Abstract: Measuring the level of HNE activity in the wound site enables diagnosing whether a chronic wound has active infection. A FRET peptide substrate containing an HNE-specific amino acid sequence is incubated with wound fluid and measured for fluorescent radiation to determine the level of HNE activity. The HNE diagnostic test is a rapid, point-of-care indicator of the level of HNE activity in wound fluid as an indicator of "active" infection.
METHODS FOR USING HUMAN NEUTROPHIL ELASTASE AS AN INDICATOR OF
ACTIVE WOUND INFECTION

CROSS-REFERENCE TO RELATED APPLICATIONS
[0001] This application claims priority from U.S. Provisional Application Serial No.
61/090,495 filed on August 20, 2008 and U.S. Provisional Application Serial No. 61/090,683
filed on August 21, 2008, each of which is incorporated herein by reference in its entirety.

INCORPORATION-BY-REFERENCE OF SEQUENCE LISTING
[0002] The Sequence Listing, which is a part of the present disclosure, includes a
computer readable file 5015878-30_ST25.TXT generated by U.S. Patent & Trademark Office
PatentIn version 3.5 software comprising nucleotide and/or amino acid sequences of the
present invention. The subject matter of the Sequence Listing is incorporated herein by
reference in its entirety.

BACKGROUND OF THE INVENTION
[0003] The present invention provides for a rapid point-of-care diagnostic system
which measures the level of Human Neutrophil Elastase (HNE) activity in biological fluid,
including chronic wound fluid to provide a quantitative indication of the level of "Active"
Infection in a chronic wound. The HNE is secreted by host neutrophils which migrate to the
wound site to neutralize the microbiological agents that are the primary cause of the active
infection. Simply measuring microbiological agents such as bacteria, fungi or the
expressions, metabolites, toxins, or cellular components or agents emitting from such
microorganisms, or components of the biofilm matrix in which bacteria and microorganisms
reside will not provide a meaningful indication of whether the existence of these
microorganisms is contributing to the failure of chronic wounds to heal. As used herein, an
"Active" infection is an infection caused by microorganisms, whether planktonic or
associated in biofilm communities that are maintaining a chronic state of wound
inflammation or contributing to wound deterioration and preventing wound healing by
continuing to stimulate the host to send large numbers of neutrophils to the wound site to
eliminate the threat. The present invention may also be applied to the diagnosis of other
active infections, for example, chronic lung infection or in chronic periodontal disease such
as gingivitis, by measuring the levels of HNE activity in sputum or gingival crevicular fluid,
respectively.

[0004] It is acknowledged that microorganisms, including bacteria and sometimes biofilm, can be present in wounds and yet not contribute to "Active" infection as defined herein, and that a diagnostic which cannot distinguish between an "Active" infection and "passive" microbial colonization is of little use as an indicator of the state of the wound with respect to whether the presence of microbes is preventing the wound from healing.

[0005] The present invention teaches that high levels of HNE in wounds are directly proportional to the massive recruitment of neutrophils to the wound (see Fig. 1) that have been activated to trigger the oxidative burst and release of protease granules, including the azurophilic granules that contain HNE, by stimuli emanating from microbial agents and colonies that are actively parasitizing wounds. The measurement of high HNE levels, therefore, will provide a useful indicator to wound care physicians of a problemmatical microbial presence that needs to be immediately addressed within the treatment options before the wound will progress towards healing.

[0006] High levels of HNE are a host response to "Active" infection, and the present invention further recognizes that these high levels of HNE contribute significantly to the further destruction of host tissue and the failure of wounds to heal. Treatment options therefore include strategies that will lower HNE activity, but this is treating the effect of the infection. A more logical way to treat the problem would be to treat the cause of the problem by eliminating the "Active" infection. Examples of how this is done today include antibiotic treatment and sharp debridement. Antibiotic treatment is only effective against planktonic bacteria and sharp debridement is a surgical procedure that must be repeatedly performed by a qualified health professional and involves removal of biofilm (along with other components of "slough" and necrotic tissue) that can re-establish within 24-48 hours.

[0007] Chronic wounds, including diabetic foot ulcers, pressure ulcers, venous leg ulcers, and ischemic leg ulcers are a grave worldwide problem. It has been estimated that 15 % of individuals with diabetes mellitus will develop lower extremity ulcers (Reiber, GE. 1996; The epidemiology of diabetic foot problems. Diabetes Med. 13 (Suppl. 1):S6-S11) and 14-24 % of diabetic patients with foot ulcers will eventually undergo amputation (American Diabetes Association. 1999; Consensus development conference on diabetic foot wound care. Diabetes Care 22:1354-1360). Approximately 100,000 limb amputations are performed in diabetic patients each year in the United States. Lower extremity ulcers were the cause of amputation in 67 of 80 patients (84 %) in a study performed by Pecoraro et al., (1990; Pathways to diabetic limb amputation basis for prevention. Diabetes Care 13: 513-521).
Pressure ulcers are a common and expensive problem in acute care, rehabilitation unit, nursing home, and home care populations. Venous leg ulcers are often painful inflections that have been estimated to affect 1% of the world's population (Trent et al., 2005; Venous ulcers: pathophysiology and treatment options. Ostomy wound manage 51(5): 38-54).

[0008] The effect of microbial infection on wound healing has been recognized for decades and the control of bioburden is recognized as an important aspect of wound management. Historically, it has been presumed that bacteria causing chronic infections exist in their planktonic state but more recent research has indicated that many chronic infections today are the result of the biofilm mode of microbial growth. Biofilm-related diseases are typically persistent infections that develop slowly, seem to be rarely resolved by immune defenses, and respond transiently to antimicrobial therapy. Recently, direct evidence of biofilm involvement in chronic wound infections was provided by James et al., (2008; Biofilms in chronic wounds. WoundRep Reg 16(1): 37-44) who found that 30 of 50 chronic wounds (60%) evaluated by microscopy contained biofilm, whereas only 1 of 16 acute wounds (6%) contained biofilm. Molecular analysis of the chronic wound specimens revealed diverse polymicrobial communities and the presence of bacteria, including strictly anaerobic bacteria, not revealed by culture (Dowd et al., 2008; Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). PLoS One 3(10): e3326).

[0009] There are presently no FDA-approved therapeutic agents that can effectively "kill" biofilm, although some are being developed that will slow down its re-establishment after sharp debridement. Regular debridement is currently the main tool for maintaining a healthy wound bed in most chronic wounds (Wolcott et al., 2009; Regular debridement is the main tool for maintaining a healthy wound bed in most chronic wounds. J Wound Care 18(2): 54-56). There is much research work going on to elucidate safe and efficacious treatments to impede biofilm re-establishment after debridement and to remove or kill biofilm communities without the need for debridement. Wolcott's group at the Southwest Regional Wound Care Center and Medical Biofilm Research Institute in Lubbock Texas is one group actively investigating new mechanisms of controlling biofilm growth in chronic inflammatory diseases, including wounds. Various agents are under investigation as anti-biofilm therapeutics, including Lactoferrin, Xylitol, EDTA, Gallium, Dispersin B, Farnesol, RNA-III Inhibitory Peptide, Furanone C30 (Wolcott and Rhoads, 2008; A study of biofilm-based wound management in subjects with critical limb ischemia. J Wound Care 17(4): 145-148, 150-152, 154-155) and bacteriophage (Rhoads et al., 2009; Bacteriophage therapy of

**DRAWINGS**

[0010] **FIGURE 1. Agents emitted from "Active" Infection recruit neutrophils to the site of infection.** Neutrophils get activated to trigger the oxidative burst, the release of proteases and the phagocytic mode to eliminate the causative agent of the "Active" infection. With the constant recruitment and activation of neutrophils, the levels of HNE become higher than the levels of natural inhibitors (e.g. α1 protease inhibitor) that normally inactivate the excess HNE such that this excess HNE also contributes to the disease by degrading host tissue (collagens, elastin, proteoglycans, growth factors) thereby stalling wound healing or causing even more host tissue damage.

[0011] **FIGURE 2. Example Diagnostic Device System in Which the HNE Activity in Biological Fluid May be Measured.** The system consists of a sterile swab for biological fluid collection (b), a plastic tube device (available from Medical Packaging Corporation) in which the chemistry of the test takes place (a), and a fluorometer (c) to measure the amount of fluorescent product released during the test.

[0012] **FIGURE 3. Steps in the Use of the Example HNE Activity Detector.**

[0013] **FIGURE 4. Schematic Representations of HNE-Specific FRET Peptide Substrates,** (a) FRET peptide substrate containing a single fluorophore and a single quencher, (b) FRET peptide substrate containing a single fluorophore and two quenchers with two HNE-specific cleavage sites.

**DETAILED DESCRIPTION OF THE INVENTION**

[0014] The present invention utilizes the discovery that increased levels of Human Neutrophil Elastase (HNE) activity provides a quantitative indication of the level of "Active" infection in a chronic wound. One embodiment incorporates this phenomenon in providing a rapid, point-of-care diagnostic as described herein.

[0015] A FRET peptide substrate containing an HNE-specific amino acid sequence, for example, Quencher-APEEIMRRQ-Fluorophore (SEQ ID NO: 1) or Quencher-AAPV-Fluorophore (SEQ ID NO: 2), will be incubated with wound fluid at ambient temperature for around 10 or more minutes. One example of a device in which the biological fluid will be incubated with an HNE-specific substrate peptide is represented schematically in Figures 2 and 3.

[0016] When ready to use this device, the practitioner opens the end of the sterile...
swab package (Fig. 2, b) opposite from the sample collection bulb. The practitioner then removes the top of the plastic device (Fig. 2, a; available from Medical Packaging Corporation) which has a slot on its underside for the hollow shaft of the sterile swab to insert into. Once the swab is inserted into the top, the wound care provider swabs the affected area of the patient and then inserts the swab into the plastic tube, replacing the top on the tube [Figure 3 (a)].

[0017] The practitioner then bends the snap valve to 45° [Figure 3 (b)] to allow the solution in the top chamber to flow down the hollow shaft of the swab and perfuse the swab bulb to elute and dilute the biological fluid sample and bathe the swab in reaction solution for around 10 or more minutes [Figure 3 (c)]. This solution also dissolves a cocktail of one or more protease inhibitors (represented by small spheres in the upper chamber), located on top of the thin foil layer in the plastic tube, to prevent non-specific cleavage of the HNE substrate peptide during the reaction phase of the test. During the 10 minute reaction time, HNE cleaves a peptide substrate to release a fluorescent signal. The more HNE activity in the biological fluid sample, the more fluorescence is released during the 10 minute incubation period. At the end of around 10 or more minutes the practitioner pushes down on the top of the device [depicted by the arrow in Figure 3 (d)] to cause the tip of the swab to perforate the thin foil membrane and allow the reaction mixture to flow to the bottom of the tube [Figure 3 (d)]. At this time an HNE neutralizing antibody, which may be located in the bottom of the tube and/or another specific HNE inhibitor (represented by the small spheres in the bottom chamber), dissolves in the reaction buffer and inhibits further HNE proteolysis of the peptide substrate such that no more fluorescence is generated. The bottom of the device is then inserted in the reading chamber of the fluorometer [Figure 2 (c)] which has been pre-calibrated using a solid fluorescent standard.

[0018] The buffer in which the HNE-specific FRET substrate peptide is dissolved in the top bulb of the device could be any buffer system conducive to the proteolysis reaction, including but not limited to Tris, phosphate, acetate, citrate, borate, cacodylate, TAPS, Bicine, Tricine HEPES, TES, MOPS, PIPES, and MES. It may contain a number of additives conducive to peptide stability of the proteolytic reaction. For example, it might contain an antioxidant to protect methionine or cysteine or fluorophore or quencher oxidation, should these amino acids or oxidisable fluorophore/quencher be included in the FRET peptide substrate. It might contain a detergent such as Brij-35 or other compound to promote solubility or proteolytic reaction. Its volume and the peptide concentration within it will be optimized to meet the specific kinetic reaction conditions that facilitate a 10 minute point-of-
care diagnostic system.

[0019] The test will be designed to be done at "room temperature", which is defined as the approximate temperature of the room in which the test is performed. We understand that a typical range of temperatures that would be found at different times of the year in hospital clinics in the various countries where this test would be sold would be 65-85 °F (-18-30 °C). This is also the temperature range within which regulatory agencies like to see robust test performance. It is not anticipated that temperature will need to be controlled for successful performance of the test, but the practitioner may be directed to perform the test within the above as the range of temperature. One embodiment of the test would be a rapid point-of-care test that the wound care professional would perform during the patient consultation to help guide their immediate decision about treatment strategy. The 10 minute time for completion of the test reflects this goal. However, it is a goal to also design into the capability of the test several alternative options for test performance to make it more flexible for the practitioner, which would mean the test would take longer to complete. For example, the practitioner may only have time to collect the sample and might choose to run the test at a later time and the option to collect a sample on a swab and run the test up to several hours later may be possible. Second, the test will include a "stop" step, where the enzymatic reaction is stopped (by the addition of a neutralizing antibody and/or protease inhibitor at a certain time point) and the practitioner will have the option to complete the test up to that point and come back up to several hours later to read the result. The central objective is to provide a rapid, point-of-care diagnostic assay that the wound care professional can use to gain biochemical information that will help guide him or her in the best treatment option at that time. Ten minutes is an optimal time point that is neither too short for adequate confidence in the result nor too long so as to preclude the capture of that piece of diagnostic information within the time frame of a typical patient consultation.

[0020] The peptide substrate may contain a quencher on the one side (e.g. the amino-terminal side) of the scissile bond and a fluorophore on the other side (e.g. the carboxyl-terminal side) of the scissile bond such that when the scissile bond is cleaved by HNE, the fluorophore signal is no longer quenched by the quencher and a fluorescent signal will occur in the sample. Examples of quenchers that could be used include: DABCYL; QXS-520; FITC; Dnp; BHQ-I (see also TABLE 2). Examples of fluorophores that could be used include: EDANS; 5-FAM; Rhodamine; Mca (see also TABLE 1).

[0021] The range of wavelengths used to achieve a fluorescent signal would depend upon the FRET pair chosen. In one example, 5-FAM is used as the fluorophore and Dabsyl as
the quencher. In this case, the excitation optics would put out light at 745 ± 15 nm (e.g. from a blue LED such as is contained in the Turner Biosystems Picofluor fluorometer. The emission optics (e.g. a photodiode detector within the Turner Biosystems Picofluor fluorometer) would measure fluorescence at 515 ± 20 nm. If Rhodamine is used as the fluorophore, an excitation wavelength of 525 ± 20 nm (e.g. using the green LED light source within the Turner Biosystems Picofluor fluorometer) would be used, and the emission optics would be set at >570 nm, again using the Turner Biosystems Picofluor fluorometer.

[0022] The Picofluor fluorometer (see http://www.turnerbiosystem.com/nslrums/PicoFluor-hand-held-tflurometer-fluorometer-DNA:ENArPlote.php) is commercially available from Turner Biosystems, Inc. The Picofluor has two dedicated optical channels that allow for the measurement of two different fluorophores in the same experiment, if required. It has an internal data logging package with interfacing software to Microsoft and Excel through a Serial Interface cable connection to a computer. The Picofluor can log up to 1,000 data points. It is CE marked for immediate use under European regulatory guidelines.

[0023] The fluorescence can be read and qualified using this portable hand-held fluorometer and recorded in patient medical records. The sequential HNE activity levels in any single wound can be followed over time to guide the physician in management of the wound. A second generation fluorometer that may be used will contain similar features with expanded firmware and software options that could automatically manage patient data over time. The fluorescence can be read and quantified in a portable hand-held fluorometer and recorded in patient medical records. The level can be followed over time to guide the physician in management of the wound.

[0024] The HNE diagnostic test will be a rapid, Point-of-care indicator of the level of Human Neutrophil Elastase activity in wound fluid. It is anticipated that the test will have utility both the first time a wound care professional measures HNE activity in a patient, and then with subsequent regular (e.g. weekly) measurements of the HNE activity over time. Both measurement types (the initial and the sequential) would add to other diagnostic parameters (including subjective sensory perception of wound size, depth, color, swelling, level of exudates, visual presence of slough, and smell) to aid the physician in their treatment strategy (the initial measurement to help choose a treatment at that time; and the sequential measurements to indicate if that treatment strategy is working or whether it needs to be changed/modified). The test provides the first objective biochemical measurement of a parameter in wound fluid that is associated with the chronicity of wounds. Specifically, a
high HNE activity is known to be associated with chronic, non-healing wounds (Yager et al., 1997. Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of protease inhibitors. Wound Rep Regen, 5(l):23-32). The present invention teaches that a high activity level of this enzyme would indicate the wound was infected and that this infection was directly contributing to the failure of the wound to heal. Wolcott and colleagues have demonstrated that biofilm often contributes to wounds being stuck in such a chronic inflammatory state (Wolcott et al., 2008, Biofilms and Chronic Wound Inflammation. J Wound Care, 17(8):333-341). A high HNE activity level, therefore, will initially alert the wound care professional to begin a treatment strategy to impede the re-establishment of biofilm after sharp (surgical) debridement (the only effective biofilm removal strategy presently available; Wolcott et al, 2009, Regular Debridement is the Main Tool for Maintaining a Healthy Wound Bed in Most Chronic Wounds. J, Wound Care, 18(6):237-238, 240-243). Regular HNE activity measurements using the test described in this invention will indicate whether the treatment strategy the wound care professional has implemented is being successful.

[0025] In one method treatment, PHI (polyhydrated ionogens, available from Greystone Pharmaceuticals, Inc.) or other wound care preparations may be administered to impede the re-establishment of biofilm after sharp debridement. See U.S. patent numbers 6,149,947 and 7,148,170, incorporated herein by reference in their entirety. One PHI-based compound used in this regard includes PHI and a hydrogel compound that also impedes the re-establishment of biofilm after debridement.

[0026] With regard to treatment strategy, the HNE indicator would guide wound care professionals in what treatment options to apply to the wound. If they had previously embarked on one treatment option, the indicator would show if that strategy was being successful. The caregiver might well change the therapeutic strategy being applied to any given wound based upon the results of the test. Treatment would not stop until full closure of the wound had occurred.

[0027] The test would have a "cut-off" or baseline level of HNE activity, above which it would indicate the presence of active infection, possibly involving biofilm, and below which there was no such infection. In one embodiment, the cut-off level is in the range of 0.25 mU/mg protein - 0.5 mU/mg protein. Yager et al, 1997 (Wound Rep Reg 5(l):23-32) found that the level of HNE activity in chronic (non-healing) wound fluid (mean = 1.095 mU.mg protein) was 14-fold higher than that in acute (healing) wound fluid (0.077 mU/mg protein). Yager et al, found levels of HNE activity to be highly variable in fluids from
chronic (non-healing) wounds, reaching maximum levels of about 2.3 mU/mg protein, or nearly 30-times the level of HNE activity in healing wounds. Elsewhere this same group reported HNE activity levels up to 100-times higher in chronic wounds than in acute wounds. Other measurement of enzyme activity formats can be used in referencing the enzyme activity of the test sample in comparison to a baseline level of HNE activity expected in a non-chronic wound. The comparative test employing a FRET pair measures fluorescence intensity which can be calibrated against a baseline level and expressed in fluorescent units.

[0028] With respect to the HNE-specific amino acid sequences used with the present invention, these can be derived in several ways: first from reproducing a portion of the natural protein substrate sequence of HNE such as those in several of the collagens, elastin, fibronectin, cartilage proteoglycans, E.Coli OmpA, and virulence factors of several bacteria such as Shigella, Salmonella, and Yersinia. The different sequences within the various natural substrates HNE cleaves could be used with the invention. For example, we might use the sequence from human liver Type-III collagen, GPLGIA GITGARGLAGP (SEQ ID NO: 3) (Mainardi et al., 1980; Specific cleavage of human type-III collagen by human polymorphonuclear leukocyte elastase. J Biol Chem 255(24): 12006-12010). A second way of designing a substrate peptide to use with the invention would be by adaptation of the inhibitory loops of natural inhibitors such as the serpin, ocl-protease inhibitor (oci-antitrypsin). Example sequences coming from this method include, but are not limited to, the inhibitory loop sequences of various serpins including ocl-proteinase inhibitor,
(LEAIPMSIPPEVKFNKPF (SEQ ID NO: 4) and truncated versions and derivatives); monocyte/neutrophil elastase inhibitor (GIATFCMLMEENFTAD (SEQ ID NO: 5) and truncated versions and derivatives); plasminogen activator inhibitor-1 (VIVSARMAPEEMIDRP (SEQ ID NO: 6) and truncated versions and derivatives); proteinase inhibitor-9 (CFVVAECMCESGPRFCA (SEQ ID NO: 7) and truncated versions and derivatives); proteinase inhibitor-6 (AIMMMRCARFVPRFCAD (SEQ ID NO: 8) and truncated versions and derivatives); cowpox virus serpin CrmA (CALVADCASTVTNEFCA (SEQ ID NO: 9) and truncated versions and derivatives); APEEIMRRQ (SEQ ID NO: 10); APEEIMDRQ (SEQ ID NO: 11); APEEIMDYQ (SEQ ID NO: 12); and VAECQC (SEQ ID NO: 13). A third way of designing amino acid substrate sequences for HNE is from the list of synthetic inhibitors that are commercially available. This latter class of sequences include AAPV (SEQ ID NO: 14), AAPA (SEQ ID NO: 15), and AAAA (SEQ ID NO: 16). To determine the sequences used with the invention, cleavage rates and specificity will be
analyzed. A sequence used with the present invention will preferably cleave rapidly at "room temperature" (see below) and be as specific for HNE as possible. One example substrate with a suitable sequence is described as follows:

[0029] The substrate is formulated as a "FRET peptide", that is, a peptide containing a fluorophore and a quencher spaced between 10-100 Å apart, and can be represented by diagrams such as are shown in Figures 4 (a) and (b):

[0030] Thus, the APEEIMRRQ (SEQ ID NO: 10), AAPV (SEQ ID NO: 14) or other sequences would be contained within the construct as a specific HNE cleavage site. Once the enzyme cleaved the sequence, the fluorophore is released from the distance constraint of the quencher and fluorescence occurs (see explanation of the principles of this process below). There may or may not be flanking sequences around the HNE recognition/cleavage sequences (Fig. 4 shows flanking sequences).

[0031] A fluorophore has the ability to absorb energy from light, transfer this energy internally, and emit this energy as light of a characteristic wavelength. Fluorescence is generated when a substance absorbs light energy at a short (higher energy) wavelength, and then emits light energy at a longer (lower energy) wavelength. Thus, for any fluorescent molecule, the wavelength of emission is always longer than the wavelength of absorption (Stokes’ Law).

[0032] The specific wavelength at which one of these molecules will most efficiently absorb energy is called the peak absorbance and the wavelength at which it will most efficiently emit energy is called the peak emission. Peak absorbance and peak emission wavelengths for some of the common fluorophores used in molecular applications, and which we might use in the present invention, are shown in TABLE 1:

**TABLE 1: Peak Absorbance and Peak Emission wavelengths, Stokes' Shifts, and Extinction Coefficients For 43 Common Fluorophores**

<table>
<thead>
<tr>
<th>Fluorophore (Dye)</th>
<th>Absorbance (nm)</th>
<th>Emission (nm)</th>
<th>Stokes Shift (nm)</th>
<th>Extinction Coefficient</th>
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<tbody>
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<td>362</td>
<td>462</td>
<td>100</td>
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</tr>
<tr>
<td>AMCA</td>
<td>353</td>
<td>442</td>
<td>89</td>
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<tr>
<td>Fluorophore (Dye)</td>
<td>Absorbance (nm)</td>
<td>Emission (nm)</td>
<td>Stokes Shift (nm)</td>
<td>Extinction Coefficient</td>
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<td>------------------</td>
<td>----------------</td>
<td>---------------</td>
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<td>------------------------</td>
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<td>Emission (nm)</td>
<td>Stokes Shift (nm)</td>
<td>Extinction Coefficient</td>
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Drawn from "Fluorescence and Fluorescence Applications" by Mark A. Behlke.


[0035] Different processes can decrease the intensity of fluorescence. Such
decreases in fluorescence intensity are called "quenching". Currently, most fluorescence detection techniques are based on quenching of fluorescence by energy transfer from one fluorophore to another fluorophore, or to a non-fluorophore. Thus, the acceptor (quencher) moiety can be either fluorescent or non-fluorescent. Until more recently, quenchers have been other fluorophores that accept a photon from energetically excited donor fluorophores. As a result, the energy level of the donor fluorophore returns to the ground state, without emitting fluorescence.

[0036] A requirement is that the fluorescence emission spectrum of the donor must overlap the absorption spectrum of the acceptor. If the acceptor is a fluorophore, the transferred energy can be emitted as fluorescence from the acceptor, but at a different wavelength that is not being measured by the fluorometer and so won’t be "seen" as fluorescence. If the acceptor is not fluorescent, the absorbed energy is lost as heat.

[0037] Consider the example of the classic FRET pair of FAM and TAMRA. Peak absorbance wavelength for FAM is 490 nm with a peak emission wavelength of 518 nm. If FAM and TAMRA are tethered at the amino-terminal and carboxyl-terminal of a peptide, respectively, and this construct is excited at 490 nm, so long as the peptide remains intact, emission will be at 580 nm (TABLE 1) and not at 518 nm (the wavelength being measured in the fluorometer) due to FAM transferring its energy to TAMRA. The fluorescence of the donor is thereby quenched through resonance energy transfer. Enzyme hydrolysis of the peptide results in spatial separation of the donor and acceptor, which leads to the recovery of the fluorescence of the donor, in this case at 518 nm, which wavelength the fluorometer has been set to measure by the practitioner of the experiment.

[0038] In recent years, TAMRA, as well as other fluorescent acceptor molecules, has been replaced with one or another of the growing family of dark quencher molecules. Dark quenchers are related to fluorophores but instead of emitting absorbed fluorescence resonance energy as light, they have the useful property of transforming the light energy to heat. Heat dissipation of fluorescence energy means that replacing a fluorescent acceptor like TAMRA with a quencher such as BHQ-I, will result in a peptide construct that has no measurable fluorescence, so long as the peptide tether remains intact. Such constructs can greatly simplify many fluorescence assays since there will be no background fluorescence. A list of quenchers used in molecular applications and which we might use in the present invention is presented in TABLE 2.
TABLE 2: Quencher Labels for Fluorescent probes

<table>
<thead>
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<th>Quencher</th>
<th>Absorption Maximum (nm)</th>
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<tr>
<td>DDQ-1</td>
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<tr>
<td>Dabcyl</td>
<td>475</td>
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<tr>
<td>Eclipse</td>
<td>530</td>
</tr>
<tr>
<td>Iowa Black FQ</td>
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<td>Iowa Black RQ</td>
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<tr>
<td>QSY-21</td>
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<tr>
<td>BHQ-3</td>
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</table>

[0039] For the design of FRET peptides, fluorophore-quencher pairs that have sufficient spectral overlap should be chosen. Fluorophores with an emission maximum between 500 and 550 nm, such as 5-FAM, TET, and HEX (TABLE 1), are best quenched by quenchers with absorption maxima between 450 and 550 nm, such as Dabcyl and BHQ-I (TABLE 2). Fluorophores with an emission maximum above 550 nm, such as Rhodamines (including Rhodamine Red, TAMRA, ROX and Texas Red) and Cy dyes (including Cy3 and Cy5), are best quenched by quenchers with absorption maxima above 550 nm (including BHQ-2 [TABLE 2]).

[0040] EXAMPLES

[0041] Example 1 - Use as a diagnostic of active infection in chronic dermal wounds. The wound care provider would remove the wound dressing from the patient's wound and take a sample of wound fluid before applying any treatment to the wound. In this way the HNE activity in the wound can be assessed at the time of consultation and would
represent the wound environment over the past 24 hours or longer. If using the Medical Packaging device shown in Figures 2(a) and 3, a soft sterile Dacron swab, designed to hold 80-100 µL of fluid, will be inserted into the top of the device. The wound care professional would then swab the patient's wound by translating the swab gently over the entire wound surface so as to collect sample from as much of the wound surface area as possible, while simultaneously gently rotating the swab so as to expose all surfaces of the swab to the wound. After sample acquisition, the practitioner will insert the swab into the elongated hollow tube of the device and replace the top on the tube [Figure 3 (a)].

[0042] The practitioner then bends the snap valve to 45° [Figure 3 (b)] to allow the solution in the top chamber to flow down the hollow shaft of the swab. This optimized volume of solution containing the FRET peptide substrate at an optimized concentration would consist of a buffer, say phosphate buffer at known pH, say 7.4, and may contain additives such as detergent (e.g. Brij-35) and anti-oxidants (e.g. glutathione, ascorbic acid or α-tocopherol). The FRET peptide could consist of any sequence cleaved by HNE, as given by example herein, for example, APEEIMRRQ (SEQ ID NO: 10) flanked on the amino-terminal side by any quencher in TABLE 2, for example DABCYL and flanked on the carboxyl-terminal end by any fluorophore in TABLE 1, for example 5-FAM. The amino-terminal and carboxy-terminal ends of the substrate can be modified to accept the fluorophore and quencher, i.e., DABCYL-GABA-APEEIMRRQK(5-FAM). The quencher and fluorophore could be attached at opposite ends of the peptide to those mentioned above, so long as they straddle the scissile bond of the peptide and are within 10-100 Å of one another in the FRET peptide. The HNE peptide substrate sequence will be optimized for enzyme kinetics, HNE specificity, and to minimize promiscuity by other proteases. If necessary, non-specific proteolytic cleavage will be eliminated by the inclusion of protease inhibitors that are specific for the interfering substances.

[0043] The FRET peptide buffer solution would perfuse the swab to elute and dilute the wound fluid sample and bathe the swab in the reaction solution for a period of time, say 10 minutes [Figure 3 (c)]. This solution also dissolves a cocktail of one or more protease inhibitors to prevent non-specific cleavage of the HNE substrate peptide during the reaction phase of the test. During the 10 minute reaction time, HNE cleaves a peptide substrate to release a fluorescent signal. The more HNE activity in the wound fluid sample, the more fluorescence would be released. At the end of the incubation time the wound care provider pushes down on the top of the device [depicted by the arrow in Figure 3 (d)] to cause the tip
of the swab to perforate the thin foil membrane and allow the reaction mixture to flow to the bottom of the tube [Figure 3 (d)]. At this time an HNE neutralizing antibody, which may be located in the bottom of the tube and/or another specific HNE inhibitor dissolves in the reaction buffer and inhibits further HNE proteolysis of the peptide substrate such that no more fluorescence is generated. The bottom of the device is then inserted in the reading chamber of the fluorometer [Figure 2 (c)] which has been pre-calibrated using a solid fluorescent standard. The readout result on the fluorometer is directly proportional to the HNE activity in the patient wound fluid and will be entered into the patient's records.

[0044] Yager et al. Wound Rep Regen, 5(1):23-32, have demonstrated elevated HNE activities in chronic wounds. Early clinical testing with this device will establish a "cutoff" level of HNE activity that distinguishes chronic from acute wounds. Using the current assessment methods for determining whether a wound is healing or non-healing, including the sensory perceptions of sight, smell, and touch, and wound area measurement, the fluorometric readout for HNE activity will be correlated with these classical diagnoses to establish the range of HNE activities typical of chronic wounds, and healing trajectory. In one analysis, the fluorometric readout will be correlated with the wound area and depth as determined by computer assisted tomography. In a second analysis, the fluorometric readout will be correlated with the presence of bacteria associated with biofilms, for example, Pseudomonas aeruginosa, Enterococcus faecalis, and Staphylococcus aureus and others (Sun et al., 2008; In vitro multispecies Lubbock chronic wound biofilm model. Wound Rep 16(6): 805-813) and whether those biofilms or planktonic bacteria are causing active infection leading to a chronic inflammatory wound. These studies will result in fluorometric levels of HNE activity that correlate with active wound infection.

[0045] The rapid test time of this point-of-care diagnostic decision will allow wound care practitioners to make real-time decisions while patients are still in their consultation rooms, as to whether the wound needs to be managed for active infection or not. If so, there are a number of agents available that, after sharp debridement, can slow the re-establishment of biofilm and treat the active infection. There is also research underway to evaluate compounds that will prevent the re-establishment of biofilm after debridement or even kill established biofilm without the need for surgical intervention. When these are characterized, the present invention may be used in conjunction with a biofilm removing agent and they could together become a "theranostic" system (diagnosis and therapy).

[0046] Example 2 - Use as a diagnostic of active infection in periodontal disease. The device would be used in the same way as for chronic dermal wounds except the
sample collected would be gingival crevicular fluid.

[0047] Example 3 - Use as a diagnostic of active infection in chronic lung disease. The device would be used in the same way as for chronic dermal wounds except the sample collected would be sputum.

[0048] In various embodiments, the present invention can also be formulated as kits. In this use, the kit could be comprised of different components that could be packaged together or separately. In one embodiment, the kit could be comprised of a pack of devices as shown in Figure 2A, a corresponding number of sterile swabs [Figure 2B], a fluorometer [Figure 2C], and a number of devices depicted in Figure 2A containing a swab that had been impregnated with known amounts of active HNE as a standard. Here, the standard could be used to either calibrate the system with respect to HNE activity or they could be for the purpose of demonstrating that the reagents in that particular manufactured lot or device were still viable at the time of use by the wound care provider and that the wound care provider could competently operate the diagnostic test system. In another embodiment, the components could be packaged separately such that a fluorometer could be obtained, or a number of test devices and swabs could be obtained, or a number of standards could be obtained.

[0049] The detailed description set forth above is provided to aid those skilled in the art in practicing the present invention. However, the invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed because these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description which do not depart from the spirit or scope of the present inventive discovery. Such modifications are also intended to fall within the scope of the appended claims.
CLAIMS

What is claimed is:

1. A method for diagnosing a patient's wound, comprising the steps of:
   a) obtaining a sample of biological fluid from the patient's wound;
   b) applying the sample to a test solution comprising an enzymatic substrate for Human Neutrophil Elastase;
   c) assaying the test solution to determine a level of enzyme activity of Human Neutrophil Elastase on the substrate in the test solution;
   d) comparing the level of enzyme activity in the test solution to a predetermined value of enzyme activity to determine whether the wound contains active infection.

2. The method according to Claim 1 in which the predetermined value of enzyme activity is calibrated to units of measurement representing a baseline value associated with an acute wound infection such that a measured level of enzyme activity in the units of measurement having a value greater than the baseline value indicates the presence of active infection in a chronic wound.

3. A method for diagnosing a patient's wound, comprising the steps of:
   a) obtaining a sample of biological fluid from the patient's wound;
   b) applying the sample to a test solution comprising an enzymatic substrate for Human Neutrophil Elastase, wherein the substrate comprises an amino acid sequence cleavable by Human Neutrophil Elastase at a scissile bond, the amino acid sequence comprising a FRET pair disposed near the scissile bond such that cleavage of the scissile
bond will separate a fluorophore and quencher comprising the FRET pair from each other to yield fluorescence;

c) exposing the test solution to a fluorometer to detect fluorescence generated by separation of the FRET pair from cleavage of the scissile bond;

d) determining a level of enzyme activity of Human Neutrophil Elastase on the substrate in the test solution by measuring the intensity of the fluorescence;

e) comparing the level of enzyme activity in the test solution to a predetermined value of enzyme activity to determine whether the wound contains active infection.

4. The method according to Claim 3 in which the predetermined value of enzyme activity is calibrated to units of measurement representing a baseline value associated with an acute wound infection such that a measured level of enzyme activity in the units of measurement having a value greater than the baseline value indicates the presence of active infection in a chronic wound.

5. The method according to Claim 4 in which the units of measurement comprise fluorescent units.

6. The method according to Claim 3 in which the amino acid sequence is selected from the group consisting of AAPV (SEQ ID NO: 14), AAPA (SEQ ID NO: 15), AAAA (SEQ ID NO: 16), APEEIMRRQ (SEQ ID NO: 10), GPLGIAGITGARGLAGP (SEQ ID NO: 3), LEAIPMSIPPEVKFNKPF (SEQ ID NO: 4), GIATFCMLMPEENFTAD (SEQ ID NO: 5), VIVSARMAPEEIMDRP (SEQ ID NO: 6), CFVVAECCMESGPRFCA (SEQ ID NO: 7), AIMMMRCARFVPRFCAD (SEQ ID NO: 8), CALVADCASTVTNEFCA (SEQ ID NO: 9), and LEAIPMSIPPEVKFNKPF (SEQ ID NO: 4).
NO: 9), APEEIMDRQ (SEQ ID NO: 11), APEEIMDYQ (SEQ ID NO: 12), and VAECCQ (SEQ ID NO: 13).

7. The method according to Claim 3 in which the fluorophore and the quencher of the FRET pair are spaced between 10-100 Å apart.

8. The method according to Claim 3 in which the fluorophore is selected from the group consisting of EDANS, 5-FAM, Rhodamine, Mca, Acridine, AMCA, BODIPY FL-Br2, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY TR, BODIPY 630/650, BODIPY 650/665, Cascade Blue, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, DABCYL, Eosin, Erythrosin, Fluorescein (FAM), TET, Joe, HEX, Lightcycler 640, Lightcycler 705, NBD, Oregon Green 488, Oregon Green 500, Oregon Green 514, Rhodamine 6G, Rhodamine Green, Rhodamine Red, Rhodol Green, TAMRA, ROX, Texas Red, NED, and VIC.

9. The method according to Claim 3 in which the quencher is selected from the group DABSYL, QSX-520, HTC, Dnp, BHQ-I, BHQ-2, BHQ-3, Eclipse, Iowa Black FQ, Iowa Black RQ, QSY-7, DDQ-II, QSY-21.

10. A kit for diagnosing the presence of active infection in a patient's wound, the kit comprising:

   a) sterile swabs for collecting a biological fluid sample from a patient's wound,

   b) an enzymatic substrate for Human Neutrophil Elastase, the substrate comprising an amino acid sequence cleavable by Human Neutrophil Elastase at a scissile bond,
c) a FRET pair comprising a fluorophore and a quencher attached to the substrate near the scissile bond such that cleavage of the scissile bond will separate a fluorophore and quencher comprising the FRET pair from each other to yield fluorescence,

d) a collection tube for mixing the biological fluid sample with the substrate to create a test solution,

whereby the relative degree of enzymatic activity by Human Neutrophil Elastase upon the substrate is measurable by the degree of fluorescence emitted in the test solution.

11. The kit according to Claim 10 in which the collection tube contains an agent to stop the generation of fluorescence at a designated time.

12. The kit according to Claim 11 in which the agent comprises a neutralizing antibody.

13. The kit according to Claim 11 in which the agent comprises an inhibitor of Human Neutrophil Elastase.

14. The kit according to Claim 10 in which the amino acid sequence is selected from the group consisting of AAPV (SEQ ID NO: 14), AAPA (SEQ ID NO: 15), AAAA (SEQ ID NO: 16), APEEIMRRQ (SEQ ID NO: 10), GPLGIAGITGARGLAGP (SEQ ID NO: 3), LEAIPMSIPPEVKFNKF (SEQ ID NO: 4), GIATFCMLMPEENFTAD (SEQ ID NO: 5), VIVSARMAPEEIMDRP (SEQ ID NO: 6), CFVVAECMESCPRFCA (SEQ ID NO: 7), AIMMMRCARFVPRF (SEQ ID NO: 8), CALVADCASTVNEFCA (SEQ ID NO: 9), APEEIMDRQ (SEQ ID NO: 11), APEEIMDYQ (SEQ ID NO: 12), and VAECCQ (SEQ ID NO: 13).

15. The kit according to Claim 10 in which the fluorophore is selected from the group consisting of EDANS, 5-FAM, Rhodamine, Mca, Acridine, AMCA, BODIPY FL-Br2,

16. The kit according to Claim 10 in which the quencher is selected from the group DABSYL, QSX-520, HTC, Dnp, BHQ-I, BHQ-2, BHQ-3, Eclipse, Iowa Black FQ, Iowa Black RQ, QSY-7, DDQ-II, QSY-21.

17. The kit according to Claim 10 in which a control swab impregnated with a predetermined amount of Human Neutrophil Elastase is provided, whereby the control swab is used as a reference to compare against the test solution.

18. The method according to Claim 1 in which the wound is a dermal wound.

19. The method according to Claim 1 in which the wound arises from chronic periodontal disease.

20. The method according to Claim 1 in which the wound arises from chronic lung infection.
Active Infection (biofilm) $\rightarrow$ Neutrophils $\rightarrow$ Human Neutrophil
Elastase
(a) 

Quencher (Acceptor) \[\text{Peptide Sequence} \quad \text{Fluorophore (Donor)}\]

containing APEEI/MRRQ (SEQ ID NO: 10)

energy (photon)

(b) 

Quencher \[\text{Peptide Sequence} \quad \text{Fluorophore} \]

containing AAPV (SEQ ID NO: 14)

Flanking sequences

Second peptide sequence also containing AAPV, for example
INTERNATIONAL SEARCH REPORT

A CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/37; GOIN 33/48 (2009 01)
USPC - 435/23; 436/63
According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/37; GOIN 33/48 (2009 01)
USPC - 435/23; 436/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/4; 23, 436/63
(Text Search)

Electronic data base consulted during the international search (name of database and, where practicable, search terms used)
PubWEST (PUBP, USPT, USOC, EPAB, IPAB), DialogPRO (Engineering), PubMed and Google Scholar
Search Terms: human neutrophil elastase, active infection, HNE, human, neutrophil elastase, active, infection, FRRET, AMC protease, swab, substrate, tube, kit, swabs, antibody, inhibit, inhibit, stop, stop, dermal, wound, periodontal, chronic lung

C DOCUMENTS CONSIDERED TO BE RELEVANT

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D Further documents are listed in the continuation of Box C

Date of the actual completion of the international search
19 October 2009 (19 10 2009)

Date of mailing of the international search report
29 OCT 2009

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Form PCT/ISA/210 (second sheet) (July 2009)