. Light of suitable wavelength(s) is used to illuminate the subject. The florescence that is emitted is detected in target and non-target organs, using (e.g.) a fluorescence detector or an endoscopic or fiber optic probe that is sensitive to fluorescent emissions. The relative amount of fluorescence in the tissues of interest can be determined using regions of interest, or using time-activity curves, using methods known to those skilled in the art. Either reflectance or tomographic images may be obtained.

[00075] For some applications, the agent may be administered repeatedly over time, to determine the changes in the fluorescent images that may have occurred. Said images may be reflective of rises or falls in proteolytic enzyme levels due to changes in disease status, an increase or decrease in inflammation etc. The differences in the images over time can be used to detect response to drug therapy.

[00076] The compositions of this invention may be administered for imaging by more than one modality. For example, the compositions may be used for imaging by optical imaging alone, or may be used for photoacoustic imaging.

EXAMPLES

Synthesis of Fluor-Enzyme cleavable substrate-Quencher constructs:

[00077] Abbreviations for Chemical Reagents, Chemical Structure Moieties and Techniques: AA – amino acid, ACN – Acetonitrile, API-ES – Atmospheric pressure ionization electrospray, Boc – tert-Butyloxycarbonyl, DCM – Dichloromethane, DIC – N, N-Diisopropylcarbodiimide, DIEA – N,N-Diisopropylethylamine, DMF – Dimethylformamide, DMSO – Dimethyl sulfoxide, Et2O – Diethyl ether, EtOAc – Ethyl acetate, Fmoc – 9-Fluorenylmethoxycarbonyl, H_2O – Water, HOBt- 1-Hydroxybenzotriazole, HBTU – O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium hexafluorophosphate,, MeOH – Methanol, Neg. ion – Negative ion, NHS – N-Hydroxysuccinimide, NMP – N-Methylpyrrolidone, Pmc – 2,2,5,7,8-Pentamethylchroman-6-sulfonyl, Pip – Piperidine, Pos. ion – Positive ion, HOBt- 1-Hydroxybenzotriazole, HBTU – O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium hexafluorophosphate, PyBOP – Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexfluorophosphate, t_R – Retention time (minutes), Reagent "B" (88:5:5:2 – TFA:H_2O:phenol:TIPS - v/v/w/v), TFA – Trifluoroacetic Acid, TIPS – Triisopropylsilane, Trt – Trityl, BHQ3® – Black Hole Quencher 3, BHQ3-OSu – Black Hole Quencher 3 Carboxylic acid Succinimidyl ester, IRDye®800CW NHS ester – Infrared Dye® 800CW Carboxylic acid Succinimidyl ester. HyLitePlus™ 750 NHS Ester – HyLitePlus™ 750 Carboxylic acid Succinimidyl ester. Abu4 = Gaba = 4-Aminobutyric acid, Cha = Cyclohexyl-L-alanine, Smc = Cys(Me) = S-Methyl-L-Cysteine, Abu2 = 2-Amino-L-butyric acid, Nva = L-Norvaline

[00078] Solvents for reactions, chromatographic purification and HPLC analyses were E. Merck Omni grade solvents from VWR Corporation (West Chester, PA). N-Methylpyrrolidinone (NMP) and N,N-dimethylformamide (DMF) were purchased from Pharmco Products Inc. (Brookfield, CT), and were peptide synthesis grade or low water/amine-free Biotech grade quality. Piperidine (sequencing grade, redistilled 99+%) and trifluoroacetic acid (spectrophotometric grade or sequencing grade) were purchased from Sigma-Aldrich Corporation (Milwaukee, WI) or from the Fluka Chemical Division of Sigma-Aldrich Corporation. N, N'-Diisopropylcarbodiimide (DIC), phenol (99%), N,N-diisopropylethylamine (DIEA) and triisopropylsilane (TIS) were purchased from Sigma-Aldrich Corporation. Fmoc-protected amino acids, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole (HOBt) were purchased from Novabiochem (San Diego, CA). BHQ3-OSu was purchased from Biosearch

Technologies, Inc (Novato, CA) and IRDye 800CW--NHS ester was purchased from LI-COR Biosciences (Lincoln, NE). HyLitePlus™ 750 NHS ester was obtained from AnaSpec, Inc (San Jose, CA).

[00079] Analytical HPLC data were generally obtained using a Shimadzu LC-10AT VP dual pump gradient system employing a Waters XTerra MS-C18 4.6 x 50 mm column, (particle size: 5 microns; 120Å pore size) and gradient or isocratic elution systems using water (0.1% TFA) and acetonitrile (0.1% TFA) as solvent A and solvent B respectively. Detection of compounds was accomplished using UV either at 220 and 254 nm (deuterium lamp) or at 701 and 790 nm (tungsten lamp).

[00080] Preparative HPLC was conducted on a Shimadzu LC-8A dual pump gradient system equipped with a SPD-10AV UV detector. Generally the solution containing the crude peptide was loaded onto a reversed phase C18 column, depending on the compound characteristics, using a third pump attached to the preparative Shimadzu LC-8A dual pump gradient system. After the compound was applied to the preparative HPLC column solvents present in the reaction mixture, such as DMF or DMSO were eluted from the column at low organic phase composition; then the desired product was eluted using a gradient elution of the stronger eluant into the weaker eluant.

[00081] Mass spectral data were obtained in-house on an Agilent LC-MSD (1100) Mass Spectrometer using API-ES in –/+ ion mode. The molecular weight of the target peptides exceeds 2000, thus the mass spectra usually exhibited doubly or triply negatively charged ion mass values rather than the molecular ion. Doubly or triply charged ion mass values of the desired peptide were generally employed for selection of fractions for collection and combination to obtain the pure peptide during HPLC purification. After careful collection of fractions by comparing MS results and HPLC purities and freeze-drying process, a small amount of the isolated fluffy solid was dissolved in water-acetonitrile (1:1) (0.25 mg/mL) and this solution was analyzed by HPLC and MS for final purity determination of the purified peptide.

General Methods for Solid Phase Peptide Synthesis (SPPS)

[00082] The linear peptides were synthesized on a 0.25 mmol scale using an ABI 433A Peptide Synthesizer which employed FastMoc™ protocols, Fmoc-Pal-Peg-PS resin (0.2 mmol/g) or NovaSyn TG Sieber resin (0.2 mmol/g), Fmoc-protected amino acids and HBTU-mediated HOBt ester activation in NMP.

[00083] Chain elongation was carried out with a 4-fold excess of each amino acid and HBTU-HOBt-DIEA reagent in NMP. In a typical coupling process, the protected amino acid

(1.0 mmol, 4 eq.) was activated with HBTU (1.0 mmol, 4 eq.), HOBt (1.0 mmol, 4 eq.) and DIEA (2.5 mmol, 10 eq.) using NMP as the solvent in an activation chamber, and transferred to the reaction vessel containing resin (0.25 mmol). After agitating the reaction mixture for 63 min, the resin was washed thoroughly with NMP.

[00084] The cleavage of the Fmoc-group was performed with 20% piperidine in NMP containing 0.1M HOBt. The final peptide on the resin with its N-terminus protected with an Fmoc group was deprotected with the cleavage cocktail, "reagent B" (TFA:water:phenol: triisopropylsilane, 88:5:5:2, v/v/w/v) (10 mL/g of resin) for 4 h. After evaporation of the volatiles under vacuum, the paste thus obtained was triturated with ether to provide a solid which was washed with ether (3x) by centrifugation and dried under vacuum to provide the required peptide as an off-white solid.

[00085] To synthesize seq004, seq007, seq011 and seq012, the preloaded Fmoc-Arg(Pmc)-NovaSyn TGT (0.2 mmol/g) resin was used. To prepare the peptides seq009, seq010, seq016 and seq017, NovaSyn TG Sieber resin (0.2 mmol/g) was employed. From these resins crude peptides were obtained after completion of the synthesis by treating with 5% TFA in dichloromethane (10 mL/g of resin) for 5 min.

[00086] The crude peptide (~100 mg) was dissolved in CH_3CN (10 mL) and the resulting solution was diluted to a final volume of 50 mL with water followed by filtration. The filtered solution was loaded onto the preparative HPLC column (Waters, XTerra® Prep MS C18, 10μ, 120Å, 50 x 250 mm) which had been pre-equilibrated with 10% acetonitrile in water (0.1% TFA). After the sample solution was applied to the column, the flow of equilibrating eluent from the gradient HPLC system was reinitiated and the composition of the eluent was then ramped to 20% CH_3CN-water (0.1%TFA) over 1 min after which a linear gradient at a rate of 0.5%/min of CH_3CN (0.1% TFA) into water (0.1% TFA) was initiated and maintained for 50 min.

[00087] Fractions (15 mL) were manually collected using UV at 220 nm as an indicator of product elution. The collected fractions were analyzed on an analytical reversed phase C18 column (Waters XTerra MS-C18, 5μ, 120Å, 4.6 x 50 mm) and product-containing fractions of >95% purity were combined and freeze-dried to afford the corresponding peptide. Typically the purification of 100 mg of crude peptide afforded 40 to 50 mg (42 to 55% yield) of the desired peptide (>95% purity). After isolation, the peptides were analyzed by HPLC and mass spectrometry to confirm identity and purity. The data for peptides seq001 to seq018 are provided in a Table 2.

Preparation of Fluorogenic Substrate Peptides

[00088] As a representative example for the synthesis of these similarly constituted fluorogenic substrate peptides, the synthesis of the seq053 is outlined as shown in Figure 5.

Step 1. Preparation of peptide sequences containing Black Hole Quencher (BHQ3)

[00089] **General Procedure:** To a solution of the pure peptide seq017 (10 mg, 0.0068 mmol) in anhydrous DMF (0.3 mL) was added BHQ3-OSu (5 mg, 0.0063 mmol) followed by DIEA (10 mg, 0.077 mmol); the mixture was stirred for 4 h at ambient temperature (flask was wrapped with aluminum foil to avoid light). The reaction mixture was diluted to 10 mL with anhydrous DMF, piperidine (0.2 mL) was added and the mixture was stirred for 30 min. After completion of the removal of the Fmoc group (as determined by HPLC and MS), the reaction mixture was diluted with water to 50 mL and loaded onto the preparative HPLC column (Waters, XTerra® Prep MS C18, 10μ, 120Å, 19 x 300 mm) which had been pre-equilibrated with 5% acetonitrile in water (0.1% TFA). During the application of the sample solution to the column, the flow of the equilibrating eluent from the preparative HPLC system was stopped.

[00090] After the sample solution was applied to the column, the flow of equilibrating eluent from the gradient HPLC system was reinitiated and the composition of the eluent was then ramped to 20% CH_3CN-water (0.1%TFA) over 1 min, after which a linear gradient at a rate of 0.5%/min of acetonitrile (0.1% TFA) into water (0.1% TFA) was initiated and maintained for 50 min. Fractions (15 mL) were manually collected using UV at 700 nm as an indicator of product elution. The collected fractions were analyzed on an analytical reversed phase C18 column (Waters XTerra MS-C18, 5μ, 120Å, 4.6 x 50 mm, System J) and product-containing fractions of >95% purity were combined and freeze-dried to afford the corresponding peptide seq035. Typically the purification of the reaction product of 10 mg of each of peptide sequences seq001 to seq018 with BHQ-OSu afforded 5 to 7 mg (40 to 50% yield) of the desired corresponding sequences seq019 to seq035 containing BHQ3 (>95% purity).

[00091] After isolation, the peptides were analyzed by HPLC and mass spectrometry to confirm identity and purity. The data are provided in Table 2 above.

Step 2. Preparation of peptide sequences containing IRDye 800CW and BHQ3

[00092] **General Procedure:** To a solution of the peptide-BHQ3 conjugate seq035 (3 mg, 0.0017 mmol) in anhydrous DMF (0.3 mL) was added IRDye800CW-NHS ester (3 mg, 0.0026 mmol) followed by DIEA (10 mg, 0.077 mmol) and the solution was stirred for 8 h at ambient temperature (flask was wrapped with aluminum foil to avoid light). After completion of the reaction (as determined by HPLC and MS), protecting groups such as Boc or Pmc group(s) on the peptide, if any, were removed as follows.

[00093] The reaction mixture was evaporated to remove the volatiles on a rotary evaporator, treated with a cleavage cocktail of TFA:water:phenol (0.5 mL, 95:3:2, v/v/w) and stirred for 2 h at ambient temperature. After deprotection was completed (as determined using HPLC and MS), the reaction mixture was diluted with water to 20 mL and loaded onto the preparative HPLC column (Waters, XTerra® Prep MS C18, 10μ, 120Å, 19 x 300 mm) which had been pre-equilibrated with 5% acetonitrile in water (0.1% TFA).

[00094] In the cases of peptide sequences not bearing protecting groups, after coupling with IRDye 800CW NHS ester the reaction mixture was diluted with water to 20 mL and loaded onto the preparative HPLC column as mentioned above. After the sample solution was applied to the column, the flow of equilibrating eluent from the gradient HPLC system was ramped to 20% CH_3CN-water (0.1%TFA) over 1 min, after which a linear gradient at a rate of 0.5%/min of acetonitrile (0.1% TFA) into water (0.1% TFA) was initiated and maintained for 50 min. Fractions (15 mL) were manually collected using UV at 790 nm as an indicator of product elution.

[00095] The collected fractions were analyzed on an analytical reversed phase C18 column (Waters XTerra MS-C18, 5μ, 120Å, 4.6 x 50 mm, System J) and product-containing fractions of >95% purity were combined and freeze-dried to afford the corresponding peptide seq035. Typically the purification of the reaction product of 3 mg of each of peptide sequences seq019 to seq036 with IRDye800CW-NHS ester afforded 2 mg - 3 mg (30 to 40% yield) of the desired corresponding sequences seq037 to seq055 bearing the IRDye800CW and BHQ3 (>95% purity) moieties. After isolation, the peptides were analyzed by HPLC and mass spectrometry to confirm identity and purity. The data are provided in Table 2 above.

[00096] A schematic of the synthesis is provided in Figure 5 for the compound having Seq ID 053.

[00097] **In vitro MMP assay**: Fluor/quencher-containing peptides were tested in an in vitro assay to determine their relative rates of cleavage by specific enzymes. The assay procedure described here was used.

[00098] **Materials:** MMPs were obtained from AnaSpec and stored in 50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$ (pH 7.5) buffer at -80 °C until use. MMP assay buffer (Cat No. 60907-500) was obtained from AnaSpec and stored at 4 °C until use. Mouse Neprilysin was obtained from R&D Systems. APMA (Aminomethylphenyl mercuric acetate, Cat No.A9563-5G) was purchased from Sigma. The 96-well plates used were NUNC MaxiSorb black plate (NUNC Cat No. 437111). The assay was performed on a Near-IRDye fluorescence plate reader (BioTek-Synergy2).

[00099] **Preparation of stock solutions:** Peptide stock solutions (500 μM) were prepared in DMSO and stored at -20 °C; an aliquot was diluted to 10 μM using Assay Buffer prior to assay. APMA solutions (10 mM) were prepared in water.

[000100] **Activation of MMPs:** MMPs (supplied at a concentration of 1 μg in 100 μL) were activated with 1 mM APMA just prior to assay. The incubation time required to activate the various MMPs are given below.

Activation time used for various MMPs

MMP	1	2	3	7	8	9	10	11	12	13	14
Time	3 h	1 h	24 h	0.3-1h	1 h	2 h	24 h	***	2 h	40 min	2-3 h

*** MMP-11 is already in active form; so no APMA treatment required

[000101] As an example, MMP-13 (supplied as 1 μg in 100 μL) was diluted to 450 μL in AnaSpec MMP assay buffer. It was mixed with APMA (10 mM, 50 μL) and incubated at 37 °C for 45 min. It was then cooled in ice (4 °C) and diluted to 1.25 mL in assay buffer to give a final enzyme concentration of 0.8 μg/mL. This solution was stored in ice until use.

[000102] **Assay:** Each IRDye800CW or HiLyte750/BHQ3 peptide (50 μL of a 10 μM solution, ~500 picomoles/well) was added to duplicate wells in a 96-well plate followed by 50 μL of assay buffer (AnaSpec). To the wells were added various activated MMPs (50 μL, 0.8 μg/mL, 40 ng/well). This provided a peptide to enzyme ratio of about 1000:1.

[000103] The plate was incubated at RT (~23°C) for up to 90 min. The progress of the enzymatic cleavage was determined by measuring the increase in relative fluorescence (RFU) using the plate reader. The plates were read at 2-5 min intervals during the first 30 min, then at every 15 min during the next 60 min.

[000104] A parallel experiment was carried out at 37°C to determine the maximum RFU emitted by the fully cleaved IRDye 800CW- or HiLyte750 dye-containing fragment from

each peptide tested. Data were normalized so they could be plotted as % cleaved, rather than as relative fluorescence units (RFU) to allow comparison between different compounds with different final fluorescence values. The normalized data was plotted as % maximum vs time. From this, the initial slope and the Y-intercept were determined to obtain the relative rate of cleavage and the background fluorescence of the fluorogenic peptide under study.

[000105] Referring now to Figure 4, typical data for cleavage of three of the Fl/Q constructs by MMP-2, -9 and 13 is shown. At t = 0 min, fluorescence was relatively minimal (i.e. the compounds did not emit significant fluorescence). After the activated MMP was added, cleavage of the peptide chain that linked the fluor and quencher occurred over time, generating fluorescence which increased until such time as 100% cleavage had occurred. Significant variations in the rate of cleavage and the sensitivity to various MMPs were observed. Of the three compounds shown in Figure 4, the compound with Seq ID 039 was cleaved fastest with MMP-2, 9 and 13, relative to the rates observed with the other two compounds. In this figure, for all three test compounds shown, MMP-13 cleaves the substrate faster than MMP-9, which in turn was faster than MMP-2. (Seq ID 039>Seq ID 37>Seq ID 040). Using such an assay, the relative sensitivity of such compounds to various enzymes can be determined.

[000106] Similar assays were performed for all test compounds, using a variety of MMPs. Data are shown in Table 3 (Appendix) for in vitro results using MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, and MMP-13.

[000107] Also shown in this table are the results obtained when a parallel assay was performed using the enzyme known as Neprilysin. This is a protease that is known to cleave a variety of peptides, especially in the brush borders of the kidney. For many applications, it is important that the cleavage of the substrate that is interposed between the fluor and the quencher be by specific enzymes and not by other non-specific enzymes in the body.

[000108] For this application, compounds that are not substantially cleaved in vivo by enzymes found in normal tissue or blood, such as for example, Neprilysin are especially preferred.

[000109] Although the compounds described herein were specifically prepared to be cleaved by MMPs, it should be obvious to those skilled in the art that other enzyme-cleavable constructs that are cleaved by other enzymes could be constructed.

[000110] In addition, it should be obvious to those skilled in the art that other NIR fluor/quencher combinations may be beneficial for this application.

Embodiments Of The Invention

[000111] The following is provided to illustrate without limitation the various embodiments of the present invention:

[000112] 1. A compound comprising:

a compound of the general formula:

F-E-Q

wherein

F is a fluorescent dye,

E is an enzyme-cleavable substrate, and

Q is a quencher and

wherein the compound fluoresces under near infrared light after cleavage of the enzyme-cleavable substrate.

[000113] 2. A compound comprising:

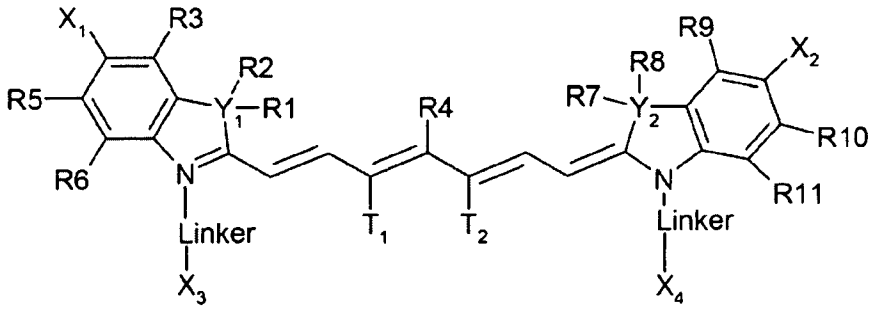
a compound of the general formula:

F-E-Q

wherein,

F is a compound selected from the group consisting of Formula I, Formula II and Formula III,

wherein Formula I comprises:



Formula I

wherein

R1, R2, R5, R6, R7, R8, R9, R10 and R11 are each independently H, Me, substituted or unsubstituted alkyl, halo, carboxy, amino, sulfonate, R12-COOH, R12OR13, R12SR13, or R12COOR13, wherein R12 is a bond or alkylene and R13 is substituted or unsubstituted alkyl

R4 is –O-aryl, NH-aryl, a substituted or unsubstituted aryl, a substituted or unsubstituted C-1-C10 alkyl, halo, S-aryl or SO2-aryl, where the aryl ring can, in all cases, be substituted or unsubstituted, or a linker that is covalently bonded (e.g. via an amine, thiol or acid functionality) to an enzymatically cleavable substrate or a linker that contains a reactive moiety suitable for coupling to an enzyme cleavable substrate.

T1 and T2 are each H, or are joined together to form a substituted or unsubstituted 5- or 6-membered ring.

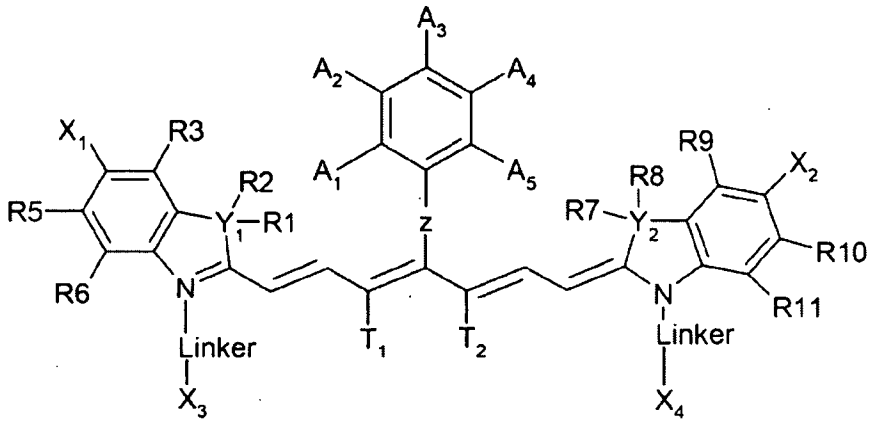
X1, X2, X3 and X4 independently represent SO3H, COOH or physiologically acceptable salts thereof, or polyethylene glycol, or one or more of X1-X4 may be a linker (e.g. a substituted or unsubstituted alkyl, substituted or unsubstituted Aryl, PEG, alkyl amine or quanidine) that is either covalently bonded (e.g. via an amine, thiol or acid functionality) to an enzymatically cleavable substrate or contains a reactive moiety suitable for coupling to an enzyme cleavable substrate,

or X1 and R3 and/or X2 and R9 can be cyclized together to form a 5- or 6 membered saturated or unsaturated ring that is optionally derivatized with H, substituted or unsubstituted C1-C6 alkyl, polyethylene glycol, substituted or unsubstituted aryl, halogen, cyano, carboxy, and/or sulfo group(s).

Y1 and Y2 are independently C, N (in which case R2 and/or R8 is absent), or O, S, Se, sulfide, sulfone or sulfoxide (in which case both R1 and R2 and/or R7 and R8 are absent), and

the Linker is a substituted or unsubstituted C1-C10 alkyl chain, a polyethylene glycol derivative, or a substituted or unsubstituted aryl group;

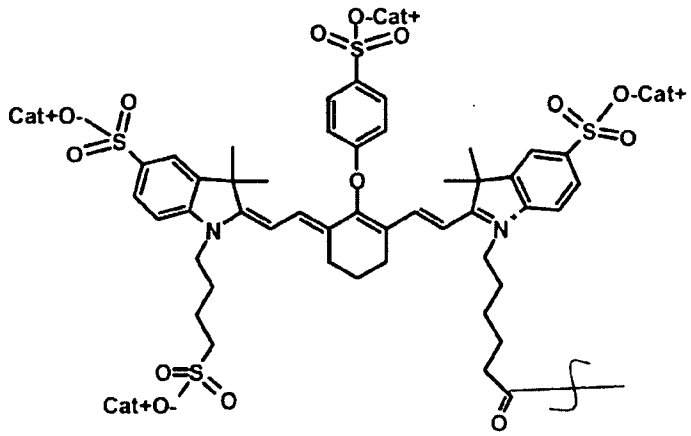
wherein Formula II comprises:



wherein

Z is –O-, -S-, -S(-O)-, -SO_2- -NH, HN-NH- or N-Alkyl, or absent and wherein A1-A5 are each independently H, alkyl, halo, carboxy, alkylcarboxy, amino, alkylamino, amino alkyl, or SO_3^-Cat+, wherein Cat^+ is a H^+ or a cation, and the remaining residues are defined as described above for Formula I; and

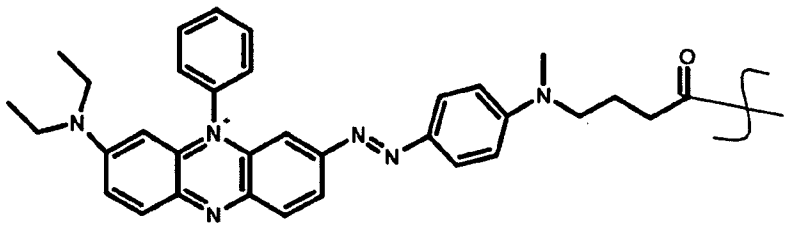
wherein Formula III comprises:



wherein Cat+ is a cation selected from the group consisting of H^+, Na^+, K^+, NH_4^+, meglumine, a protonated organic base or a physiologically acceptable cation,

E is an enzyme-cleavable substrate, and

Q is



and

wherein the compound fluoresces under near infrared light after cleavage of the enzyme-cleavable substrate.

3. The compound of embodiment 2 wherein E is a MMP cleavable substrate.

4. The compound of embodiment 1 wherein said compound is selected from the group comprising SEQ ID Nos. 037, 038, 039, 040, 041, 042, 043, 044, 046, 047, 048, 051, 052, 053, and 054.

5. The compound of embodiment 3, wherein E is not substantially cleavable by enzymes found in normal tissue or blood such as Neprilysin.

6. The compound of embodiment 1, wherein F is IRDye800CW.

7. The compound of embodiment 1, wherein Q is Black Hole Quencher 3.

8. The compound of embodiment 1, wherein the enzyme cleavable substrate is selectively cleaved by a proteolytic enzyme that is overexpressed in a disease state.

Table 3: Relative rate of cleavage of fluorogenic peptides by various MMPs and Neprilysin

Seq ID	Peptides (X=IRDye800CW; C-Terminal –NH_2 unless otherwise indicated)	Neprilysin		MMP-1 collagenase		MMP-2 Gelatinase		MMP-3 Stromelysin		MMP-7 Matrilysin		MMP-8 Neutrophil collagenase		MMP-9 Gelatinase		MMP-10 Strome-lysin2		MMP-13 Collagenase3	
		Slope	*inter*	Slope	*inter*	Slope	*inter*	Slope	*inter*	Slope	*inter*	Slope	*inter*	Slope	*inter*	Slope	*inter*	Slope	*inter*
Seq037	X-K(ivDde)PLGL-K(BHQ3)AR	NC		0.67		0.53	*5.03*	0.19	5.08	1.12	*4.66*	0.2	7.17	1.61	*5.54*	0.19	*4.58*	5.21	*3.93*
Seq038	X-GPLGL-K(BHQ3)AR	NC		0.16	1.44	0.53	*2.56*	0.81	1.81	1.51	*2.33*	0.11	1.16	1.21	*3.85*	0.16	*1.43*	3.94	*2.53*
Seq039	X-PLGL-K(BHQ3)AR	NC		0.42	2.84	1.18	*3.44*	0.91	2.04	3.32	*2.54*	0.36	1.84	2.77	*3.89*	0.43	*2.26*	6.72	*2.98*
Seq040	X-KPLGLK(BHQ3)AR-OH	NC		0.11	1.55	0.15	*1.44*	0.60	3.17	0.79	*3.27*	1.35	1.69	0.39	*2.32*	0.61	*3.19*	1.80	*1.60*
Seq041	X-Abu4-PQGLK(BHQ3)AR	NC		0.16	1.04	0.77	*1.45*	0.21	0.69	0.55	*0.94*	0.24	0.91	1.30	*1.19*	0.04	*0.53*	3.38	*0.17*
Seq042	X-P-Cha-G-Smc-HAK(BHQ3)	0.17	2.8	1.10	3.39	0.84	*2.83*	0.16	2.24	0.23	*2.64*	0.49	4.22	3.87	*3.36*	0.64	*2.51*	3.86	*2.00*
Seq043	X-PLAL-K(BHQ3)-AR-OH	NC		0.79	24.7	0.46	*25.8*					0.32	24.9	1.25	*26.6*			2.47	*26.5*
Seq044	X-P-Cha-G-Nva-HAK(BHQ3)	0.28	2.6	0.27	3.97	0.91	*3.63*	0.05	3.84	0.16	*4.29*	0.16	3.51	2.22	*4.53*	0.24	*3.80*	2.94	*4.00*
Seq046	X-Abu4-P-Cha-Abu2-Smc-HAK(BHQ3)AK	NC		0.07	21.6	0.22	*24.8*	0.30	20.1	0.67	*20.4*	0.01	21.4	0.94	*24.2*	0.20	*20*	0.28	*26.3*
Seq047	X-PLGM-K(BHQ3)-SR	NC		0.19	1.49	2.18	*1.83*	0.25	1.93	0.32	*2.18*	0.07	0.77	3.45	*1.94*	0.27	*0.60*	5.71	*2.85*
Seq048	X-PLAY-K(BHQ3)AR-OH	NC		0.21	3.39	0.55	*2.66*	0.56	2.77	0.16	*2.92*	0.27	2.84	1.89	*2.99*	0.32	*2.15*	3.67	*2.48*
Seq051	X-Abu4-PQGLAK(BHQ3)	PC		NC		0.11	*5.52*	NC		NC		NC		0.52	*7.14*	NC		0.35	*5.79*
Seq052	X-Abu4-PQGLK(BHQ3)AK	NC		0.09	0.49	0.63	*0.68*	0.15	0.97	0.13	*0.80*	0.11	0.34	1.11	*0.62*	0.03	*0.50*	2.33	*0.95*
Seq053	X-KPLGL-K(BHQ3)AR	NC		0.13	0.69	0.32	*1.17*	0.04	1.07	0.03	*1.06*	0.01	0.86	0.70	*2.01*	0.01	*1.14*	2.32	*2.53*
Seq054	*HyLitePlus750*-KPLGL-K(BHQ3)AR	NC		0.23	1.05	0.23	*1.05*	0.02	1.11	0.08	*1.22*	0.02	1.01	0.54	*1.93*	0.01	*1.10*	2.09	*3.29*

NC: No Cleavage; LC: Little cleavage; PC: Partial cleavage

We claim :

1. A compound comprising:

   a compound of the general formula:

   F-E-Q

   wherein

   F is a near infrared fluorescent dye,

   E is an enzyme-cleavable substrate, and

   Q is a quencher and

   wherein the compound fluoresces under near infrared light after cleavage of the enzyme-cleavable substrate.

2. A compound of claim 1 wherein F is selected from the group consisting of Cy 7, IRDye800CW, HyLitePlus750, Alexa Fluor 750. DyLight 750, and DyLight 800.

3. A compound comprising:

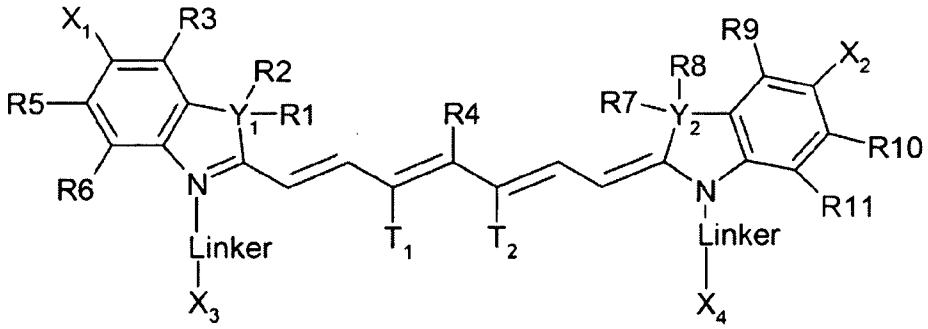
   a compound of the general formula:

   F-E-Q

   wherein,

   F is a near infrared fluorescent dye selected from the group consisting of Formula I, Formula II and Formula III,

   wherein Formula I comprises:



Formula I

   wherein

   R1, R2, R3, R5, R6, R7, R8, R9, R10 and R11 are each independently H, Me, substituted or unsubstituted alkyl, halo, carboxy, amino, sulfonate, R12-COOH, R12OR13, R12SR13, or R12COOR13,

   wherein

R12 is a bond or alkylene and R13 is substituted or unsubstituted alkyl,

R4 is –O-aryl, NH-aryl, a substituted or unsubstituted aryl, a substituted or unsubstituted C-1-C10 alkyl, halo, S-aryl or SO2-aryl, where the aryl ring can, in all cases, be substituted or unsubstituted, or a linker that is covalently bonded (e.g. via an amine, thiol or acid functionality) to an enzymatically cleavable substrate or a linker that contains a reactive moiety suitable for coupling to an enzyme cleavable substrate:

T1 and T2 are each H, or are joined together to form a substituted or unsubstituted 5- or 6-membered ring:

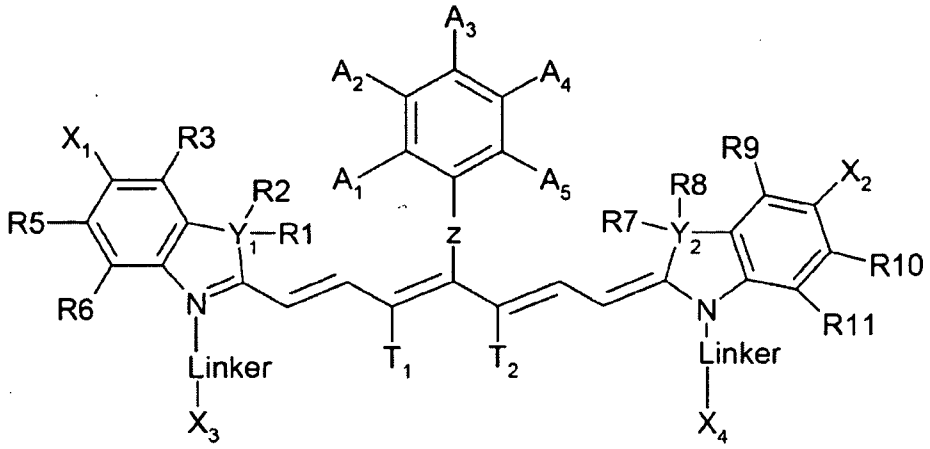
X1, X2, X3 and X4 independently represent SO3H, COOH or physiologically acceptable salts thereof, or polyethylene glycol, or one or more of X1-X4 may be a linker (e.g. a substituted or unsubstituted alkyl, substituted or unsubstituted Aryl, PEG, alkyl amine or quanidine) that is either covalently bonded (e.g. via an amine, thiol or acid functionality) to an enzymatically cleavable substrate or contains a reactive moiety suitable for coupling to an enzyme cleavable substrate,

or X1 and R3 and/or X2 and R9 can be cyclized together to form a 5- or 6 membered saturated or unsaturated ring that is optionally derivatized with H, substituted or unsubstituted C1-C6 alkyl, polyethylene glycol, substituted or unsubstituted aryl, halogen, cyano, carboxy, and/or sulfo group(s);

Y1 and Y2 are independently C, N (in which case R2 and/or R8 is absent), or O, S, Se, sulfide, sulfone or sulfoxide (in which case both R1 and R2 and/or R7 and R8 are absent), and

Linker is a substituted or unsubstituted C1-C10 alkyl chain, a polyethylene glycol derivative, or a substituted or unsubstituted aryl group;

wherein Formula II comprises:



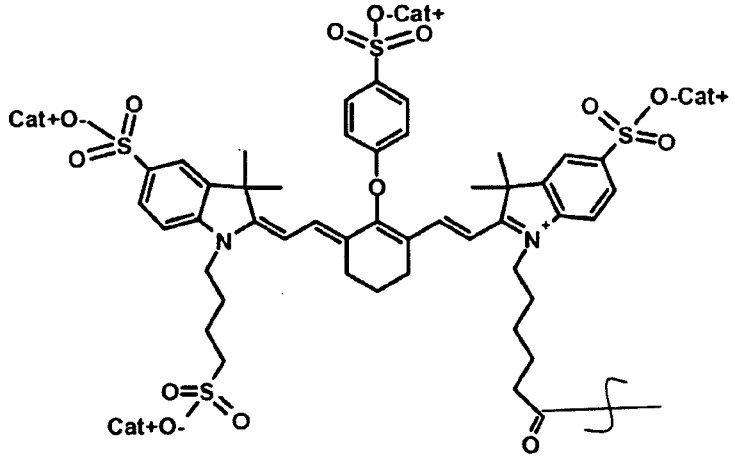
wherein

Z is –O-, -S-, -S(-O)-, -SO_2- -NH, HN-NH- or N-Alkyl, or absent and

wherein A1-A5 are each independently H, alkyl, halo, carboxy, alkylcarboxy, amino, alkylamino, amino alkyl, or SO_3^-Cat+,

wherein Cat^+ is a H^+ or a cation, and the remaining residues are defined as described above for Formula I; and

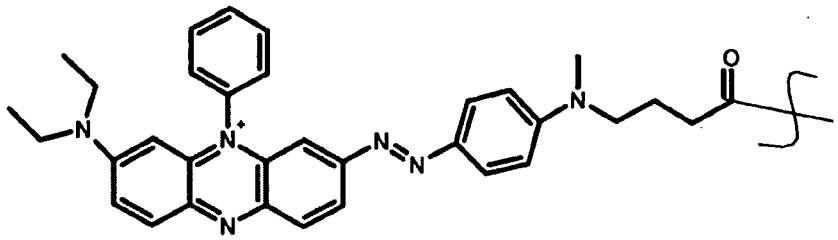
wherein Formula III comprises:



wherein Cat+ is a cation selected from the group consisting of H^+, Na^+, K^+, NH_4^+, meglumine, a protonated organic base or a physiologically acceptable cation,

E is an enzyme-cleavable substrate, and

Q is



and

wherein the compound fluoresces under near infrared light after cleavage of the enzyme-cleavable substrate.

4. The compound of claim 2 wherein Q is selected from the group consisting of Deep Dark Quencher, Dabcyl, Dabsyl, Epoche Eclipse, Iowa Black FQ, Black Hole Quencher 1, Black Hole Quencher 2, Black Hole Quencher 3, Deep Dark Quencher II , Blackberry Quencher 650, Iowa Black RQ, QSY-7, QSY-21.
5. A compound of claim 3 wherein F is IRDye800CW.
6. A compound of claim 3 wherein E is a MMP cleavable substrate.
7. The compound of claim 3, wherein E is attached to Q and is selected from the group consisting of:

-Lys(ivDde)-Pro-Leu-Gly-Leu-Lys(BHQ-3)-Ala-Arg-NH_2;

-Gly-Pro-Leu-Gly-Leu-Lys(BHQ-3)-Ala-Arg-NH_2;

-Pro-Leu-Gly-Leu-Lys(BHQ-3)-Ala-Arg-NH_2;

-Pro-Cha-Gly-Smc-His-Ala-Lys(BHQ-3)-NH_2;

-Pro-Leu-Ala-Leu-Lys(BHQ-3)-Ala-Arg-OH;

-Pro-Cha-Gly-Nva-His-Ala-Lys(BHQ-3)-NH_2;

-Pro-Cha-Gly-Smc-His-Ala-Lys(BHQ3)-NH_2;

-Abu4-Pro-Cha-Abu2-Smc-His-Ala-Lys(BHQ-3)-Ala-Lys-NH_2;

-Pro-Leu-Gly-Met-Lys(BHQ-3)-Ser-Arg-OH;

-Pro-Leu-Ala-Tyr-Lys(BHQ-3)-Ala-Arg-OH;

-Abu4-Pro-Gln-Gly-Leu-Lys(BHQ-3)-Ala-Lys-NH_2;

-Lys-Pro-Leu-Gly-Leu-Lys(BHQ-3)-Ala-Arg-NH_2;

-Gly-Pro-Leu-Gly-Leu-Lys(BHQ-3)-Ala-Arg-NH_2; and

-Pro-Leu-Gly-Leu-Lys(BHQ-3)-Ala-Arg-NH_2.

8. The compound of claim 1 wherein said compound is selected from the group comprising SEQ ID Nos. 037, 038, 039, 040, 041, 042, 043, 044, 046, 047, 048, 051, 052, 053, 054 and 55.
9. The compound of claim 3, wherein E is not substantially cleavable by enzymes found in normal tissue or blood such as Neprilysin.
10. The compound of claim 1, wherein F is selected from the group consisting of: Alexa Fluor 750, HiLytePlus750 and IRDye 800CW.
11. The compound of claim 11, wherein F is IRDye800CW.
12. The compound of claim 1, wherein Q is selected from the group consisting of: Deep Dark Quenchers, Dabcyl, Eclipse, Iowa Black FQ and RQ, Blackberry Quencher 650, QSY-7, QSY-21, and Black Hole Quenchers 0, 1, 2, and 3.
13. The compound of claim 12, wherein Q is Black Hole Quencher 3.
14. The compound of claim 1, wherein the enzyme cleavable substrate is selectively cleaved by a proteolytic enzyme that is overexpressed in a disease state.
15. A method of imaging comprising administering to a subject a compound of any one of claims 1, 2 or 3 and imaging the fluorescence that is generated.
16. A method of diagnosing or staging arthritis comprising administering to a subject a compound of any one of claims 1, 3, 6, or 7 and imaging the fluorescence that is generated.
17. A method of claim 17 further comprising comparing imaging results obtained over time.
18. A method of performing an in vitro assay for the presence of MMPs, comprising the in vitro incubation of a compound of claim 3, 6 or 7 with a bodily fluid, synovial fluid sample or ex vivo tissue sample and detecting the fluorescence that is generated.
19. A method of performing an in vitro assay for the presence of MMPs, comprising the in vitro incubation of a compound of claim 3, 6 or 7 with a sample of interest and detecting the fluorescence that is generated.
20. A method of performing an in vitro assay for the presence of an enzyme, comprising the in vitro incubation of a compound of claim 1 with a sample of interest and detecting the fluorescence that is generated.

FIG. 1

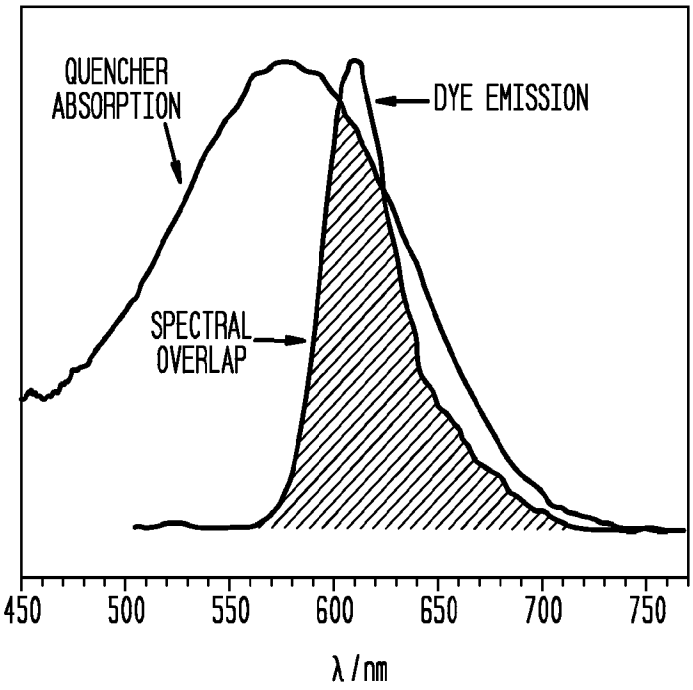

QUENCHER ABSORPTION
DYE EMISSION
SPECTRAL OVERLAP
450
500
550
600
650
700
750
λ/nm

FIG. 2

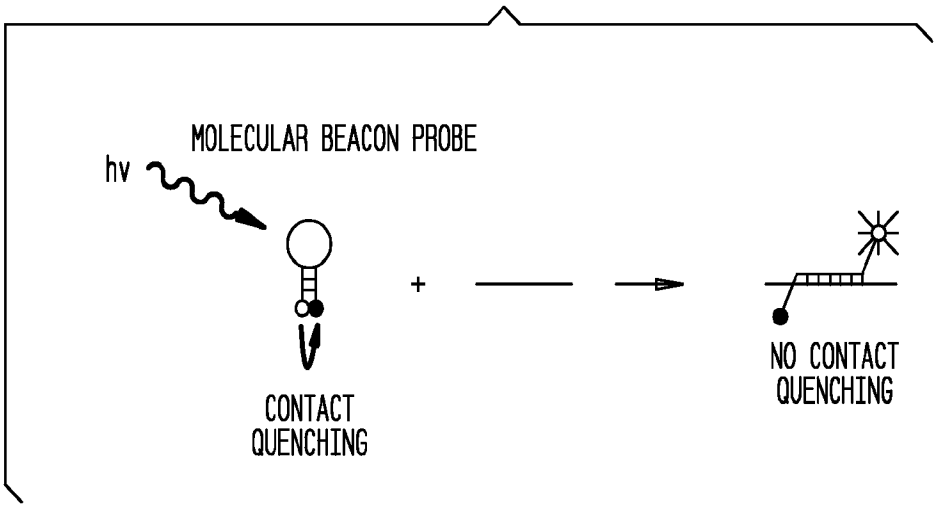

MOLECULAR BEACON PROBE
hv
+
CONTACT QUENCHING
NO CONTACT QUENCHING

FIG. 3

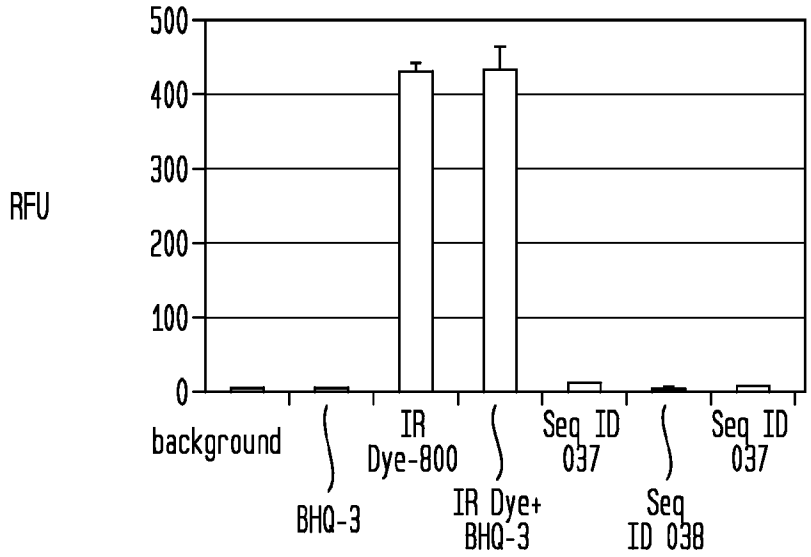

RFU
500
400
300
200
100
0
background
BHQ-3
IR Dye-800
IR Dye+ BHQ-3
Seq ID 037
Seq ID 038
Seq ID 037

FIG. 4

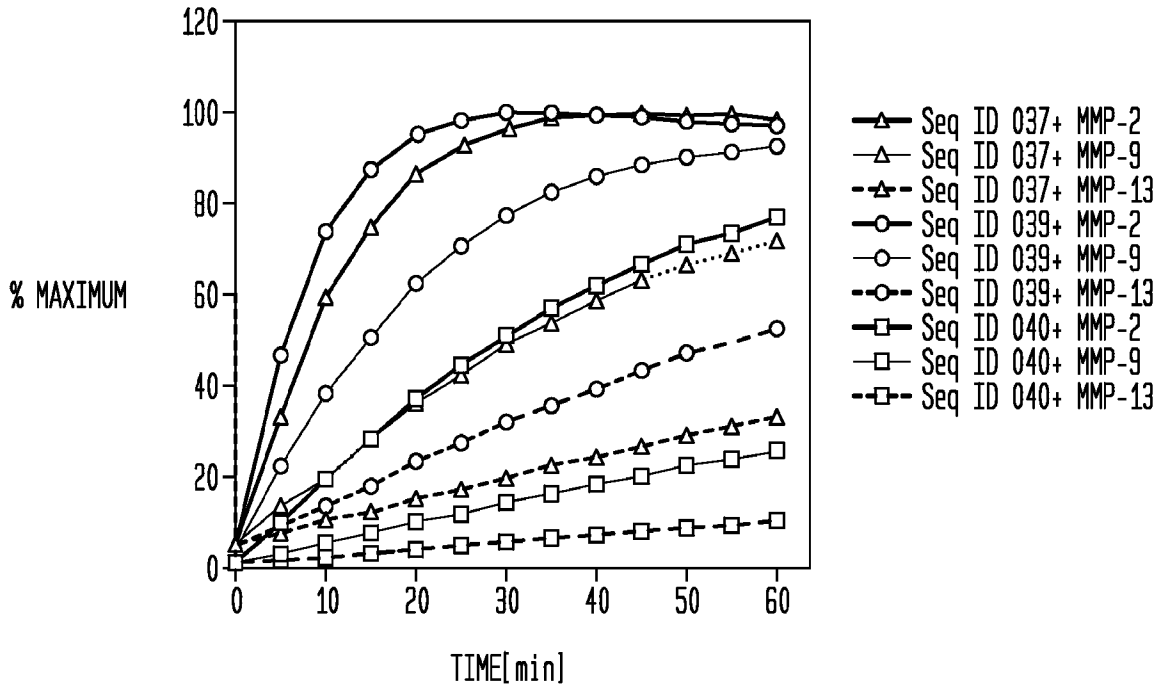
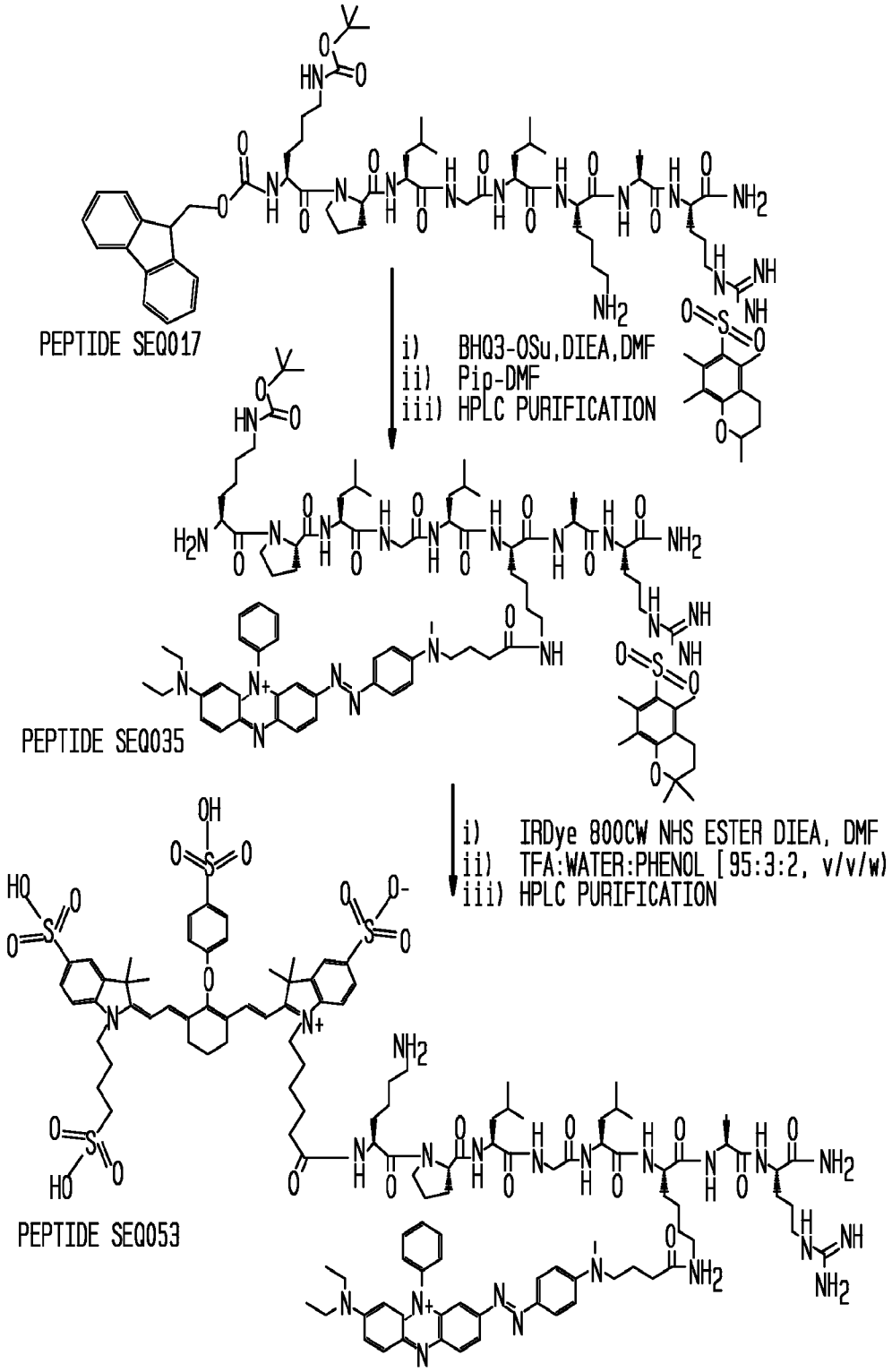

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100
80
60
40
20
0
0
10
20
30
40
50
60
TIME[min]
Seq ID 037+ MMP-2
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Seq ID 037+ MMP-13
Seq ID 039+ MMP-2
Seq ID 039+ MMP-9
Seq ID 039+ MMP-13
Seq ID 040+ MMP-2
Seq ID 040+ MMP-9
Seq ID 040+ MMP-13

FIG. 5



IRDye 800CW-Lys-Pro-Leu-Gly-Leu-Arg(BHQ3)-Ala-Arg-NH_2