



TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

DETECTING MICROBIAL INFECTION IN WOUNDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application Nos. 62/315,546, filed March 30, 2016, and U.S. 62/315,556, filed March 30, 2016, which disclosures are incorporated herein by reference in their entireties and made a part hereof.

TECHNICAL FIELD

[0002] Embodiments described herein generally relate to wound healing, and in particular to compositions and methods for the detection and treatment of wounds.

BACKGROUND

[0003] In mammals, dermal injury triggers an organized complex cascade of cellular and biochemical events that result in a healed wound. Wound healing is a complex dynamic process that results in the restoration of anatomic continuity and function: an ideally healed wound is one that has returned to normal anatomic structure, function, and appearance. A typical wound heals via a model consisting of four stages - 'exudative' phase, proliferative phase, reparative phase and epithelial maturation (Hatz et al., *Wound Healing and Wound Management*, Springer-Verlag, Munich, 1994) or hemostatic, inflammatory, proliferative and remodeling phase (Nwomeh et al., *Clin. Plast. Surg.* 1998, 25, 341). The inflammatory phase is particularly important to the wound healing process, wherein biochemical reactions at the wound situs facilitate healing but also cause tissue breakdown due to production of excess proteases.

[0004] Infection of the wound results in either a slower, or an arrested healing process. For example, pathogens in a wound can produce toxins (e.g., *Clostridium* species), generate noxious metabolites like ammonia that raise pH (e.g., *Proteus* species), activate or produce tissue lytic enzymes like proteases, or promote tissue invasion, thereby leading to an increase in the size or seriousness of the wound. In a worst case, pathogens can leave the wound and cause sepsis.

[0005] In order to keep the chronicity of wounds in check, a variety of assessment techniques and/or tools are employed in the clinical and veterinary setting. Current methods of assessing an infected wound are based primarily on assaying for a variety of parameters associated with the wound. For instance, a wound may be assessed visually, length and depth measurements may be taken, digital photography may be used where available to track the visual condition and size of a wound (Krasner et al., *supra*). In clinical practice, diagnosis of infection is based on measurement of secondary parameters, such as, odor, presence of local pain, heat, swelling, discharge, and redness. Many of these clinical indicators, such as inflammation and discharge have a low predictive value of infection in wounds. In other

instances, the number(s) and type(s) of pathogenic flora at the wound situs may be determined using laboratory and/or clinical diagnostic procedures. Swabbing of a wound followed by microbiology testing in the hospital laboratory is an option for confirmation of bacterial colonization and identification of the strains associated with infection, thus allowing for the prescription of correct antibiotic course. However, this process is time consuming and labor intensive. Delay in diagnosis of infection can delay the administration of antibiotics and may increase the risk of developing sepsis.

[0006] One of the biggest drawbacks associated with existing clinical diagnostics is a lag associated with the onset of infection and the timing of detection. For instance, positive identification of infection using swabbing procedures often depends on attainment of a “critical mass” of microorganisms at the wound site and so early detection cannot be made until a detectable level is reached. Also, the swabs may be contaminated with the flora of the surrounding tissue, thereby complicating the diagnostic procedure. Other drawbacks include, *e.g.*, sampling errors, delays in transport of the swabs, errors in analytical procedures, and/or errors in reporting. See, the review by Bowler et al., *Clin Microbiol Rev.* 14(2): 244–269, 2001.

[0007] There is therefore an imminent but unmet need for diagnostic reagents and methods that enable early diagnosis of clinical infection, preferably, which permit clinical diagnosis prior to manifestation of clinical symptoms of infection. There is also a need for compositions and methods that would assist in predicting clinical infection of a wound prior to the manifestation of clinical symptoms. Such a prognostic aid would allow early intervention with suitable treatment (*e.g.*, antimicrobial treatment) before the wound is exacerbated and surgery or other drastic intervention is required to prevent further infection. Additionally, if clinicians could respond to wound infection as early as possible, the infection could also be treated with minimal antibiotic usage. This would reduce the need for hospitalization and would reduce the risk of secondary infections, *e.g.*, as a result of contact with other diseased subjects.

SUMMARY

[0008] The technology disclosed herein provides for compositions and methods of detecting infected and/or chronic wounds. The disclosed technology improves upon exiting assays by: increasing the sensitivity, precision and specificity of detection of infected wounds; providing for the ability of qualitative and quantitative measurements; and, increasing the speed of detection of infected wounds *in situ* and in real-time. The assays and methods described herein are partly based on the use of specific reagents that detect biomarkers and/or probes which are present in infected or chronic wounds. The detection process may involve use of reagents that are specific to the markers present in infected wounds but not non-infected or non-chronic

wounds and the detection step may involve qualitative or quantitative measurements of the signal(s) that are generated when the probe is acted upon by the marker. In embodiments wherein the detection method involves detection of enzymes present in wounds, the probes comprise modified enzyme substrates that are specific to the enzyme, which generate signals that may be optionally amplified. This greatly improves efficiency and specificity of detection. Moreover, a plurality of detection probes, each specific for one or more targets, *e.g.*, enzymes that are specific to the wounds, may be employed. This greatly helps to maximize both efficiency and accuracy of diagnostic assays while minimizing the incidence of false positives (*e.g.*, due non-specific interactions and/or target redundancy). Furthermore, the experimental results disclosed herein confirm that the novel probes and the assay techniques based thereon are capable of detecting and characterizing various types of wounds. Finally, the reagents of the disclosed technology may be used together with therapeutic molecules such as antibiotics, antifungal agents, etc. to monitor and evaluate treatment and management of chronic wounds.

[0009] Embodiments described herein are based, in part, on the discovery that cells of the immune system, including enzymes generated thereby, may serve as markers in the early diagnosis of wounds. These cells, *e.g.*, neutrophils, are recruited at the wound situs to combat infection, do so by engulfing bacteria (and other pathogens) and/or neutralizing them with enzymes. Some enzymes are specific towards proteins (*e.g.*, elastase, cathepsin G), others are specific towards cell wall components (*e.g.*, lysozyme) and yet others mediate protein denaturation (*e.g.*, NADPH oxidase, xanthine oxidase, myeloperoxidase (MPO) and other peroxidases). These cells, *e.g.*, neutrophils, are generally only short-lived and when they lyse in the area of the infection, they release the contents of their lysosomes including the enzymes, which can then be detected to provide a reliable measurement of the status of the wound.

[0010] Accordingly, various embodiments described herein utilize the detection of enzyme markers, which are indicative of the presence of myeloid cells, and neutrophils in particular, in a biological sample of interest, for example, wound tissue. Increased level or activity of such enzymes in the wound fluid, therefore, corresponds to a heightened bacterial challenge and a manifestation of disturbed host/bacteria equilibrium in favor of the invasive bacteria.

[0011] In one embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region.

[0012] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I), wherein the anchor A is covalently associated with the indicator I via a covalent interaction to form a recognition site S.

[0013] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I), wherein the anchor A is covalently associated with the

indicator I via a covalent interaction to form a recognition site S, and wherein the recognition site (S) is specific for a wound-specific hydrolase. Under this embodiment, the hydrolase is a glycosidase or a protease. Particularly under this embodiment, the protease is elastase, cathepsin G or myeloperoxidase.

[0014] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a polysaccharide, a cellulose, a polyacrylate, a polyethyleneimine, a polyacrylamide, a peptidoglycan, or a chitosan, or a monomer thereof, an oligomer thereof, a derivative thereof, a mixture or a combination thereof.

[0015] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises chitosan or a monomer thereof, an oligomer thereof, a derivative thereof, a mixture or a combination thereof.

[0016] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a monomer of chitosan comprising D-glucosamine or N-acetyl-D-glucosamine, an oligomer thereof, or a combination thereof.

[0017] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises at least two units of D-glucosamine, N-acetyl-D-glucosamine or a combination thereof.

[0018] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a randomly substituted partial N-, partial O-acetylated chitosan, chitosan oligosaccharide, carboxymethyl chitosan, or hydroxyalkyl chitosan or a derivative thereof.

[0019] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a randomly substituted partial N-, partial O-acetylated chitosan.

[0020] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a randomly substituted partial N-, partial O-acetylated chitosan, wherein the acetylated chitosan comprises a degree of acetylation (DA) between about 40% to about 90%.

[0021] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein

the anchor A comprises a randomly substituted partial N-, partial O-acetylated chitosan, wherein the acetylated chitosan comprises a degree of acetylation (DA) of greater than 50%.

[0022] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a randomly substituted partial N-, partial O-acetylated chitosan, wherein the chitosan is halogenated.

[0023] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises chitosan or a monomer thereof, an oligomer thereof, a derivative thereof, a mixture or a combination thereof.

[0024] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor; and I is an indicator region, wherein the anchor A comprises a chitosan compound or a derivative thereof selected from the group consisting of chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitooligosaccharide; 2-acetamido-2-deoxy-D-glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; an oligomer thereof; or a combination thereof.

[0025] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) or a motif therein is conjugated to the anchor and the conjugate is a substrate for a glycosidase.

[0026] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) or a motif therein is conjugated to the anchor and the conjugate is a substrate for a glycosidase.

[0027] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) or a motif therein is conjugated to the anchor and the conjugate is a substrate for lysozyme.

[0028] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) or a motif therein is conjugated to the anchor via a glycosidic bond at the 1 α -carbon of chitosan or a monomer thereof, an oligomer thereof, or a derivative thereof.

[0029] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) further comprises a dye containing a sulfonylethyl-hydrogensulphate-reactive-group or a dye containing a dichlorotriazine reactive-group.

[0030] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) further comprises a dye which is reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16 or a combination thereof.

[0031] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) further comprises a dye which is reactive blue 4, reactive red 120, reactive blue 2, reactive green 19, or reactive brown 10, or a combination thereof.

[0032] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator (I) comprises a detectable label selected from the group consisting of a luminescent molecule, a chemiluminescent molecule, a fluorochrome, a fluorescent quenching agent, a lipid, a colored molecule, a radioisotope, a scintillant, biotin, avidin, streptavidin, protein A, protein G, an antibody or a fragment thereof, a polyhistidine, Ni²⁺, a Flag tag, a myc tag, a heavy metal, and an enzyme.

[0033] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region and the anchor is directly conjugated to the indicator via a glycosidic linkage.

[0034] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region and the anchor is directly conjugated to the indicator via a glycosidic linkage, wherein the anchor comprises a compound selected from the group consisting of chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitooligosaccharide; 2-acetamido-2-deoxy-D-glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; or a combination thereof.

[0035] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A-I (Formula I) wherein, A is an anchor and I is an indicator region and the anchor is directly conjugated to the indicator via a glycosidic linkage, wherein the indicator is selected from the group consisting of reactive black 5, remazol brilliant blue, reactive violet 5 or

reactive orange 16, reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10, or a combination thereof.

[0036] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region and the anchor is directly conjugated to the indicator via a glycosidic linkage, wherein the anchor comprises a compound selected from the group consisting of chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitooligosaccharide; 2-acetamido-2-deoxy-D-glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; or a combination thereof; and wherein the indicator is selected from the group consisting of reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16, reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10, or a combination thereof.

[0037] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator comprises a hydrophilic binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*) or the hydrophobic binding module (PDB) from Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*) or a chimeric variant thereof.

[0038] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator comprises a hydrophilic binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*) or the hydrophobic binding module (PDB) from Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*) or a chimeric variant thereof, wherein the anchor comprises cellulose or a derivative thereof or polyethylene terephthalate or a derivative thereof.

[0039] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator comprises a hydrophilic binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*) or the hydrophobic binding module (PDB) from Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*) or a chimeric variant thereof, wherein the anchor comprises cellulose or a derivative thereof or polyethylene terephthalate or a derivative thereof.

[0040] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator comprises a hydrophilic binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*) or a chimeric variant thereof and the anchor comprises cellulose or a derivative thereof, wherein the indicator is hydrophilically associated with the anchor.

[0041] In another embodiment, provided herein is a chemical entity comprising a compound with the structure A–I (Formula I) wherein, A is an anchor and I is an indicator region, wherein the indicator comprises Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*) and the anchor comprises polyethylene terephthalate or a derivative thereof, wherein the indicator is hydrophobically associated with the anchor.

[0042] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a histidine-tag and an enterokinase cleavage site, or a portion thereof.

[0043] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) gene, a spacer sequence encoding a histidine-tag and an enterokinase cleavage site, or a portion thereof.

[0044] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof. Particularly, under this embodiment, the spacer sequence encodes a hexahistidine (His6) tag.

[0045] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys).

[0046] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence. Particularly, under this embodiment, the *elasub1* sequence is located subsequent to the enterokinase cleavage site. Especially under this embodiment, the *elasub1* sequence encodes for functional amino acids selected from the group consisting of cysteine, lysine, arginine, glutamine, asparagine, glutamic acid, aspartic acid, serine, threonine or tyrosine.

[0047] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence.

[0048] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an

enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence.

[0049] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites.

[0050] In another embodiment, provided herein is a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites.

[0051] In another embodiment, provided herein is a vector comprising an expression control sequence and a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites.

[0052] In another embodiment, provided herein is a vector comprising an expression control sequence and a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites.

[0053] In another embodiment, provided herein is a host cell comprising a vector comprising an expression control sequence and a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites. Particularly under this embodiment, the host cell is a bacterial cell or an insect cell or a mammalian cell.

[0054] In another embodiment, provided herein is a host cell comprising a vector comprising an expression control sequence and a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites. Particularly under this embodiment, the host cell is a bacterial cell or an insect cell or a mammalian cell.

[0055] In another embodiment, provided herein is a method of making the protein encoded by a chimeric construct comprising polynucleotide sequences encoding (a) a *trxA* (thioredoxin) or a portion thereof; (b) a spacer sequence encoding a histidine-tag; (c) an enterokinase cleavage site or a portion thereof; (d) an *elasub1* sequence subsequent to the enterokinase cleavage site; (e) a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence; and (f) an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites in the *elasub1* sequence; comprising culturing a host cell comprising the chimeric construct under conditions sufficient to induce expression of the chimeric construct and obtaining the chimeric protein from the cell culture; and optionally purifying the construct by His-tag affinity purification.

[0056] In another embodiment, provided herein is a method of making the protein encoded by a chimeric construct comprising polynucleotide sequences encoding (a) a *trxA* (thioredoxin) or a portion thereof; (b) a spacer sequence encoding a histidine-tag; (c) an enterokinase cleavage site or a portion thereof; (d) an *elasub1* sequence subsequent to the enterokinase cleavage site; (e) a hydrophobic *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence; and (f) an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites in the

elasub1 sequence; comprising culturing a host cell comprising the chimeric construct under conditions sufficient to induce expression of the chimeric construct and obtaining the chimeric protein from the cell culture; and optionally purifying the construct by His-tag affinity purification.

[0057] In another embodiment, provided herein is a polypeptide encoded by a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites. Under this embodiment, provided herein is a composition comprising the CBM sequence containing polypeptide (“CBM polypeptide”) and a dye or a label. Further provided herein under this embodiment is a chemical entity comprising an anchor and an indicator region comprising the composition comprising the CBM polypeptide and the dye/label. Especially under this embodiment, provided herein is a chemical entity comprising an anchor which is cellulose or a derivative thereof and the indicator region comprising the composition comprising the CBM polypeptide and the dye/label.

[0058] In another embodiment, provided herein is a polypeptide encoded by a chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a poly-histidine-tag and an enterokinase cleavage site, or a portion thereof and an enterokinase cleavage site (Asp Asp Asp Lys) and an *elasub1* sequence and a *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence, wherein the *elasub1* sequence comprises nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites. Under this embodiment, provided herein is a composition comprising the PDM sequence containing polypeptide (“PDM polypeptide”) and a dye or a label. Further provided herein under this embodiment is a chemical entity comprising an anchor and an indicator region comprising the composition comprising the PDM polypeptide and the dye/label. Especially under this embodiment, provided herein is a chemical entity comprising an anchor which is polyethylene terephthalate or a derivative thereof and the indicator region comprising the composition comprising the PDM polypeptide and the dye/label.

[0059] In another embodiment, provided herein is a method for the determining the presence or absence of an enzyme selected from the group consisting of HLE and CatG in a biological sample, comprising contacting the biological sample with the composition containing the CBM

polypeptide and the dye/label or a chemical entity comprising the anchor and the indicator comprising the CBM polypeptide and the dye/label; and detecting the label.

[0060] In another embodiment, provided herein is a method for the determining the presence or absence of an enzyme selected from the group consisting of HLE and CatG in a biological sample, comprising contacting the biological sample with the composition containing the PDM polypeptide and the dye/label or a chemical entity comprising the anchor and the indicator comprising the PDM polypeptide and the dye/label; and detecting the label.

[0061] In another embodiment, provided herein is a method for diagnosing an infected or a chronic wound, comprising, contacting the wound with the composition containing the CBM polypeptide and the dye/label or a chemical entity comprising the anchor and the indicator comprising the CBM polypeptide and the dye/label; and detecting the label. Under this embodiment, the wound is present in a tissue, e.g., skin tissue, of a subject in need of such diagnosis, e.g., a human subject. Particularly under this embodiment, the detection is made *in situ*. Especially under this embodiment, the diagnosis can be at multiple time points, based on visual and/or electronic valuation of the dye.

[0062] In another embodiment, provided herein is a method for diagnosing an infected or a chronic wound, comprising, contacting the wound with the composition containing the PDM polypeptide and the dye/label or a chemical entity comprising the anchor and the indicator comprising the CBM polypeptide and the dye/label; and detecting the label. Under this embodiment, the wound is present in a tissue, e.g., skin tissue, of a subject in need of such diagnosis, e.g., a human subject. Particularly under this embodiment, the detection is made *in situ*. Further, under this embodiment, the diagnosis can be at multiple time points, based on visual and/or electronic valuation of the dye.

[0063] In another embodiment, provided herein is a method for treating an infected or a chronic wound, comprising, contacting the wound with any of the foregoing compositions, wherein the composition comprises at least one antibiotic and optionally together with a healing agent. Under this embodiment, the wound to be treated is present in a tissue, e.g., skin tissue, of a subject in need of such diagnosis, e.g., a human subject. Further, under this embodiment, the wound is treated *in situ* and may be accompanied by pre-treatment or post-treatment diagnosis.

[0064] In one embodiment, provided herein is a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is independently any amino acid, y is each, independently, an integer between 0 and 200, L is a linking moiety, and Z comprises a detectable label.

[0065] In one embodiment, provided herein is a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is independently any amino acid, y is each, independently, an integer between 1 and 50, L is a linking moiety, and Z comprises a detectable label.

[0066] In one embodiment, provided herein is a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is independently any amino acid, y is each, independently, an integer between 1 and 10, L is a linking moiety, and Z comprises a detectable label.

[0067] In one embodiment, provided herein is a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is independently any amino acid, y is each, independently, an integer between 1 and 6, L is a linking moiety, and Z comprises a detectable label.

[0068] In one embodiment, provided herein is a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is independently any amino acid, y is each, independently, an integer between 0 and 200, L is a linking moiety, and Z comprises a detectable label, wherein each of the peptides comprising the sequence X_yAAPX_y-Z (SEQ ID NO: 1), X_yAAPX_y-L-Z (SEQ ID NO: 2), $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3) or $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), are each, individually, labile to elastase. Under this embodiment, each of the polypeptides may be optionally protected with an amine protecting group, e.g., an amine protection group which is fluorenylmethyloxycarbonyl (Fmoc).

[0069] In one embodiment, provided herein is a composition comprising a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is independently any amino acid, y is each, independently, an integer between 0 and 200, L is a linking moiety, and Z comprises a detectable label, and a carrier.

[0070] In one embodiment, provided herein is a chemical entity comprising an anchor region (A) and an indicator region (I) comprising a polypeptide comprising the sequence set forth in (a) X_yAAPX_y-Z (SEQ ID NO: 1), (b) X_yAAPX_y-L-Z (SEQ ID NO: 2), (c) $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3), or (d) $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), wherein each X is

independently any amino acid, y is each, independently, an integer between 0 and 200, L is a linking moiety, and Z comprises a detectable label.

[0071] In one embodiment, provided herein is a polypeptide comprising the amino acid sequence set forth in (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X is each, independently any amino acid; y is each, independently, a number selected from 0 to 6; N^4 is selected from alanine, glycine, valine, and glutamine; N^3 is selected from alanine, glycine, proline, lysine, and serine; N^2 is selected from proline, alanine, and glycine; N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; L is a linking moiety; and Z comprises a detectable label.

[0072] In one embodiment, provided herein is a polypeptide comprising the amino acid sequence set forth in (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X is each, independently any amino acid; y is each, independently, a number selected from 1 to 3; N^4 is selected from alanine, glycine, valine, and glutamine; N^3 is selected from alanine, glycine, proline, lysine, and serine; N^2 is selected from proline, alanine, and glycine; N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; L is a linking moiety; and Z comprises a detectable label.

[0073] In one embodiment, provided herein is a polypeptide comprising the amino acid sequence set forth in (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X is each, independently any amino acid; y is each, independently, a number selected from 0 to 6; N^4 is selected from alanine, glycine, valine, and glutamine; N^3 is selected from alanine, glycine, proline, lysine, and serine; N^2 is selected from proline, alanine, and glycine; N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; L is a linking moiety; and Z comprises a detectable label, wherein the polypeptides comprising the sequence (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), are each, individually, labile to cathepsin G. Under this embodiment, each of the polypeptides may be optionally protected with an amine protecting group, e.g., an amine protection group which is fluorenylmethyloxycarbonyl (Fmoc).

[0074] In one embodiment, provided herein is a composition comprising a polypeptide comprising the amino acid sequence set forth in (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X is each, independently any amino acid; y is each, independently, a number selected from 0 to 6; N^4 is selected from alanine, glycine, valine, and glutamine; N^3 is selected from alanine, glycine, proline, lysine, and serine; N^2 is selected from proline, alanine, and glycine; N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; L is a linking moiety; and Z comprises a detectable label and a carrier.

[0075] In one embodiment, provided herein is a chemical entity comprising an anchor region (A) and an indicator region (I) comprising a polypeptide comprising the amino acid sequence set forth in (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X is each, independently any amino acid; y is each, independently, a number selected from 0 to 6; N^4 is selected from alanine, glycine, valine, and glutamine; N^3 is selected from alanine, glycine, proline, lysine, and serine; N^2 is selected from proline, alanine, and glycine; N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; L is a linking moiety; and Z comprises a detectable label.

[0076] It is understood that other embodiments and configurations of the subject technology will become readily apparent to those skilled in the art from the following detailed description, wherein various configurations of the subject technology are shown and described by way of example or illustration. As will be realized, the subject technology is capable of other and different configurations and its several details are capable of modification in various other respects, all without departing from the scope of the subject technology. Accordingly, the figures and detailed description are to be regarded as illustrative in nature and not as restrictive.

BRIEF DESCRIPTION OF THE FIGURES

[0077] To understand the present disclosure, it will now be described by way of example, with reference to the accompanying figures in which embodiments and examples of the disclosures are illustrated and, together with the descriptions below, serve to explain the principles of the disclosure.

[0078] **FIG. 1:** Infrared spectrum of Example 2. Chitosan derivatives with varying degree of acetylation (DA) were produced, but only material with a DA of 48 % was further used.

[0079] **FIG. 2:** Infrared spectrum and NMR spectrum of Example 2. Chitosan derivatives with varying DA were produced, but only material with a DA of 48 % was further used.

[0080] **FIG. 3:** Infrared spectrum of chitooligosaccharides of Example 5.

[0081] **FIG. 4:** Infrared spectrum of chitooligosaccharides of Example 6.

[0082] **FIG. 5:** Time course of dye release from dye conjugated acetyl chitosan when incubated with 5000 units per mL lysozyme.

[0083] **FIG. 6:** (A) Time course of dye release from dye conjugated acetyl chitosan when incubated with 5000 units per mL lysozyme in phosphate buffer when the chitosan has varying concentrations of dye conjugated; (B) Time course of dye release from dye conjugated acetyl chitosan when incubated with 5000 units per mL lysozyme in either phosphate buffer of artificial wound fluid containing 2% protein as BSA.

[0084] **FIG. 7:** Color response of immobilized nitrazine yellow and bromocresol purple of Example 11 varies depending on the pH at which the preparation is dried.

[0085] **FIG. 8:** Comparison of different lysozyme responsive dye releases of different staining degrees.

[0086] **FIG. 9:** Influence of the proportion between reactive dye and PG.

[0087] **FIG. 10:** Elastase responsive dye release.

[0088] **FIG. 11:** Multiple Sequence Alignment of the Various Constructs. Asterisk (*) indicates identity; semi-colon (;) indicates conservative substitution; and period (.) indicates semi-conservative substitution.

DETAILED DESCRIPTION

[0089] Various aspects now will be described more fully hereinafter. Such aspects may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey its scope to those skilled in the art.

[0090] Throughout this disclosure, various patents, patent applications and publications are referenced. The disclosures of these patents, patent applications and publications in their entireties are incorporated into this disclosure by reference in order to more fully describe the state of the art as known to those skilled therein as of the date of this disclosure. This disclosure will govern in the instance that there is any inconsistency between the patents, patent applications and publications cited and this disclosure.

[0091] I. Definitions

[0092] Where a range of values is provided, it is intended that each intervening value between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the disclosure. For example, if a range of 1 μm to 8 μm is stated, it is intended that 2 μm , 3 μm , 4 μm , 5 μm , 6 μm , and 7 μm are also explicitly disclosed, as well as the range of values greater than or equal to 1 μm and the range of values less than or equal to 8 μm .

[0093] The singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a “polymer” includes a single polymer as well as two or more of the same or different polymers, reference to an “excipient” includes a single excipient as well as two or more of the same or different excipients, and the like.

[0094] The word “about” when immediately preceding a numerical value means a range of plus or minus 10% of that value, *e.g.*, “about 50” means 45 to 55, “about 25,000” means 22,500

to 27,500, etc., unless the context of the disclosure indicates otherwise, or is inconsistent with such an interpretation. For example in a list of numerical values such as “about 49, about 50, about 55, “about 50” means a range extending to less than half the interval(s) between the preceding and subsequent values, *e.g.*, more than 49.5 to less than 52.5. Furthermore, the phrases “less than about” a value or “greater than about” a value should be understood in view of the definition of the term “about” provided herein.

[0095] “Substantially” or “essentially” means nearly totally or completely, for instance, 80%–95% or greater of some given quantity, *e.g.*, at least 85%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.9%, or more % by weight or volume or any other parameter being measured. “Substantially free” means nearly totally or completely absent of some given quantity such as being present at a level of less than about 1% to about 20% of some given quantity, *e.g.*, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, or less % by weight or volume or any other parameter being measured. In some embodiments, “substantially free” means presence at a level of less than or equal to 1-5% by weight of the pharmaceutical composition.

[0096] II. Overview

[0097] Provided herein are compositions and systems for the therapy and diagnosis of wounds and wound management, wherein the compositions, when in use, indicate the presence of elevated enzyme levels in a wound *in situ*.

[0098] As used herein, a “wound” refers to physical disruption of the continuity or integrity of tissue structure. “Wound healing” refers to the restoration of tissue integrity. It will be understood that this can refer to a partial or a full restoration of tissue integrity. Treatment of a wound thus refers to the promotion, improvement, progression, acceleration, or otherwise advancement of one or more stages or processes associated with the wound healing process.

[0099] The wound may be acute or chronic. Chronic wounds, including pressure sores, venous leg ulcers and diabetic foot ulcers, can simply be described as wounds that fail to heal. Whilst the exact molecular pathogenesis of chronic wounds is not fully understood, it is acknowledged to be multi-factorial. As the normal responses of resident and migratory cells during acute injury become impaired, these wounds are characterized by a prolonged inflammatory response, defective wound extracellular matrix (ECM) remodeling and a failure of re-epithelialization.

[00100] The wound may be any internal wound, *e.g.*, where the external structural integrity of the skin is maintained, such as in bruising or internal ulceration, or external wounds, particularly

cutaneous wounds, and consequently the tissue may be any internal or external bodily tissue. In one embodiment the tissue is skin (such as human skin), *i.e.* the wound is a cutaneous wound, such as a dermal or epidermal wound.

[00101] The human skin is composed of two distinct layers, the epidermis and the dermis, below which lies the subcutaneous tissue. The primary functions of the skin are to provide protection to the internal organs and tissues from external trauma and pathogenic infection, sensation and thermoregulation. The skin tissue of most mammals is structured similarly.

[00102] The outermost layer of skin, the epidermis, is approximately 0.04 mm thick, is avascular, is comprised of four cell types (keratinocytes, melanocytes, Langerhans cells, and Merkel cells), and is stratified into several epithelial cell layers. The inner-most epithelial layer of the epidermis is the basement membrane, which is in direct contact with, and anchors the epidermis to, the dermis. All epithelial cell division occurring in skin takes place at the basement membrane. After cell division, the epithelial cells migrate towards the outer surface of the epidermis. During this migration, the cells undergo a process known as keratinization, whereby nuclei are lost and the cells are transformed into tough, flat, resistant non-living cells. Migration is completed when the cells reach the outermost epidermal structure, the stratum corneum, a dry, waterproof squamous cell layer which helps to prevent desiccation of the underlying tissue. This layer of dead epithelial cells is continuously being sloughed off and replaced by keratinized cells moving to the surface from the basement membrane. Because the epidermal epithelium is avascular, the basement membrane is dependent upon the dermis for its nutrient supply.

[00103] The dermis is a highly vascularized tissue layer supplying nutrients to the epidermis. In addition, the dermis contains nerve endings, lymphatics, collagen protein, and connective tissue. The dermis is approximately 0.5 mm thick and is composed predominantly of fibroblasts and macrophages. These cell types are largely responsible for the production and maintenance of collagen, the protein found in all animal connective tissue, including the skin. Collagen is primarily responsible for the skin's resilient, elastic nature. The subcutaneous tissue, found beneath the collagen-rich dermis, provides for skin mobility, insulation, calorie storage, and blood to the tissues above it.

[00104] Wounds can be classified in one of two general categories, partial thickness wounds or full thickness wounds. A partial thickness wound is limited to the epidermis and superficial dermis with no damage to the dermal blood vessels. A full thickness wound involves disruption of the dermis and extends to deeper tissue layers, involving disruption of the dermal blood vessels. The healing of the partial thickness wound occurs by simple regeneration of epithelial tissue. Wound healing in full thickness wounds is more complex. Cutaneous wounds contemplated herein may be either partial thickness or full thickness wounds.

[00105] Wounds contemplated herein include cuts and lacerations, surgical incisions or wounds, punctures, grazes, scratches, compression wounds, abrasions, friction wounds (*e.g.*, nappy rash, friction blisters), decubitus ulcers (*e.g.*, pressure or bed sores); thermal effect wounds (burns from cold and heat sources, either directly or through conduction, convection, or radiation, and electrical sources), chemical wounds (*e.g.* acid or alkali burns) or pathogenic infections (*e.g.*, viral, bacterial or fungal) including open or intact boils, skin eruptions, blemishes and acne, ulcers, chronic wounds, (including diabetic-associated wounds such as lower leg and foot ulcers, venous leg ulcers and pressure sores), skin graft/transplant donor and recipient sites, immune response conditions, *e.g.*, psoriasis and eczema, stomach or intestinal ulcers, oral wounds, including a ulcers of the mouth, damaged cartilage or bone, amputation wounds and corneal lesions.

[00106] Chemical Entities and Compositions Thereof

[00107] Embodiments described herein provide chemical entities, which may be used to diagnose and/or treat chronic wounds. The chemical entities and compositions thereof, as described herein, are used in methods to detect the level of one or more enzymes in a mammalian wound. In some embodiments, the chemical entities and compositions thereof, as described herein, are used in methods to diagnose a chronic wound in a mammal. In some embodiments, the chemical entities and compositions thereof described herein are used in methods to diagnose an infected wound in a mammal. In other embodiments, the chemical entities and compositions thereof described herein are used in methods to treat a wound in a mammal. In further embodiments, the chemical entities and compositions thereof described herein are used in methods to treat an infected or a chronic wound in a mammal.

[00108] In one embodiment, provided herein is a chemical entity capable of detecting enzyme activity from a body fluid, the chemical entity comprising: an anchor region (A) and an indicator region (I). Under this embodiment, the chemical entity has a basic chemical structure A-I (Formula I), wherein A is an anchor region and I is an indicator region.

[00109] In some embodiments, the anchor region (A) is associated with the indicator region (I) via an enzyme recognition site (S). Under this embodiment, the enzyme recognition site is a structure or a motif that allows binding to an enzyme.

[00110] In one embodiment, the enzyme recognition site (S) is naturally present in the anchor region. In another embodiment, the enzyme recognition site (S) is introduced in the anchor region via chemical modification. Alternately, the enzyme recognition site (S) may be naturally present in the indicator region (I) or synthetically introduced in the indicator region (I) via one or more chemical modifications.

[00111] In one embodiment, the chemical entity of Formula I comprises an anchor (A) which is associated with the indicator (I), either covalently or non-covalently. Particularly, the association between the anchor region (A) and the indicator region (I) is mediated via a covalent interaction. As is understood in the art, covalent bonds involve sharing of electrons between the bonded atoms. In contrast, non-covalent bonds may include, for example, ionic interactions, electrostatic interactions, hydrogen bonding interactions, physiochemical interactions, van der Waal forces, Lewis-acid/Lewis-base interactions, or combinations thereof.

[00112] In one embodiment, the anchor A is associated with the indicator I via a covalent interaction to form the recognition site S. In another embodiment, the anchor A is associated with the indicator I via a covalent interaction that is not a part of the recognition site S.

[00113] In some embodiments, the chemical entity further comprises an enzyme-labile or enzyme-reactive region (R). In one embodiment, the reactive region (R) is a part of the anchor region. In another embodiment, the reactive region (R) is a part of the indicator region (I). Still further, the reactive region (R) is a part of the enzyme recognition site (S).

[00114] In one embodiment, the reactive region (R) interacts with one or more target enzymes selected from the group consisting of elastase, lysozyme, cathepsin G, and myeloperoxidase, or a combination thereof.

[00115] Anchor region (A)

[00116] In some embodiments of the chemical entity of Formula I, the anchor region comprises a compound which is a polysaccharide, cellulose, polyacrylate, polyethyleneimine, polyacrylamide, peptidoglycan, or chitosan, or a monomer thereof, a derivative thereof, a mixture or a combination thereof.

[00117] In one embodiment, the anchor A comprises a compound which is a chitosan or a monomer thereof, a derivative thereof, a mixture or a combination thereof. Non-covalent bonds may include, for example, ionic interactions, electrostatic interactions, hydrogen bonding inetyl-D-glucosamine (acetylated unit). Accordingly, chitosan monomer may comprise D-glucosamine and N-acetyl-D-glucosamine. In another embodiment, the chitosan may comprise at least 2, at least 3, at least 4, at least 5, or more units of D-glucosamine or N-acetyl-D-glucosamine or a combination thereof. Chitosan, including, shorter fragments thereof, is generally manufactured by treating chitin with an alkaline substance, *e.g.*, sodium hydroxide, and optionally hydrolyzing the glycosidic linkages between the individual monomer units.

[00118] In another embodiment, the anchor A comprises a chitosan derivative. Example chitosan or chitosan derivatives include chitosan salts, water-soluble chitosan, water-soluble, randomly substituted partial N-, partial O-acetylated chitosan, chitosan oligosaccharide, carboxymethyl chitosan, and hydroxyalkyl chitosan. The hydroxyalkyl substituents of the

hydroxyalkyl, chitosans and the carboxymethyl substituents of the carboxymethyl chitosans could be attached to any of the pendant nitrogen or oxygen groups on the chitin or chitosan ring subunit. Representative hydroxyalkyl chitosans include but are not limited to, hydroxyethyl chitosan (also known as glycol chitosan), hydroxypropyl chitosan, dihydroxypropyl chitosan, hydroxybutyl chitosan and dihydroxybutyl chitosan.

[00119] In one embodiment, the chitosan derivative is a randomly substituted partial N-, partial O-acetylated chitosan. The acetylated chitosan derivatives are generally defined by a degree of acetylation or degree of acetylation. As is understood in the art, the degree of acetylation (DA) represents the proportion of N-acetyl-d-glucosamine units with respect to the total number of units in the chitosan molecule. See, Chatelet et al., *Biomaterials*, 22(3):261-8, 2001. By the term “degree of deacetylation” is meant the percentage of free amino groups on the water soluble, chitosan or chitosan derivative. The percent of N-acetylation can be calculated from the deacetylation value. The terms N-acetylation or O-acetylation are also referred to as the degree of substitution with C(O)CH₃ on either N or O. As is understood in the art, a chitosan derivative having a DA value greater than 50% N-acetylation is sometimes described as a chitin. However, the term “chitosan” is used throughout the disclosure herein to include chitosans and, if the N-acetylation is greater than 50%, to include chitins. See, U.S. Patent No. 7,683,039.

[00120] In one embodiment, the chitosan derivative has a DA of at least about 40%, about 41%, about 42%, about 43%, about 44%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or greater %, e.g., between about 45% to 95%, particularly between about 60% to about 80%. Particularly, the chitosan is at least 48% deacetylated, especially at least 75% deacetylated.

[00121] In one embodiment, “chitosan derivative” as used herein includes salts, amides, esters, enol ethers, enol esters, acetals, ketals, orthoesters, hemiacetals, hemiketals, acids, bases, solvates, hydrates or prodrugs of the chitosan. Such derivatives may be readily prepared by those of skill in this art using known methods for such derivatization. In certain embodiments, the derivatives may be administered to animals or humans without substantial toxic effects and either are pharmaceutically active or are prodrugs. Representative types of chitosan derivatives are described in U.S. Patent Nos. 9,012,429; 5,773,608; and 3,911,116.

[00122] In another embodiment, the derivative is a salt of the polymeric compound, *e.g.*, salts of Li^+ , Na^+ , K^+ , Rb^+ , Mg^{2+} , Ca^{2+} , Sr^{2+} , or Ba^{2+} , preferably Na^+ , K^+ , Mg^{2+} , Ca^{2+} . Salts of chitin and chitosan, such as sodium or calcium salts, are known in the art. See, US patent No. 5,599,916.

[00123] In some embodiments, the derivative anchor compound is a halogenated anchor compound, *e.g.*, halogenated polysaccharide, halogenated cellulose, halogenated polyacrylate, halogenated polyethyleneimine, halogenated polyacrylamide, halogenated peptidoglycan, or halogenated chitosan, or a monomer thereof, *e.g.*, halogenated D-glucosamine and/or halogenated N-acetyl-D-glucosamine. The halogen is selected from the group consisting of Cl, Br, I, particularly, the halogen is Cl.

[00124] In some embodiments, the derivative compound is an isomer of the anchor compound. term “isomer” includes compounds with the same formula but a different arrangement of atoms in the molecule. In embodiments, isomers of the compounds are “tautomers” or “stereoisomers” of the compounds. The term “stereoisomer” refers to compounds that differ in the chirality of one or more stereocenters. Stereoisomers include enantiomers and diastereomers. The term “tautomer” refers to alternate forms of a compound that differ in the position of a proton, such as enol-keto and imine-enamine tautomers, or the tautomeric forms of the anchor compound.

[00125] In some embodiments, the anchor compound may contain a combination or mixture of one or more of the aforementioned compounds. The term “combination” includes compounds containing more than one component, which may be conjugated or non-conjugated to one another. In one embodiment, the anchor compound comprises a combination of one or more of the aforementioned compounds which are conjugated to each other, *e.g.*, via covalent or non-covalent interaction. As a particular example, the anchor may comprise a combination of chitosan and oxidized cellulose. See, U.S. Patent Application Publication No. 2014/0045761.

[00126] In some embodiments, the compounds include mixtures of the aforementioned polymeric compounds. The term “mixture” refers to a mingling together of two or more substances without the occurrence of a reaction by which they would lose their individual properties. For instance, a mixture of compound A and compound B may contain any weight ratio of compound A and compound B, such that the total weight of the mixture would amount to 100%, *e.g.*, 99:1 weight ratio of compound A/compound B or 1:99 weight ratio of compound A/compound B. A typical mixture may contain about 2, 3, 4, 5, or more of the aforementioned polymer compounds.

[00127] In some embodiments, the anchor A further comprises an ionic chemical group, a material with a hydrophilic moiety, or a material with a hydrophobic moiety, *e.g.*, an aliphatic

chain or an aliphatic alcohol. In embodiments wherein the anchor comprises an ionic chemical group, the ionic chemical group may be positively or negatively charged. In some embodiments, the anchor region comprises a reactive moiety for covalent attachment to a support material such as a photoactive phenylazide or an epoxide group. See, U.S. Patent Application Publication No. 2016/0159777.

[00128] Methods of introducing reactive groups into chitosan and/or other glycosidic compounds such as polysaccharide, cellulose, glycans, etc., are known in the art. For example, U.S. Patent No. 7,125,968 discloses functionalized chitosan derivatives, which comprise a chitin/chitosan and incorporated therein at least one of a carbohydrate, a photo-reactive functional group, an amphipathic group, *e.g.*, a polyoxyethylene alkyl ether, and a glycosaminoglycan. Such techniques can be used to derivatize types of other anchor compounds.

[00129] Particularly, the anchor region A comprises chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitooligosaccharide; 2-acetamido-2-deoxy-D-glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; or a combination thereof.

[00130] Indicators

[00131] In some embodiments, the chemical entities comprise one or more indicators, *e.g.*, at least 1, at least 2, at least 3, at least 4, or more of indicators. Such compositions may include, for example, a plurality of substrates conjugated to the same gel polymer or different gel polymers.

[00132] In certain embodiments, the indicators are labeled. The term “label,” as used herein, refers to any substance attached to an epitope binding agent, or other substrate material, in which the substance is detectable by a detection method. Non-limiting examples of suitable labels include luminescent molecules, chemiluminescent molecules, fluorochromes, fluorescent quenching agents, colored molecules, radioisotopes, scintillants, biotin, avidin, streptavidin, protein A, protein G, antibodies or fragments thereof, polyhistidine, Ni²⁺, Flag tags, myc tags, heavy metals, and enzymes (including alkaline phosphatase, peroxidase, and luciferase). Methods for attaching the labels to the anchor compounds are described in the Examples.

[00133] In certain embodiments, the indicators are labeled with a label which is a detectable label. A detectable label is a moiety, the presence of which can be ascertained directly or indirectly. Generally, detection of the label involves the creation of a detectable signal such as for example an emission of energy. The label may be of a chemical, peptide or nucleic acid nature although it is not so limited. The nature of label used will depend on a variety of factors, including the nature of the analysis being conducted, the type of the energy source and detector

used and the type of polymer, analyte, probe and primary and secondary analyte-specific binding partners.

[00134] In a particular embodiment, the label is sterically and chemically compatible with the constituents to which it is bound, e.g., the anchor region. In particular, the label is of the shape and size that it does not hinder enzyme recognition site (S) and/or enzyme-reactive region (R).

[00135] In one embodiment, the indicator or a motif therein attached to the anchor is a substrate for a glycosidase. Particularly, the indicator or motif therein attached to the anchor is a substrate for lysozyme.

[00136] In another embodiment, the indicator or a motif therein attached to the anchor is a substrate for a protease selected from the group consisting of elastase, cathepsin G or myeloperoxidase (MAO), or a combination thereof.

[00137] In another embodiment, the indicator or a motif therein attached to the anchor is a substrate for a glycosidase which is lysozyme and a protease selected from the group consisting of elastase, cathepsin G or myeloperoxidase (MAO), or a combination thereof.

[00138] In one embodiment, the indicator (I) or a motif therein attached to the anchor is a peroxidase substrate, an arylamine, an amino phenol, an aminophenyl ether, an indoxyl, a neutral dye, a charged dye, a nanoparticle, or a colloidal gold particle.

[00139] In some embodiments, the indicator (I) or a motif therein attached to the anchor is a peroxidase substrate. In some embodiments, the peroxidase substrate is selected from *p*-aminophenol, ABTS (2,2-inophenol, ABTS (strate. In some embodiments, acid) diammonium salt), 3,3'-diaminobenzidine, 3,4 diaminobenzoic acid, DCPIP, *N,N*-dimethyl-*p*-phenylenediamine, *o*-dianisidine, *p*-phenylenediamine, 4-chloro-1-naphthol, *o*-phenylenediamine *N*-(4-aminobutyl)-*N*-ethylisoluminol, 3-amino-9-ethylcarbazole, 4-aminophthalhydrazide, 5-aminosalicylic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), indoxyl, indigo, Fast Blue RR, 4-chloro-7-nitrobenzofurazan. In some embodiments, the indicator (I) or a label attached thereto is an arylamine. In some embodiments, the indicator (I) or a label attached thereto is an amino phenol. In some embodiments, the indicator (I) or a label attached thereto is an aminophenol ether. In some embodiments, the indicator (I) or a label attached thereto is an indoxyl. In some embodiments, the indicator (I) or a label attached thereto is a neutral dye. In some embodiments, the indicator (I) or a label attached thereto is a charged dye. In some embodiments, the charged dye is selected from remazole brilliant blue, toluidine blue, reactive black 5, remazol brilliant blue, reactive violet 5, and reactive orange 16, or a hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is remazole brilliant blue, or a hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is toluidine blue. In some embodiments, the charged dye is

reactive black 5, or a hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is reactive violet 5, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is reactive orange 16, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the indicator (I) or a label attached thereto is a dichlorotriazine-based reactive dye such as reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10. In some embodiments, the dichlorotriazine-based reactive dye appears black.

[00140] In some embodiments, the indicator (I) or a label attached thereto is a reactive dye containing a sulfonylethyl-hydrogensulphate-reactive-group. In some embodiments, the reactive dye is reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16. In some embodiments, the reactive dye is reactive black 5. In some embodiments, the reactive dye is remazol brilliant blue. In some embodiments, the reactive dye is reactive violet 5. In some embodiments, the reactive dye is reactive orange 16. In some embodiments, the reactive dye is reactive black 5, remazol brilliant blue, or reactive violet 5. In some embodiments, the reactive dye is reactive black 5 or remazol brilliant blue.

[00141] In some embodiments, the indicator (I) or a label attached thereto is a nanoparticle. In some embodiments, the indicator (I) or a label attached thereto is a colloidal gold particle. In some embodiments, the indicator (I) or a label attached thereto is a charged dye, an indole derivative, or a luminol derivative.

[00142] Particularly, the indicator or a motif therein attached to the anchor comprises a dye containing a sulfonylethyl-hydrogensulphate-reactive-group, e.g., reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16, or a combination thereof; or a dye containing a dichlorotriazine reactive-group, e.g., reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10, or a combination thereof.

[00143] Anchor-Indicator conjugates

[00144] In various enzymes an anchor A is conjugated with the indicator I directly, e.g., via an glycosidic linkage. The anchor portion of the conjugate is selected from the group consisting of, e.g., chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitooligosaccharide; 2-acetamido-2-deoxy-D-glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; or a combination thereof. Likewise, the indicator is selected from the group consisting of reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16, reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10, or a combination thereof. A glycosidic linkage is formed between a hydroxyl group of the anchor

compound with a reactive group in the indicator compound. In order to mimic the natural substrate of glycosidase, the 1 α -carbon in the sugar backbone of the anchor molecule is involved in the glycosidic linkage.

[00145] Markers

[00146] Embodiments described herein may utilize chemical moieties that assay for various biological markers present in a chronic or infected wound. In one embodiment, the marker is a wound-specific marker, which is an enzyme selected from the group consisting of hydrolases, proteases, esterases, and peroxidases.

[00147] As used herein, a “wound specific enzyme” is an enzyme that is differentially expressed in a wound. By “differential expression” it is meant that the level or the activity of the enzyme is higher or lower in the wound microenvironment compared to other sites, *e.g.*, normal tissue or surrounding tissue. Particularly, differential expression implies higher level of expression or activity of the enzyme in the wound microenvironment compared to normal or unwounded tissue. Differential expression of enzyme may be analyzed by routine means. For example, levels of enzyme in a sample may be analyzed by ELISA assays or other immunoassays. Activities of the enzyme may be analyzed by measuring rates of loss of a substrate and/or rates of formation of the product, *e.g.*, using mass spectroscopy or HPLC. Such techniques are known in the art and are described in the Examples section.

[00148] In one embodiment, the marker is a hydrolase. As used herein, a “hydrolase” or “hydrolytic enzyme” is an enzyme that catalyzes the hydrolysis of a chemical bond, *e.g.*, esterases and nucleases (break ester bonds); glycolases (break glycosidic linkers); peptidases (break peptide bonds), etc.

[00149] In one specific embodiment, the wound-specific glycoside hydrolase is lysozyme. Lysozyme (UNIPROT accession Nos. P61626 [human] and P08905 [mouse]) is a glycoside hydrolase and its main function is to destroy the cell walls of bacteria. It hydrolyses the (1 $\rightarrow$ 4)- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and also between N-acetyl-D glucosamine residues in chitodextrin. The natural substrate for lysozyme is the peptidoglycan layer of bacterial cell walls. However, a variety of low molecular mass substrates including murein degradation products as well as synthetic compounds have been used for various photometric, isotopic, and immunological lysozyme assays. Hölte et al., *EXS*, 75:105-10, 1996. See also Sigma Catalog Number M5639 and Sigma Catalog Number N8638.

[00150] In one embodiment, the individual components of the chemical moiety have been adapted for recognition by wound-specific hydrolase, *e.g.*, a wound-specific lysozyme.

[00151] Alternately or additionally, the individual components of the chemical moiety can be modified for recognition by other wound specific enzymes. In one embodiment, the additional wound specific enzyme is a protease. As used herein, a “wound specific protease” is a protease that is differentially expressed in a wound. By “differential expression” it is meant that the level or the activity of the protease is higher or lower in the wound microenvironment compared to other sites, *e.g.*, normal tissue or surrounding tissue. Particularly, differential expression implies higher level of expression or activity of the protease in the wound microenvironment compared to unwounded tissue. Differential expression of proteases may be analyzed by routine means. For example, levels of proteases in a sample may be analyzed by ELISA assays or other immunoassays. Activities of the proteases may be analyzed by measuring rates of loss of a peptide substrate and/or rates of formation of the product, *e.g.*, using mass spectroscopy or HPLC. Such techniques are known in the art and are described in the Examples section.

[00152] In one embodiment, the wound-specific protease is cathepsin G (UNIPROT accession Nos. P08311 [human] and P28293 [mouse]), which is one of the three serine proteases of the chymotrypsin family that are stored in the azurophil granules. Cathepsin G-specific substrates have the sequence Ala-Ala-Pro-Phe or Ala-Ala-Pro-Met (Sigma Aldrich Catalog Nos. S7388 and M7771).

[00153] In another embodiment, the wound specific protease is elastase (*e.g.*, human neutrophil elastase or HNE) (UNIPROT accession Nos. P08246 [human] and Q3UP87 [mouse]). HNE is a serine proteinase in the same family as chymotrypsin and has broad substrate specificity. Secreted by neutrophils and macrophages during inflammation, it destroys bacteria and host tissue. In one embodiment, the substrate for detecting HNE has a core sequence Alanine-Alanine-Proline-Valine (AAPV). In another embodiment, the substrate for HNE is Ala-Pro-Glu-Glu-Ile/Met-Arg-Arg-Gln (APEEI/MRRQ) (Kasperkiewicz et al., *PNAS USA*, 111(7): 2518–2523, 2014; Korkmaz et al., *Methods Mol Biol.*, 844:125–138, 2012).

[00154] Still in a further embodiment, the wound-specific enzyme is peroxidase, more specifically, a myeloperoxidase (MPO). MPO (UNIPROT accession Nos. P05164 [human] and P11247 [mouse]) is a peroxidase found in neutrophil granulocytes. In the presence of hydrogen peroxide (H₂O₂) and a halide (most commonly chloride) it produces the antimicrobial substances hypochlorite, singlet oxygen (1O₂), chlorine (Cl₂) and hydroxyl radicals (OH•). MPO can be detected using tetramethylbenzidine or 4-Benzoylamino-2,5-dimethoxyaniline. See, Andrews et al., *Anal Biochem*, 127(2):346-50, 1982; Klebanoff et al., *J. Leukocyte Biol.*, 77, 598-625, 2005.

[00155] Enzyme recognition site (S)

[00156] Insofar as embodiments disclosed herein relate to the specific detection of wound-specific markers, disclosed herein are substrates containing enzyme recognition sites (S) for the wound-specific markers. Thus, in one embodiment, the chemical moiety comprises an anchor region A or an indicator (I) comprising a recognition site for a wound-specific enzyme, *e.g.*, an enzyme cleavage site.

[00157] In one embodiment, the enzyme recognition site comprises glycosidic bonds. As used herein, a “glycosidic bond” is formed between the hemiacetal or hemiketal group of a saccharide (or a molecule derived from a saccharide) and the hydroxyl group of some compound such as an alcohol. A substance containing a glycosidic bond is a glycoside. The term “glycoside” is now extended to also cover compounds with bonds formed between hemiacetal (or hemiketal) groups of sugars and several chemical groups other than hydroxyls, such as -SR (thioglycosides), -SeR (selenoglycosides), -NR₁R₂ (N-glycosides), or even -CR₁R₂R₃ (C-glycosides).

[00158] In one embodiment, the chemical moieties disclosed herein contain one or more glycosidic bonds which are cleaved by glycolases. In one specific embodiment, the chemical moieties comprise a glycosidic bond linking anchor A and the indicator I, either directly or via another group. Particularly, the anchor A and the indicator I are directly linked via one or more glycosidic bonds, in which case, the chemical entity is cleaved by the glycolase and therefore can be used in detecting the glycolase.

[00159] In one embodiment, the indicator molecule comprises an enzymatically-cleavable peptide comprising a peptide bond. As used herein, a “peptide bond” is formed by the condensation reaction between two amino acids, wherein the acid moiety of one reacts with the amino moiety of the other to produce a peptide bond (-CO-NH-) between the two amino acids. The individual peptides provide a motif for the recognition by a sequence-specific protease. As used herein, the term “sequence-specific protease” means a protease recognizing a specific sequence of a peptide for its digesting (for example, caspase), and is distinguished from a generic protease (for example, trypsin) that sequentially decomposes a peptide from one end thereof or digest a peptide in a sequence-nonspecific manner. For sequence specificity, the amino acid sequence of the peptide substrate may comprise four or more amino acid (a.a.) residues.

[00160] As used herein, the term “peptide” includes a natural peptide comprising a linear chain or branched amino acids, peptidomimetics, as well as pharmaceutically acceptable salts thereof. Typically, a peptide comprises a plurality of amino acid residues, *e.g.*, 2, 3, 4, 5, 6, 8, 10, or more amino acid residues which are bonded to each other via covalent bonds, *e.g.*, a peptide bond. “Amino acid residue” means the individual amino acid units incorporated into the

peptides of the disclosure. As used herein, the term “amino acid” means a naturally occurring or synthetic amino acid, as well as amino acid analogs, stereoisomers, and amino acid mimetics that function similarly to the naturally occurring amino acids. Included by this definition are natural amino acids such as: (1) histidine (His) (2) isoleucine (Ile) (3) leucine (Leu) (4) lysine (Lys) (5) methionine (Met) (6) phenylalanine (Phe) (7) threonine (Thr) (8) tryptophan (Trp) (9) valine (Val) (10) arginine (Arg) (11) cysteine (Cys) (12) glutamine (Gln) (13) glycine (Gly) (14) proline (Pro) (15) serine (Ser) (16) tyrosine (Tyr) (17) alanine (Ala) (18) asparagine (Asn) (19) aspartic acid (Asp) (20) glutamic acid (Glu) (21) selenocysteine (Sec); including unnatural amino acids: (a) citrulline; (b) cystine; (c) gamma-amino butyric acid (GABA); (d) ornithine; (f) theanine and amino acid derivatives such as betaine; carnitine; carnosine creatine; hydroxytryptophan; hydroxyproline; N-acetyl cysteine; S-Adenosyl methionine (SAM-e); taurine; tyramine. Among these, amino acids containing reactive side chains, *e.g.*, cysteine, serine, threonine, lysine, arginine, aspartate/asparagine, glutamate/glutamine, glycine, alanine, etc. are particularly employed for modification of the substrate.

[00161] Enzyme-reactive site (R)

[00162] In some embodiments, the chemical entities contain one or more enzyme-labile or enzyme-reactive regions (R) for the detection of wound-specific enzymes.

[00163] In one embodiment, wherein the enzyme is a glycosidase such as lysozyme, the enzyme-labile or enzyme-reactive region comprises an acyl chitosan of at least 3 glucosamine or *N*-acetylglucosamine or peptidoglycan units, which are optionally acetylated. The enzyme reactive site may contain, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20 or more units of glucosamine or *N*-acetylglucosamine or peptidoglycan units. In one embodiment, the R comprises at least 3 glucosamine or *N*-acetylglucosamine or a combination thereof, wherein the glucosamine and/or *N*-acetylglucosamine are optionally acetylated. In another embodiment, the enzyme-labile or enzyme-reactive region comprises peptidoglycan, wherein the peptidoglycan is optionally acetylated.

[00164] In some embodiments, the chemical moieties comprise enzyme reactive sites (R) for one or more wound-specific protease disclosed above, *e.g.*, cathepsin G, and myeloperoxidase, elastase or a combination thereof. As used herein, the term “reactive site for a protease” means a peptide comprising an amino acid sequence of a protein, which is recognized by the protease as a substrate for its protease activity, *e.g.*, as a substrate that can be cleaved into one or more products. In some embodiments, the chemical entities comprise a peptide region comprising a peptide sequence comprising a plurality of amino acids. The term “plurality” means two or more units, *e.g.*, amino acids, although the individual units need not be structurally and/or functionally

different. Typically, the indicator region (I) of the chemical entity comprises the peptide which serves as the enzyme reactive site for the wound-specific protease.

[00165] In one embodiment, the enzyme-labile or enzyme-reactive region comprises a peptide that is labile to elastase, cathepsin G, myeloperoxidase or a combination thereof.

[00166] In one embodiment, the enzyme-labile region comprises a peptide that is liable to elastase. Under this embodiment, the chromogenic indicator for elastase would be high contrast and thus serve as a clear indicator when used in situ in medicinal products.

[00167] The ideal substrate would make a blue, violet or deep green colour. It would also be fixed in a sterically permissible position with high turnover. The state of the art is the opposite. Available substrates contain a p-nitrophenol group, which is low molecular weight but gives rise to a yellow soluble chromophore. Most skilled investigators regard that the substrate should be soluble in water, reasoning that this is the most likely way that the substrate will find its way to the active site.

[00168] In contrast the embodiments described herein depart from that general rationale. It was contemplated that elastase digests a solid phase substrate, namely structural proteins, which are, by definition, not soluble, that a substrate specific to it would have to be adapted accordingly. As such, both the color of the indicator and the systems that they could be employed with, e.g., electronically detection, were adapted to the wound environment.

[00169] Therefore, contrary to the art teachings to employ soluble substrates, embodiments described herein contemplate use of a low water soluble, elastase substrates that give rise to Blue, violet or Green colors.

[00170] In one embodiment, the enzyme-labile or enzyme-reactive region comprises a peptide comprising an amino acid sequence of:

[00171] X_yAAPX_z-Z (SEQ ID NO: 1),

[00172] wherein each X is independently any amino acid,

[00173] y is each, independently, an integer between 0 and 200, and

[00174] Z comprises a detectable label.

[00175] In one embodiment, the enzyme-labile or enzyme-reactive region comprises a peptide comprising an amino acid sequence of:

[00176] X_yAAPX_z-L-Z (SEQ ID NO: 2),

[00177] wherein each X is independently any amino acid,

[00178] y is each, independently, an integer between 0 and 200, and

[00179] Z comprises a detectable label.

[00180] In another embodiment, the enzyme-labile or enzyme-reactive region comprises a peptide comprising an amino acid sequence of:

- [00181] $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3),
- [00182] wherein each X is independently any amino acid,
- [00183] y is each, independently, an integer between 0 and 200, and
- [00184] Z comprises a detectable label.
- [00185] In yet another embodiment, the enzyme-labile or enzyme-reactive region comprises a peptide comprising an amino acid sequence of:
- [00186] $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4),
- [00187] wherein each X is independently any amino acid,
- [00188] y is each, independently, an integer between 0 and 200,
- [00189] L is a linking moiety, and
- [00190] Z comprises a detectable label.
- [00191] In another specific embodiment, the reactive region R comprises the peptide sequence $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4), wherein X, L and Z are each, as described above, and y is, each, independently an integer from 1 to 50.
- [00192] Still in a further embodiment, the reactive region R comprises the peptide sequence $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4), wherein X, L and Z are each, as described above, and y is, each, independently an integer from 1 to 10.
- [00193] Particularly, the reactive region R comprises the peptide sequence $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4), wherein X, L and Z are each, as described above, and y is, each, independently an integer from 1 to 6.
- [00194] In one embodiment, each of the aforementioned peptides comprising the sequence $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4), are each, individually, labile to elastase.
- [00195] In some embodiments, one or more of the amino acids in the amino acid sequence $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4) is protected, e.g., with an amine protection group, for example, fluorenylmethyloxycarbonyl (Fmoc).
- [00196] In general, the elastase substrates have the formula a-b-c-d-e-f, wherein
- [00197] In one embodiment,
- [00198] a is selected from: A, V, F, G, M, R, L
- [00199] b is selected from: no amino acid, or independently, P, F, A, R, L, G
- [00200] g is selected from: no amino acid, or independently, P, A, R, L, G

- [00201] d is selected from: no amino acid, or independently, P, A, R, L, G, V
- [00202] e is selected from: no amino acid, or independently, P, A, R, L, G, V, E
- [00203] f is selected from: no amino acid, or independently, P, A, R, L, G.
- [00204] In another embodiment, elastase substrates have the formula a-b-c-d-e-f, wherein
- [00205] a is selected from: A, V, F, G, M, R, L
- [00206] b is selected from: no amino acid, or independently, P, F, A, G
- [00207] g is selected from: no amino acid, or independently, P, A, R, L, G
- [00208] d is selected from: no amino acid, or independently, P, A, R, L, G, V
- [00209] e is selected from: no amino acid, or independently, P, A, G, V, E
- [00210] f is selected from: no amino acid, or independently, P, A, G.
- [00211] In another embodiment, elastase substrates have the formula a-b-c-d-e-f, wherein
- [00212] a is selected from: A, V, F, G, M, R, L
- [00213] b is selected from: no amino acid, or independently, P, F, A, G
- [00214] g is selected from: no amino acid, or independently, P, A, L, G
- [00215] d is selected from: no amino acid, or independently, P, A, L, G, V
- [00216] e is selected from: no amino acid, or independently, P, A, G, V, E
- [00217] f is selected from: no amino acid, or independently, P, A, G.
- [00218] In another embodiment, elastase substrates have the formula a-b-c-d-e-f, wherein
- [00219] a is selected from: A, V, F, G
- [00220] b is selected from: no amino acid, or independently, P, F, A, G
- [00221] g is selected from: no amino acid, or independently, P, A, G
- [00222] d is selected from: no amino acid, or independently, P, A, G,
- [00223] e is selected from: no amino acid, or independently, A, G, V,
- [00224] f is selected from: no amino acid, or independently, A, G.
- [00225] Especially, in another embodiment, elastase substrates have the formula a-b-c-d-e-f, wherein
- [00226] a is selected from: A, V, F
- [00227] b is selected from: no amino acid, or independently, P, F, A,
- [00228] g is selected from: no amino acid, or independently, A, G
- [00229] d is selected from: no amino acid, or independently, A, G,
- [00230] e is selected from: no amino acid, or independently, A, G,
- [00231] f is selected from: no amino acid, or independently, A, G.
- [00232] Preferably, elastase substrates have the formula a-b-c-d-e-f, wherein
- [00233] a is selected from: A, V, F
- [00234] b is selected from: no amino acid, or independently, P, F, A,

- [00235] g is selected from: no amino acid, or independently, A,
- [00236] d is selected from: no amino acid, or independently, A,
- [00237] e is selected from: no amino acid, or independently, A,
- [00238] f is selected from: no amino acid, or independently, A.
- [00239] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a peptide that is labile to cathepsin G.
- [00240] In one embodiment, the enzyme-labile or enzyme-reactive region comprises a peptide comprising an amino acid sequence of:
- [00241] $X_y N^4 N^3 N^2 N^1 X_y - Z$ (SEQ ID NO: 5), wherein
- [00242] X is each, independently, any amino acid;
- [00243] y is each independently a number selected from 0 to 6;
- [00244] N^4 is selected from alanine, glycine, valine, and glutamine;
- [00245] N^3 is selected from alanine, glycine, proline, lysine, and serine;
- [00246] N^2 is selected from proline, alanine, and glycine;
- [00247] N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; and
- [00248] Z comprises a detectable label; and the peptide is labile to cathepsin G.
- [00249] In some embodiments, one or more of the amino acids in the amino acid sequence is protected. In some embodiments, one or more of the amino acids in the amino acid sequence is protected with an fmoc group. In some embodiments, one of the amino acid in the amino acid sequence is protected with an fmoc group.
- [00250] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a peptide comprising an amino acid sequence of
- [00251] $X_y N^4 N^3 N^2 N^1 X_y - L - Z$ (SEQ ID NO: 6), wherein
- [00252] X is each, independently any amino acid;
- [00253] y is each, independently, a number selected from 0 to 6;
- [00254] N^4 is selected from alanine, glycine, valine, and glutamine;
- [00255] N^3 is selected from alanine, glycine, proline, lysine, and serine;
- [00256] N^2 is selected from proline, alanine, and glycine;
- [00257] N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; and
- [00258] L is a linking moiety; and
- [00259] Z comprises a detectable label.

[00260] In one embodiment, each of the aforementioned peptides comprising the sequence $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5) and $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), are each, individually, labile to cathepsin G.

[00261] In some embodiments, one or more of the amino acids in the amino acid sequence $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5) and $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6) is protected, e.g., with an amine protection group, for example, fluorenylmethyloxycarbonyl (Fmoc).

[00262] Detectable label Z

[00263] In some embodiments, Z is a peroxidase substrate, an arylamine, an amino phenol, an aminophenyl ether, an indoxyl, a neutral dye, a charged dye, a nanoparticle, or a colloidal gold particle.

[00264] In some embodiments, Z is a peroxidase substrate selected from *p*-aminophenol, ABTS (2,2'-azino-3,6-dibenzothiazole-6-sulfonic acid), the peroxidase substrate acid) diammonium salt), 3,3'-diaminobenzidine, 3,4-diaminobenzoic acid, DCPIP, *N,N*-dimethyl-*p*-phenylenediamine, *o*-dianisidine, *p*-phenylenediamine, 4-chloro-1-naphthol, *o*-phenylenediamine *N*-(4-aminobutyl)-*N*-ethylisoluminol, 3-amino-9-ethylcarbazole, 4-aminophthalhydrazide, 5-aminosalicylic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), indoxyl, indigo, Fast Blue RR, 4-chloro-7-nitrobenzofurazan.

[00265] In some embodiments, Z is an arylamine, an amino phenol, an aminophenyl ether, an indoxyl, a neutral dye, a charged dye selected from remazole brilliant blue, toluidine blue, reactive black 5, remazol brilliant blue, reactive violet 5, and reactive orange 16, or a hydrolytic or ammonolytic derivatives thereof. Particularly, Z is a charged dye selected from remazole brilliant blue; toluidine blue; reactive black 5 or a hydrolytic or an ammonolytic derivative thereof.

[00266] In some embodiments, Z is a dichlorotriazine-based reactive dye such as reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10. In some embodiments, the dichlorotriazine-based reactive dye appears black. In some embodiments, Z is a reactive dye containing a sulfonylethyl-hydrogensulphate-reactive-group.

[00267] In some embodiments, Z is a nanoparticle. In some embodiments, Z is a colloidal gold particle.

[00268] In some embodiments, Z is a charged dye, an indole derivative, or a luminol derivative.

[00269] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a phenol, an amino phenol, an aminophenyl ether, an indoxyl, or a quinone. In some embodiments, the enzyme-labile or enzyme-reactive region comprises a phenol. In some embodiments, the enzyme-labile or enzyme-reactive region comprises an amino phenol. In some

embodiments, the enzyme-labile or enzyme-reactive region comprises an amino phenol ether. In some embodiments, the enzyme-label or enzyme-reactive region comprises an indoxyl. In some embodiments, the enzyme-labile or enzyme-reactive region comprises a quinone. In some embodiments, the enzyme-labile or enzyme-reactive region reacts with myeloperoxidase but does not react with heme.

[00270] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a peroxidase substrate, an arylamine, an amino phenol, a neutral dye, a charged dye, a nanoparticle, or a colloidal gold particle. In some embodiments, the enzyme-labile or enzyme-reactive region comprises a peroxidase substrate. In some embodiments, the peroxidase substrate is selected from *p*-aminophenol, ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), 3,3'-diaminobenzidine, 3,4 diaminobenzoic acid, DCPIP, *N,N*-dimethyl-*p*-phenylenediamine, *o*-dianisidine, *p*-phenylenediamine, 4-chloro-1-naphthol, *o*-phenylenediamine *N*-(4-aminobutyl)-*N*-ethylisoluminol, 3-amino-9-ethylcarbazole, 4-aminophthalhydrazide, 5-aminosalicylic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and 4-chloro-7-nitrobenzofurazan, Fast Blue RR, N-(2-hydroxy)tetradecyl-Fast Blue RR. In some embodiments, the enzyme-labile or enzyme-reactive region comprises an arylamine. In some embodiments, the enzyme-labile or enzyme-reactive region comprises an amino phenol. In some embodiments, the enzyme-labile or enzyme-reactive region comprises a neutral dye. In some embodiments, the enzyme-labile or enzyme-reactive region comprises a charged dye. In some embodiments, the charged dye is selected from remazole brilliant blue, toluidine blue, reactive black 5, remazol brilliant blue, reactive violet 5, and reactive orange 16, or hydrolytic or ammonolytic derivatives of each of these. In some embodiments, the charged dye is remazole brilliant blue, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is toluidine blue. In some embodiments, the charged dye is reactive black 5, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is reactive violet 5, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the charged dye is reactive orange 16, or hydrolytic or ammonolytic derivatives thereof.

[00271] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a dichlorotriazine-based reactive dye such as reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10.

[00272] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a nanoparticle. In some embodiments, Z is a colloidal gold particle.

[00273] In some embodiments, the enzyme-labile or enzyme-reactive region comprises a charged dye, an indole derivative, or a luminol derivative. In some embodiments, the enzyme-

labile or enzyme-reactive region comprises an indole derivative. In some embodiments, the enzyme-labile or enzyme-reactive region comprises a luminol derivative.

[00274] In some embodiments, the indicator region comprises a dye that presents a visible color change in normal ambient lighting. In some embodiments, the dye has a contrasting color to wound products, which are commonly red, yellow, or brown. In further embodiments, the dye is violet, blue or dark green. In some embodiments, the dye is violet. In some embodiments, the dye is blue. In some embodiments, the dye is dark green. In some embodiments, the dye has low molecular weight, is charged, contains reactive or linkable groups, is stable to gamma irradiation, and is deeply colored. In some embodiments, the dye is selected from cibracron series dyes, azo dyes, and remazol dyes, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the dye is selected from cibracron series dyes. In some embodiments, the dye is selected from azo dyes. In some embodiments, the dye is selected from remazol dyes, or hydrolytic or ammonolytic derivatives thereof. In some embodiments, the dye is selected from rhodamine, coumarin, cyanine, xanthene, polymethine, pyrene, dipyrromethene borondifluoride, naphthalimide, a phycobiliprotein, peridinium chlorophyll proteins, fluorescein, 6-FAM, rhodamine, Texas Red, California Red, iFluor594, tetramethylrhodamine, a carboxyrhodamine, carboxyrhodamine 6F, carboxyrhodol, carboxyrhodamine 110, Cascade Blue, Cascade Yellow, coumarin, Cy2®, Cy3®, Cy3.5®, Cy5®, Cy5.5®, Cy7®, Cy-Chrome, DyLight 350, DyLight 405, DyLight 488, DyLight 549, DyLight 594, DyLight 633, DyLight 649, DyLight 680, DyLight 750, DyLight 800, phycoerythrin, PerCP (peridinin chlorophyll-a Protein), PerCP-Cy5.5, JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), NED, ROX (5-(and-6)-carboxy-X-rhodamine), HEX, Lucifer Yellow, Marina Blue, Oregon Green 488, Oregon Green 500, Oregon Green 514, Alexa Fluor® 350, Alex Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, 7-amino-4-methylcoumarin-3-acetic acid, BODIPY® FL, BODIPY® FL-Br₂, BODIPY® 530/550, BODIPY® 558/568, BODIPY® 630/650, BODIPY® 650/665, BODIPY® R6G, BODIPY® TMR, BODIPY® TR, and dimethylaminoazobenzenesulfonic acid (dabsyl), or conjugates thereof, or combinations thereof.

[00275] In some embodiments, the indicator region comprises a dichlorotriazine-based reactive dye such as reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10. In some embodiments, the dichlorotriazine-based reactive dye appears black.

[00276] In some embodiments, the indicator region comprises the reaction product of a reactive dye containing a sulfonylethyl-hydrogensulphate-reactive-group. In some embodiments, the reactive dye is reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16. In some embodiments, the reactive dye is reactive black 5. In some embodiments, the

reactive dye is remazol brilliant blue. In some embodiments, the reactive dye is reactive violet 5. In some embodiments, the reactive dye is reactive orange 16. In some embodiments, the reactive dye is reactive black 5, remazol brilliant blue, or reactive violet 5. In some embodiments, the reactive dye is reactive black 5 or remazol brilliant blue.

[00277] In some embodiments, the indicator region comprises a particle (*e.g.*, colloidal metal or quantum dots) that present color changes in normal ambient lighting. In some embodiments, the indicator region comprises a nanoparticle. In some embodiments, the indicator region comprises a colloidal gold particle.

[00278] In some embodiments, the indicator region comprises a dye that presents a visible color change under UV light. In some embodiments, the indicator region comprises a dye that is fluorescent. In some embodiments, the indicator region comprises a dye that is luminescent.

[00279] In some embodiments, the indicator region comprises an enzyme-reactive moiety. In some embodiments, the enzyme-reactive moiety interacts with an accessory enzyme to produce a product that is visible to the naked eye or detectable by electronic means. In some embodiments, the enzyme-reactive moiety interacts with an accessory enzyme to produce a product that is visible to the naked eye. In some embodiments, the enzyme-reactive moiety interacts with an accessory enzyme to produce a product that is detectable by electronic means. In some embodiments, the indicator region comprises an indoxyl glycoside that is cleaved by hexaminidase, glucuronidase, glucosidase or galactosidase depending on the terminal sugar used, to produce indigo. In some embodiments, the indicator region comprises a phenol that is oxidized by an accessory enzyme to produce a visible product. In some embodiments, the indicator region comprises a phenol that is oxidized by laccase to produce a visible product. In some embodiments, the indicator region comprises a metallo motif that is detectable by electronic means. In some embodiments, the indicator region comprises a ferrocene or ferrocene analog that is detectable by electronic means. In some embodiments, the accessory enzyme is selected from lipase, esterase, hexosaminidase, peroxidase, oxidase, glycosidase, glucosidase, and laccase. In some embodiments, the accessory enzyme is not present in the wound fluid. In some embodiments, the accessory enzyme is present in the wound fluid. In some embodiments, the enzyme-reactive moiety interacts with an accessory enzyme to produce a product that is visible under UV light.

[00280] Chemical entities containing a plurality of enzyme recognition sites (S) and reaction sites (R)

[00281] In further embodiments, disclosed herein are chemical entities containing the anchor A, the indicator I, which individually or together comprise a plurality of enzyme recognition sites (S) and enzyme reaction group (R). Typically, such chemical entities are employed to assay

for a plurality of enzymes, e.g., a combination comprising at least one protease and at least one glycosidase.

[00282] In one embodiment, disclosed herein are chemical entities containing the anchor A, the indicator I, which individually or together comprise a plurality of enzyme recognition sites (S) and enzyme reaction sites (R), wherein at least one reactive site is specific for a glycosidase, e.g., lysozyme; and at least one enzyme reaction site is specific for a protease selected from the group consisting of elastase, cathepsin G, myeloperoxidase or a combination thereof. The individual reaction sites and recognition sites for these enzymes have been described previously.

[00283] In one embodiment, disclosed herein are chemical entities containing the anchor A, the indicator I, which individually or together comprise a plurality of enzyme recognition sites (S) and enzyme reaction sites (R), wherein at least one reactive site is specific for a glycosidase, e.g., lysozyme; and at least one enzyme reaction site is specific for a protease selected from the group consisting of elastase. The individual reaction sites and recognition sites for these enzymes have been described previously.

[00284] In one embodiment, disclosed herein are chemical entities containing the anchor A, the indicator I, which individually or together comprise a plurality of enzyme recognition sites (S) and enzyme reaction sites (R), wherein at least one reactive site is specific for a glycosidase, e.g., lysozyme; and at least one enzyme reaction site is specific for a protease selected from the group consisting of cathepsin G. The individual reaction sites and recognition sites for these enzymes have been described previously.

[00285] In one embodiment, disclosed herein are chemical entities containing the anchor A, the indicator I, which individually or together comprise a plurality of enzyme recognition sites (S) and enzyme reaction sites (R), wherein at least one reactive site is specific for a glycosidase, e.g., lysozyme; and at least one enzyme reaction site is specific for a protease selected from the group consisting of myeloperoxidase (MPO). The individual reaction sites and recognition sites for these enzymes have been described previously.

[00286] Owing to the greater predictive power of employing a combination of enzyme substrates, it is contemplated that the diagnostic utility of chemical entities comprising a plurality of reaction and recognition sites, as outlined above, will be greatly enhanced compared to entities comprising unitary (e.g., single type) of reaction and recognition sites. At the very least, entities comprising a plurality of reaction/recognition sites will permit diagnosis of at least 2, at least 3, at least 4 or more markers simultaneously. By the way of example, lysosomal and protease activity at the wound situs may be detected and monitored simultaneously using the multiplex chemical entities disclosed herein.

[00287] Support material

[00288] In some embodiments, the anchor region (A) of the chemical entity binds the chemical entity to a support material, e.g., via covalent interaction, ionic interaction, hydrophobic interaction, electrostatic interactions, hydrogen bonding interactions, physiochemical interactions, van der Waal forces, Lewis-acid/Lewis-base interactions, or combinations thereof.

[00289] In some embodiments, the support matrix comprises dextran, agarose, silica, synthetic polymer, or dextran, agarose, silica, or synthetic polymer covalently coupled to an antibody, ligand, or epitope tag.

[00290] In some embodiments, the anchor region is a polystyrene bead, silica gel bead, polysaccharide bead, polyacrylamide bead, cellulose bead, polysaccharide, derivatized cellulose, polyacrylate, polyethyleneimine, polyacrylamide, UV-activatable reactive group, peptidoglycan, or chitosan derivative, or a combination thereof. In some embodiments, the anchor region binds to a support material after a short period of UV irradiation.

[00291] In some embodiments, the chemical entity is printed on or in a support material such as filter paper or a woven or non-woven material that is capable of being wet by a wound fluid and which displays capillary action. In some embodiments, the reporting entity or chemical entity is chemically bonded onto or into a support material such as filter paper or a woven or non-woven material that is capable of being wet by a wound fluid and which displays capillary action that is similar in all dimensions. In some embodiments, the chemical entity is ionically bound onto or into a support material such as filter paper or a woven or non-woven material that is capable of being wet by a wound fluid and which displays capillary action. In some embodiments, the chemical entity is covalently bound onto or into a support material such as filter paper or a woven or non-woven material that is capable of being wet by a wound fluid and which displays capillary action. Support material includes, but is not limited to, cellulose, polyamide, polyester, polyacrylate and other similar polymers that are useful as fibers. In some embodiments, the support material is cellulose. In some embodiments, the support material is polyamide. In some embodiments, the support material is polyester. In some embodiments, the support material is polyacrylate.

[00292] Additional moieties

[00293] In some instances, the pH of a wound can influence many factors of wound healing, such as angiogenesis, protease activity, oxygen release, and bacterial toxicity. Chronic non-healing wounds may have an elevated alkaline environment. As the wound progresses towards healing, the pH of the wound moves to neutral and then becomes acidic. Monitoring of the pH of

the wound may provide a method to assess the condition of the wound (*e.g.*, infection or no infection) and aid in determining a wound's response to treatment.

[00294] Accordingly, in some embodiments, the chemical entity for the detection of infection in a wound comprises an indicator region comprising a pH-sensitive moiety that presents a visible color change. In one embodiment, the pH-sensitive moiety presents a visible color change at alkaline pH, *e.g.*, a pH = 7.2-9.5; pH = 7.2-9.0; pH = 7.2-8.5; pH = 7.2-8.0; pH = 7.5-8.5; pH = 7.5-9.0; pH = 8.0-9.0. In other embodiments, the pH-sensitive moiety presents a visible color change at pH = 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, or 9.5, or 0.1 increments thereof.

[00295] In some embodiments, the pH-sensitive moiety presents a visible color change at neutral pH range, *e.g.*, at pH = 6.9, 7.0, or 7.1, or 0.05 increments thereof.

[00296] In some embodiments, the pH-sensitive moiety presents a visible color change at acidic pH, *e.g.*, pH = 4.5-6.8; pH = 4.5-6.5; pH = 5.0-6.8; pH = 5.4-6.8; pH = 5.4-6.5. In other embodiments, the pH-sensitive moiety presents a visible color change at pH = 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, or 6.9, or 0.1 increments thereof.

[00297] In some embodiments, the pH-sensitive moiety is selected from the group consisting of bromothymol blue, phenol red, bromophenol red, chlorophenol red, thymol blue, bromocresol green, bromocresol purple; nitrazine yellow; and sulfophthalein dyes or a combination thereof.

[00298] Compositions:

[00299] Embodiments described herein further relate to compositions containing the compounds of Formula I. Such compositions may be prepared using conventional methods.

[00300] Once formulated, the resulting stock composition of compounds of Formula I may be further modified into desired form, *e.g.*, gels, balms, lotions, cream, paste, ointments, etc. using conventional methods, *e.g.*, using carriers, gelling agents, emollients, surfactants, humectants, viscosity enhancers, emulsifiers, etc. See, *e.g.*, WO 2013/004953.

[00301] Carriers for use in the composition may include, but are not limited to, water, glycerin, diglycerin, glycerin derivatives, glycols, glycol derivatives, sugars, ethoxylated and/or propoxylated esters and ethers, urea, sodium PCA, alcohols, ethanol, isopropyl alcohol, and combinations thereof. In one embodiment, the carrier is propylene glycol. Typically, the composition contains a carrier in an amount from about 1 % by weight of the composition to about 99.9% by weight of the composition, more typically from about 2% by weight of the composition to about 95% by weight of the composition, and more typically from about 5% by weight of the composition to about 90% by weight of the composition.

[00302] Thermo-reversible gelling agents are defined as ingredients that are soluble, partially soluble, or miscible in a hydrophilic carrier at elevated temperatures, such as 50°C, wherein the agents have the ability to thicken the carrier when cooled to 25°C, but will be less viscous at 50°C when application to a substrate is necessary. Suitable hydrophilic carriers include water, glycols, *e.g.*, propylene glycol. Thermo-reversible gelling agents for use in the composition may include salts of fatty acids such as sodium stearate, sodium palmitate, potassium stearate. These salts can be added to the composition or can be created in-situ by addition of the fatty acid and neutralizing with appropriate base. An example of in-situ formation of the composition is to provide stearic acid and sodium hydroxide to produce sodium stearate. Other common thermos-reversible gelling agents could include, *e.g.*, polyethylene glycols and derivatives such as PEG-20, PEG-150 distearate, PEG-150 pentaerythrityl tetrastearate, disteareth-75 IPDI, disteareth-100 IPDI, fatty alcohols, *e.g.*, cetyl alcohol, fatty acids such as stearic acid, hydroxystearic acid and its derivatives, and combinations thereof.

[00303] In addition to the carrier and thermos-reversible gelling agent, the composition can contain various other ingredients and components. Examples of other ingredients that may be included within the composition are emollients, sterols or sterol derivatives, natural and synthetic fats or oils, viscosity enhancers, rheology modifiers, polyols, surfactants, alcohols, esters, silicones, clays, starch, cellulose, particulates, moisturizers, film formers, slip modifiers, surface modifiers, skin protectants, humectants, sunscreens, and the like.

[00304] Pharmaceutical Compositions and/or Preparations:

[00305] Embodiments described herein further relate to pharmaceutical compositions and/or preparations comprising one or more of the aforementioned compounds of Formula I and a carrier. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, salts, compositions, dosage forms, etc., which are--within the scope of sound medical judgment--suitable for use in contact with the tissues of human beings and/or other mammals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. In some aspects, "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government, or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals (*e.g.*, animals), and more particularly, in humans.

[00306] The pharmaceutical compositions may be prepared by any suitable means known in the art. Examples of such compositions include those adapted for: (a) topical application, *e.g.*, articles (*e.g.*, gauzes, pads, swabs, dressings), creams, ointments, gels, lotions, etc.; (b) parenteral administration, *e.g.*, subcutaneous, intramuscular or intravenous injection as a sterile solution or suspension; (c) oral administration, external application (*e.g.* drenches including

aqueous and non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pellets for admixture with feedstuffs, pastes for application to the tongue, etc.

[00307] In certain embodiments, the pharmaceutical compositions may comprise one or more antibiotic agents. As used herein, the term “antibiotic” or “antimicrobial agent” refers to a substance that inhibits the growth of or destroys microorganisms. Preferably, the antibiotic is useful in curbing the virulence of an infectious agent and/or treating an infectious disease. Antibiotic also refers to semi-synthetic substances wherein a natural form produced by a microorganism, *e.g.*, yeast or fungus is structurally modified.

[00308] Preferably, the antibiotic is selected from the group consisting of β -lactams (including, β -lactamase inhibitors and cephalosporins), fluoroquinolones, aminoglycosides, tetracyclines and/or glycylicyclines and/or polymyxins. Any combination of antimicrobial agents may also be employed, *e.g.*, at least one β -lactam and at least one fluoroquinolone; at least one aminoglycoside and one cephalosporin; at least one β -lactam and one β -lactamase inhibitor, optionally together with an aminoglycoside, etc.

[00309] As used herein, the term “ β -lactam” inhibitor includes natural and semi-synthetic penicillins and penicillin derivatives, *e.g.*, benzathine penicillin, benzylpenicillin (penicillin G), phenoxymethylpenicillin (penicillin V), procaine penicillin and oxacillin; methicillin, dicloxacillin and flucloxacillin; temocillin; amoxicillin and ampicillin; azlocillin, carbenicillin, ticarcillin, mezlocillin and piperacillin; biapenem, doripenem, ertapenem, imipenem, meropenem, panipenem and PZ-601; cephalexin, cephalothin, cefazolin, cefaclor, cefuroxime, cefamandole, cefotetan, cefoxitin, cefotaxime, and cefpodoxime; cefepime and ceftazidime; cefadroxil, cefixime, cefprozil, cephalexin, cephalothin, cefuroxime, cefamandole, cefepime and ceftazidime; cefoxitin, cefotetan, cefmetazole and flomoxef; tigemonam, nocardicin A and tabtoxin; clavulanic acid, moxalactam and flomoxef. Fluoroquinolones include, ciprofloxacin, garenoxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin. Aminoglycosides include, for *e.g.*, kanamycin, amikacin, tobramycin, dibekacin, gentamicin, sisomicin, netilmicin, neomycin B, neomycin C, neomycin E (paromomycin) and streptomycin, including synthetic derivatives clarithromycin and azithromycin. Tetracyclines include naturally-occurring compounds (*e.g.*, tetracycline, chlortetracycline, oxytetracycline, demeclocycline) or semi-synthetic agents (*e.g.*, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline). Glycylicyclines (*e.g.*, minocycline/tigecycline) are derived from tetracyclines. Polymyxins include, *e.g.*, polymyxin B and polymyxin E (colistin).

[00310] In certain embodiments, the compositions may contain an antibiotic at a concentration of 0.1 mg/mL, 0.5 mg/L, 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14

mg/mL, 15 mg/mL, 16 mg/mL, 17 mg/mL, 18 mg/mL, 19 mg/mL, 20 mg/mL, 21 mg/mL, 22 mg/mL, 23 mg/mL, 24 mg/mL, 25 mg/mL, 26 mg/mL, 27 mg/mL, 28 mg/mL, 29 mg/mL, 30 mg/mL, 31 mg/mL, 32 mg/mL, 33 mg/mL, 34 mg/mL, 35 mg/mL, 36 mg/mL, 37 mg/mL, 38 mg/mL, 39 mg/mL, 40 mg/mL, 41 mg/mL, 42 mg/mL, 43 mg/mL, 44 mg/mL, 45 mg/mL, 50 mg/mL, 60 mg/mL, 70 mg/mL, 80 mg/mL, 90 mg/mL, 100 mg/mL, 150 mg/mL, 200 mg/mL, 250 mg/mL, 300 mg/mL, 400 mg/mL, 500 mg/mL, or more. For example, imipenem and ertapenem may be used in the concentrations of 50, 30, 20, 15, 10, 5 and 1 mg/mL.

[00311] Wound dressings:

[00312] Disclosed herein, in certain embodiments, are wound dressings comprising wound dressing materials as described herein, *e.g.*, compounds of Formula I. In some embodiments, the wound dressings consist essentially of the wound dressing materials as described herein, *e.g.*, a compound of Formula I.

[00313] In one embodiment, the wound dressing disclosed herein are biocompatible, biodegradable, non-immunogenic and readily commercially available.

[00314] In one embodiment, the compounds of Formula I are provided in the form of particles, such as fiber particles or powder particles, optionally containing a medicament. In particular, the materials preferably contain CMC fibers.

[00315] The compositions may preferably comprise an intimate mixture of the dressing material and other compounds. For instance, in one embodiment, the intimate mixture comprises a mixed solution or dispersion of the dressing material and a suitable vehicle, such as a solvent, or a solid composition produced by removing solvent from such a solution or dispersion. Under this embodiment, the dressing material makes up at least 5%, more preferably at least 10%, 20%, 30%, 50%, 75%, 90% or greater % by weight of the material. In certain preferred embodiments, the material consists essentially of the dressing material.

[00316] Other components of the material may include 0-25% by weight, for example from about 1 to about 20% by weight, of one or more other biocompatible polysaccharides, for example alginates such as sodium alginate or calcium alginate, starch derivatives such as sodium starch glycolate, cellulose derivatives such as methyl cellulose or carboxymethyl cellulose, or glycosaminoglycans such as hyaluronic acid or its salts, chondroitin sulfate or heparan sulfate. The materials may also comprise up to about 25% by weight, for example from about 1 to about 20% by weight, of one or more structural proteins selected from the group consisting of fibronectin, fibrin, laminin, elastin, collagen and mixtures thereof. Preferably the protein comprises collagen, and more preferably it consists essentially of collagen. The materials may also comprise up to about 20% by weight, preferably from about 2% to about 10% by weight of

water. The materials may also contain 0-40% by weight, for example from about 5 to about 25% by weight, of a plasticizer, preferably a polyhydric alcohol such as glycerol or sorbitol.

[00317] In certain embodiments, the materials may also comprise up to about 10% by weight, for example from about 0.01 to about 5% by weight, typically from about 0.1 to about 2% by weight of one or more therapeutic wound healing agents, such as non-steroidal anti-inflammatory drugs (*e.g.*, acetaminophen), steroids, local anesthetics, antimicrobial agents, or growth factors (*e.g.*, fibroblast growth factor or platelet derived growth factor). The antimicrobial agent may, for example, comprise an antiseptic, an antibiotic, or mixtures thereof. Preferred antibiotics include tetracycline, penicillins, terramycins, erythromycin, bacitracin, neomycin, polymyxin B, mupirocin, clindamycin and mixtures thereof. Preferred antiseptics include silver, including colloidal silver, silver salts including salts of one or more of the anionic polymers making up the material, silver sulfadiazine, chlorhexidine, povidone iodine, triclosan, sucralfate, quaternary ammonium salts and mixtures thereof. These medicated wound dressing materials according to the disclosed technology provide sustained release of the therapeutic agents as the wound dressing material breaks down in use.

[00318] All of the above percentages are on a dry weight basis. Preferably, the weight ratio of the wound dressing material to other auxiliary agents and materials is from about 1:99 to about 99:1. More preferably, the weight ratio is in the range about 1:9 to about 9:1, more preferably it is in the range about 4:1 to about 1:4, still more preferably in the range about 2:1 to about 1:2.

[00319] The material may be in any convenient form, such as a powder, microspheres, flakes, a mat or a film.

[00320] In certain embodiments, the material is in the form of a semisolid or gel ointment for topical application.

[00321] In certain embodiments, the material is in the form of a freeze-dried or solvent-dried bioabsorbable sponge for application to a chronic wound. Preferably, the average pore size of the sponge is in the region of 10-500 μm , more preferably about 100-300 μm . A suitable sponge has been made by freeze-drying or solvent drying an aqueous dispersion comprising compounds of Formula I, together with suitable therapeutic agents.

[00322] In yet other embodiments, the material is in the form of a flexible film, which may be continuous or interrupted (*e.g.* perforated). The flexible film preferably comprises a plasticizer to render it flexible, such as glycerol.

[00323] The ready availability of both gel forming polymers, *e.g.*, cellulose derivatives, having a range of controllable properties means that the properties of the compositions the

disclosed technology can be controlled to an exceptional degree. In particular, the rate of biological absorption, porosity and density of the materials can be controlled.

[00324] In one embodiment, provided herein are wound dressing materials in sheet form, comprising an active layer of a composition comprising compounds of Formula I. The active layer would normally be the wound contacting layer in use, but in some embodiments it could be separated from the wound by a liquid-permeable top sheet. In one embodiment, the area of the active layer is from about 1 cm² to about 400 cm², particularly from about 4 cm² to about 100 cm².

[00325] In another embodiment, the wound dressing material further comprises a backing sheet extending over the active layer opposite to the wound facing side of the active layer. Preferably, the backing sheet is larger than the active layer such that a marginal region of width 1 mm to 50 mm, preferably 5 mm to 20 mm extends around the active layer to form a so-called island dressing. In such cases, the backing sheet is preferably coated with a pressure sensitive medical grade adhesive in at least its marginal region.

[00326] In embodiments wherein the dressing material comprises a backing sheet, the back sheet is substantially liquid-impermeable. In another embodiment, the backing sheet is semipermeable, *e.g.*, the backing sheet is preferably permeable to water vapor, but not permeable to liquid water or wound exudate. Preferably, the backing sheet is also microorganism-impermeable. Suitable continuous conformable backing sheets will preferably have a moisture vapor transmission rate (MVTR) of the backing sheet alone of 300 to 5000 g/m²/24 hrs, preferably 500 to 2000 g/m²/24 hrs at 37.5° C at 100% to 10% relative humidity difference. The backing sheet thickness is preferably in the range of 10 to 1000 micrometers, more preferably 100 to 500 micrometers.

[00327] Suitable polymers for forming the backing sheet include polyurethanes and polyalkoxyalkyl acrylates and methacrylates. Preferably, the backing sheet comprises a continuous layer of a high density blocked polyurethane foam that is predominantly closed-cell. A suitable backing sheet material is a polyurethane film.

[00328] In wound dressings comprising a backing layer comprising an adhesive, the adhesive layer should be moisture vapor transmitting and/or patterned to allow passage of water vapor. The adhesive layer is preferably a continuous moisture vapor transmitting, pressure-sensitive adhesive layer of the type conventionally used for island-type wound dressings, for example, a pressure sensitive adhesive based on acrylate ester copolymers, polyvinyl ethyl ether and polyurethane. Polyurethane-based pressure sensitive adhesives may be selectively used.

[00329] In another embodiment, the dressing may comprise further layers of a multilayer absorbent article may be built up between the active layer and the protective sheet. For example,

these layers may comprise an apertured plastic film to provide support for the active layer in use, in which case the apertures in the film are preferably aligned in register with the apertures in the hydrogel layer.

[00330] Still further, in other embodiments, the dressing may comprise an absorbent layer between the active layer and the protective sheet, especially if the dressing is for use on exuding wounds. The optional absorbent layer may be any of the layers conventionally used for absorbing wound fluids, serum or blood in the wound healing art, including gauzes, nonwoven fabrics, superabsorbents, hydrogels and mixtures thereof. Preferably, the absorbent layer comprises a layer of absorbent foam, such as an open celled hydrophilic polyurethane foam. In other embodiments, the absorbent layer may be a nonwoven fibrous web, for example a carded web of viscose staple fibers.

[00331] In certain embodiments, the wound dressing may be protected by a removable cover sheet. The cover sheet is normally formed from flexible thermoplastic material. Suitable materials include polyesters and polyolefins. Preferably, the adhesive-facing surface of the cover sheet is a release surface. That is to say, a surface that is only weakly adherent to the active layer and the adhesive on the backing sheet to assist peeling of the hydrogel layer from the cover sheet. For example, the cover sheet may be formed from a non-adherent plastic such as a fluoropolymer, or it may be provided with a release coating such as a silicone or fluoropolymer release coating.

[00332] In one embodiment, the wound dressing is sterile and packaged in a microorganism-impermeable container.

[00333] Kits:

[00334] In certain embodiments, the disclosed technology provides kits comprising, in one or separate compartments, the compounds of Formula I, optionally together with an excipient, carrier or oil. The kits may further comprise additional ingredients, *e.g.*, gelling agents, emollients, surfactants, humectants, viscosity enhancers, emulsifiers, etc., in one or more compartments. The kits may optionally comprise instructions for formulating an article for diagnosing, detecting or treating wounds, *e.g.*, chronic or infected wounds. The kits may also comprise instructions for using the components, either individually or together, in the treatment of wounds.

[00335] In a related embodiment, the disclosed technology provides kits comprising a package and at least one absorbent article (described above) comprising the aforementioned compositions. Alternately, the kits may comprise the individual components separately, optionally together with secondary information, useable in or with the package.

[00336] Other embodiments disclosed herein relate to the use of the composition for the preparation of a dressing for the treatment of a wound. Preferably, the wound is a chronic wound, for example a wound selected from the group consisting of venous ulcers, decubitis ulcers and diabetic ulcers.

[00337] Surfaces:

[00338] Embodiments of the disclosed technology further provide for surfaces comprising the aforementioned compounds of Formula I, wherein the reporter or peptide is oriented to permit binding to a partner, *e.g.*, an enzyme. Preferably, the surface is a surface of a solid support. Numerous and varied solid supports are known to those in the art. Useful solid supports include natural polymeric carbohydrates and their synthetically modified, cross-linked or substituted derivatives, such as agar, agarose, cross-linked alginic acid, substituted and cross-linked guar gums, cellulose esters, especially with nitric acid and carboxylic acids, mixed cellulose esters, and cellulose ethers; natural polymers containing nitrogen, such as proteins and derivatives, including cross-linked or modified gelatins; natural hydrocarbon polymers, such as latex and rubber; synthetic polymers which may be prepared with suitably porous structures, such as vinyl polymers, including polyethylene, polypropylene, polystyrene, polyvinylchloride, polyvinylacetate and its partially hydrolyzed derivatives, polyacrylamides, polymethacrylates, copolymers and terpolymers of the above polycondensates, such as polyesters, polyamides, and other polymers, such as polyurethanes or polyepoxides; porous inorganic materials such as sulfates or carbonates of alkaline earth metals and magnesium, including barium sulfate, calcium sulfate, calcium carbonate, silicates of alkali and alkaline earth metals, aluminum and magnesium; and aluminum or silicon oxides or hydrates, such as clays, alumina, talc, kaolin, zeolite, silica gel, or glass (these materials may be used as filters with the above polymeric materials); and mixtures or copolymers of the above classes, such as graft copolymers obtained by initializing polymerization of synthetic polymers on a pre-existing natural polymer.

[00339] In one embodiment, the support is a well of an array plate, *e.g.*, a microarray. Methods for constructing such arrays are known in the art, *e.g.*, Cao et al., *Appl Environ Microbiol.*, 77(23): 8219–8225, 2011. Each compound of Formula I (or the peptide indicators alone) may be spotted in triplicate to eliminate irregular data due to physical defects in the array.

[00340] Systems:

[00341] Embodiments of the disclosed technology further provide for diagnostic systems comprising the aforementioned compositions and/or kits.

[00342] The various components of the diagnostic systems may be provided in a variety of forms. For example, the compounds of Formula I (*e.g.*, compounds containing peptide reporters) may be provided as a lyophilized reagent. These lyophilized reagents may be pre-mixed before

lyophilization so that when reconstituted they form a complete mixture with the proper ratio of each of the components ready for use in the assay. In addition, the diagnostic systems of the disclosed technology may contain a reconstitution reagent for reconstituting the lyophilized reagents of the kit.

[00343] Nucleic acids

[00344] In one embodiment, included herein are nucleic acids encoding the following peptides:

[00345] X_yAAPX_y-Z (SEQ ID NO: 1), X_yAAPX_y-L-Z (SEQ ID NO: 2), $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3) or $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4); $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5) or $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above.

[00346] In another embodiment, included herein are nucleic acids encoding the polypeptide sequences for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable fragment thereof and/or an immunogenic fragment thereof.

[00347] In particular embodiment, included herein are nucleic acids encoding the polypeptide sequences set forth in Table 4 (for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM_His, CatGSub1_CBM_His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM_His, CatGSub2_CBM_His) or an enzyme-cleavable fragment thereof and/or an immunogenic fragment thereof.

[00348] The phrases “nucleic acid” or “nucleic acid sequence,” as used herein, refer to an oligonucleotide, nucleotide, polynucleotide, or any fragment thereof, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material. In this context, “fragments” refers to those nucleic acid sequences which are greater than about 10 nucleotides in length, and most preferably are at least about 40 nucleotides, at least about 100 nucleotides, or at least about 300 nucleotides in length.

[00349] Embodiments disclosed herein further relate to variants of the aforementioned polynucleotides.

[00350] In one embodiment, included herein are variants of aforementioned nucleic acids which comprise, or alternatively consist of, a nucleotide sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%, or greater % identity to, for example, the nucleic acids encoding the following peptides: X_yAAPX_y-Z (SEQ ID NO: 1), X_yAAPX_y-L-Z (SEQ ID NO:

2), $X_y\text{AAP}(\text{V/F/A})X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP}(\text{V/F/A})X_y\text{-L-Z}$ (SEQ ID NO: 4); $X_y\text{N}^4\text{N}^3\text{N}^2\text{N}^1X_y\text{-Z}$ (SEQ ID NO: 5) or $X_y\text{N}^4\text{N}^3\text{N}^2\text{N}^1X_y\text{-L-Z}$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above.

[00351] In one embodiment, included herein are variants of aforementioned nucleic acids which comprise, or alternatively consist of, a nucleotide sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%, or greater % identity to, for example, the nucleotide coding sequence in ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM His, or the complementary strand thereto, or the RNA equivalent thereof, or a complementary RNA equivalent thereof.

[00352] In a related embodiment, included herein are nucleotide sequence variants of aforementioned nucleic acids which comprise, or alternatively consist of, a nucleotide sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%, or greater % identity to, for example, the nucleotide encoding the polypeptide sequences set forth in Table 4 (for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM_His, CatGSub1_CBM_His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM_His, CatGSub2_CBM_His), or the complementary strand thereto, or the RNA equivalent thereof, or a complementary RNA equivalent thereof. One skilled in the art can use routine software, e.g., Three-to-One Sequence Manipulation Suite (which generates three potential nucleic acid sequences for each inputted polypeptide sequence), to arrive at the encoding nucleic acid sequences. The Three-to-One software is available freely from [bioinformatics\(dot\)org](http://bioinformatics(dot)org).

[00353] The phrases “percent identity” or “% identity” refer to the percentage of sequence similarity found in a comparison of two or more amino acid or nucleic acid sequences. Percent identity can be determined electronically, e.g., by using the MEGALIGN program (LASERGENE software package, DNASTAR). The MEGALIGN program can create alignments between two or more sequences according to different methods, e.g., the Clustal Method. (Higgins, D. G. and P. M. Sharp (1988) *Gene* 73:237-244.) The Clustal algorithm groups sequences into clusters by examining the distances between all pairs. The clusters are aligned pairwise and then in groups. The percentage similarity between two amino acid sequences, e.g., sequence A and sequence B, is calculated by dividing the length of sequence A, minus the number of gap residues in sequence A, minus the number of gap residues in sequence B, into the sum of the residue matches between sequence A and sequence B, times one hundred. Gaps of low or of no homology between the two amino acid sequences are not included in determining percentage similarity. Percent identity between nucleic acid sequences can also be

calculated by the Clustal Method, or by other methods known in the art, such as the Jotun Hein Method. (See, e.g., Hein, J. (1990) *Methods Enzymol.* 183:626-645.) Identity between sequences can also be determined by other methods known in the art, e.g., by varying hybridization conditions.

[00354] In another embodiment, included herein are variant polynucleotides which hybridize to one or more nucleic acid molecules under stringent hybridization conditions or lower stringency conditions. "Hybridization," as the term is used herein, refers to any process by which a strand of nucleic acid bonds with a complementary strand through base pairing. For example, hybridization under high stringency conditions could occur in about 50% formamide at about 37° C. to 42° C. Hybridization could occur under reduced stringency conditions in about 35% to 25% formamide at about 30° C. to 35° C. In particular, hybridization could occur under high stringency conditions at 42° C. in 50% formamide, 5×SSPE, 0.3% SDS, and 200 µg/ml sheared and denatured salmon sperm DNA. Hybridization could occur under reduced stringency conditions as described above, but in 35% formamide at a reduced temperature of 35° C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

[00355] The term "hybridization complex" as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary bases. A hybridization complex may be formed in solution or formed between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed).

[00356] In another embodiment, included herein are variants which are polynucleotide fragments of the aforementioned nucleic acids.

[00357] Also included herein are oligonucleotides, e.g., PCR primers, which hybridize to one or more nucleic acids. The term "oligonucleotide," as used herein, refers to a nucleic acid sequence of at least about 6 nucleotides to 60 nucleotides, preferably about 15 to 30 nucleotides, and most preferably about 20 to 25 nucleotides, which can be used in PCR amplification or in a hybridization assay or microarray. As used herein, the term "oligonucleotide" is substantially equivalent to the terms "amplimers," "primers," "oligomers," and "probes," as these terms are commonly defined in the art.

[00358] Also included herein are modified nucleic acids such as PNA. "Peptide nucleic acid" (PNA), as used herein, refers to an antisense molecule or anti-gene agent which comprises an

oligonucleotide of at least about 5 nucleotides in length linked to a peptide backbone of amino acid residues ending in lysine. The terminal lysine confers solubility to the composition. PNAs preferentially bind complementary single stranded DNA and RNA and stop transcript elongation, and may be pegylated to extend their lifespan in the cell. (See, e.g., Nielsen, P. E. et al. (1993) *Anticancer Drug Des.* 8:53-63.)

[00359] Vectors

[00360] Also included herein are vectors which contain one or more of the aforementioned nucleic acids. In one embodiment, the vector comprises at least one protein encoding nucleic acid, e.g., nucleic acids encoding the polypeptide sequences for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable fragment thereof and/or an immunogenic fragment thereof, in operable linkage with one or more additional sequences. The additional sequences may be synthetic in nature. The terms “operably associated” or “operably linked,” as used herein, refer to functionally related nucleic acid sequences. A promoter is operably associated or operably linked with a coding sequence if the promoter controls the transcription of the encoded polypeptide. While operably associated or operably linked nucleic acid sequences can be contiguous and in reading frame, certain genetic elements, e.g., repressor genes, are not contiguously linked to the encoded polypeptide but still bind to operator sequences that control expression of the polypeptide.

[00361] Codon optimized sequences

[00362] Included herein are codon-optimized sequences of the aforementioned nucleic acid sequences and vectors. Codon optimization for expression in a host cell, e.g., bacteria such as *E. coli* or insect Hi5 cells, may be routinely performed using Codon Optimization Tool (CodonOpt), available freely from Integrated DNA Technologies, Inc., Coralville, Iowa.

[00363] Host cells

[00364] Included herein are host cells containing the aforementioned nucleic acid sequences and vectors. In one embodiment, the host cell is capable of recombinantly expressing the gene sequence contained in the vector under standard culture conditions to generate the polypeptide product, e.g., polypeptide sequences for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable

fragment thereof and/or an immunogenic fragment thereof. In one specific embodiment, the host cell is *E. coli*.

[00365] Polypeptides

[00366] In one embodiment, included herein are polypeptides comprising the following amino acid sequences: $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP(V/F/A)}X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP(V/F/A)}X_y\text{-L-Z}$ (SEQ ID NO: 4); $X_yN^4N^3N^2N^1X_y\text{-Z}$ (SEQ ID NO: 5) or $X_yN^4N^3N^2N^1X_y\text{-L-Z}$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above.

[00367] In another embodiment, included herein are the polypeptide sequences for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable fragment thereof and/or an immunogenic fragment thereof.

[00368] In particular embodiments, included herein are the polypeptide sequences set forth in Table 4 (for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His) or an enzyme-cleavable fragment thereof and/or an immunogenic fragment thereof.

[00369] In another embodiment, included herein are variants of aforementioned polypeptides which comprise, or alternatively consist of, an amino acid sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%, or greater % identity to, for example, the following polypeptide sequences: $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP(V/F/A)}X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP(V/F/A)}X_y\text{-L-Z}$ (SEQ ID NO: 4); $X_yN^4N^3N^2N^1X_y\text{-Z}$ (SEQ ID NO: 5) or $X_yN^4N^3N^2N^1X_y\text{-L-Z}$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above.

[00370] In another embodiment, included herein are variant polypeptides which comprise, or alternatively consist of, an amino acid sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater % identity to, for example, the polypeptide sequences for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable fragment thereof. Particularly, the fragment comprises a minimal structural motif for the enzyme recognition site (S) or enzyme-reactive site (R) for the hydrolase enzymes described herein, e.g., lysozyme, elastase, cathepsin G, MAO, or

a combination thereof. Alternately or additionally, the fragment peptides are immunogenic molecules that can be recognized by antibodies or antigen-binding domains thereof.

[00371] Homologs

[00372] In another embodiment, included herein are homologs to the aforementioned peptides and polynucleotides. The term “homology,” as used herein, refers to a degree of complementarity. There may be partial homology or complete homology. The word “identity” may substitute for the word “homology.” A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to as “substantially homologous.” The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization, and the like) under conditions of reduced stringency. A substantially homologous sequence or hybridization probe will compete for and inhibit the binding of a completely homologous sequence to the target sequence under conditions of reduced stringency. This is not to say that conditions of reduced stringency are such that non-specific binding is permitted, as reduced stringency conditions require that the binding of two sequences to one another be a specific (i.e., a selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% homology or identity). In the absence of non-specific binding, the substantially homologous sequence or probe will not hybridize to the second non-complementary target sequence.

[00373] Mutants

[00374] In another embodiment, included herein are variant peptides comprising a mutation in the core polypeptide sequence for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable fragment thereof.

[00375] In one embodiment, the mutation is a substitution, deletion, addition of 1-3 amino acids. Preferably, the mutation does not change the enzyme recognition sites in the mutant peptides so formed. If the mutation results in a change in the composition of the recognition site or cleavage site, then it is contemplated that the mutation is due to a conserved amino acid substitution,

[00376] The words “insertion” or “addition,” as used herein, refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, to the sequence found in the naturally occurring molecule. A

“substitution,” as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

[00377] Antibodies

[00378] Embodiments disclosed herein further include antibodies which bind specifically to one or more of the aforementioned immunogenic peptides.

[00379] In one embodiment, the antibodies bind to polypeptides comprising the following amino acid sequences: X_yAAPX_y-Z (SEQ ID NO: 1), X_yAAPX_y-L-Z (SEQ ID NO: 2), $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3) or $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4); $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5) or $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above. In another embodiment, the antibodies bind to fragment of these polypeptides. Contemplated herein are antigen-binding fragments of such antibodies, e.g., F(ab) domain, F(ab)₂ domains, scFv domains, including synthetically generated antibodies (using, e.g., phase display technology).

[00380] In one embodiment, the antibodies bind to polypeptide sequences for ElaSub1_CBM, CatGSub1_CBM, ElaSub1_CBM His, CatGSub1_CBM His, ElaSub2_CBM, CatGSub2_CBM, ElaSub2_CBM His, CatGSub2_CBM His, ElaSub1_PDM, CatGSub1_PDM, ElaSub1_PDM His, CatGSub1_PDM His, ElaSub2_PDM, CatGSub2_PDM, ElaSub2_PDM His, CatGSub2_PDM or an enzyme-cleavable fragment thereof and/or an immunogenic fragment thereof. Contemplated herein are antigen-binding fragments of such antibodies, e.g., F(ab) domain, F(ab)₂ domains, scFv domains, including synthetically generated antibodies (using, e.g., phase display technology).

[00381] Purified molecules

[00382] Included herein are purified biomolecules, e.g., nucleic acids, proteins, peptides, and/or antibody molecules, including, conjugates thereof. The term “substantially purified,” as used herein, refers to nucleic acids, amino acids or antibodies that are removed from their natural environment and are isolated or separated, and are at least about 60% free, preferably about 75% free, and most preferably about 90% free from other components with which they are naturally associated.

[00383] In embodiments described herein, the biomolecules may be altered by combining with various components of the chemical entities, e.g., anchor region and/or indicator region, such that their form and/or functionality is significantly changed compared to any natural counterparts.

[00384] Methods of Making Compounds of Formula I:

[00385] Embodiments provided herein further relate to methods of making compounds of Formula I, including precursors thereof. The term “precursor” includes any compound which is employed as a reactant to generate an intermediary or a final product.

[00386] In one embodiment, provided herein is a method of making a compound of Formula I comprising the structure A–I, wherein, A is an anchor as described above and I is an indicator as described above, comprising, conjugating the anchor with the indicator molecule, *e.g.*, via covalent bond. In one embodiment, the anchor or the indicator may comprise a recognition site (S) or a reaction/labile site (R) for a wound-specific marker, *e.g.*, a wound-specific enzyme such as a hydrolase, and more specifically a protease or glycosidase, as described before. Under this embodiment, the substrate for the wound-specific marker comprises, for example, a hydrolysable substrate, *e.g.*, an amino acid, a sugar, a peptide, a polysaccharide, a nucleic acid, a lipid, or a combination thereof.

[00387] In one embodiment, the anchor is conjugated to the reporter molecule via a peptide linkage, a glycosidic linkage, an amide linkage, an ester linkage, an ether linkage, an anhydride linkage or a similar linkage. As used herein, a “peptide bond” is formed by the condensation reaction between two amino acids, wherein the acid moiety of one reacts with the amino moiety of the other to produce a peptide bond (–CO–NH–) between the two amino acids. In one embodiment, the peptide bond is cleaved with a wound-specific protease, *e.g.*, elastase, cathepsin G or MAO, or a combination thereof. As used herein, a “glycosidic bond” is formed between the hemiacetal or hemiketal group of a saccharide (or a molecule derived from a saccharide) and the hydroxyl group of some compound such as an alcohol. In one embodiment, the peptide bond is cleaved with a wound-specific glycosidase, *e.g.*, lysozyme.

[00388] Methods for conjugating reactive moieties to generate glycosidic, peptide, ester, oxyester, amide, amido, oxyamido, ether, sulfonyl, sulfinyl, sulfonamide, or other linkages such as alkoxy, alkylthio, alkylamino, etc. are known in the art and are further described in the examples.

[00389] In another embodiment, provided herein is a method of making a compound of Formula I comprising the structure A–I, wherein, A and I are each, as described previously.

[00390] In one embodiment, the A is conjugated to the I via a glycosidic linkage.

[00391] In another embodiment, the A is conjugated to the I via a hydrophilic or hydrophobic linkage.

[00392] In one embodiment, the compound of Formula I having the structure A–I is synthesized by first conjugating the anchor region A with the indicator region I to generate the compound of Formula I.

[00393] In another embodiment, the indicator is first synthesized via genetic recombinant technology, e.g., expressing a nucleic acid encoding the indicator region in a suitable host cell, and combining the indicator with the anchor region. Under this embodiment, in one instance, the indicator region is designed to contain nucleic acid sequences which bind to the anchor region, e.g., hydrophilically or hydrophobically. One representative example of a hydrophilic interaction comprises use of an anchor containing polar groups, e.g., partially deacetylated (e.g., DA of <30%) chitosan, cellulose or carboxymethyl cellulose (or a derivative thereof), which interacts with a hydrophilic carbohydrate binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*). Another representative example of a hydrophobic interaction comprises use of an anchor containing non-polar groups, e.g., polyethylene terephthalate (or a derivative thereof), which interacts with a or the hydrophobic binding module from *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDB).

[00394] In one embodiment, peptide indicators, e.g., polypeptides comprising the following amino acid sequences: $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP(V/F/A)}X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP(V/F/A)}X_y\text{-L-Z}$ (SEQ ID NO: 4); $X_yN^4N^3N^2N^1X_y\text{-Z}$ (SEQ ID NO: 5) or $X_yN^4N^3N^2N^1X_y\text{-L-Z}$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above, (including variant polypeptides) may be synthesized via host-cell expression systems. Such a method may comprise, for example, generating a construct encoding one or more of the aforementioned polypeptides or variants, placing said construct in a suitable vector, e.g., plasmid vector or baculovirus vector, transfecting a host cell, e.g., E coli or insect Hi5 cells, with the vector; culturing the host cells under suitable conditions to allow expression of said vector; and optionally purifying the expressed polypeptide from the culture.

[00395] In another embodiment, peptide indicators, e.g., polypeptides comprising the following amino acid sequences: $X_y\text{AAP}X_y\text{-Z}$ (SEQ ID NO: 1), $X_y\text{AAP}X_y\text{-L-Z}$ (SEQ ID NO: 2), $X_y\text{AAP(V/F/A)}X_y\text{-Z}$ (SEQ ID NO: 3) or $X_y\text{AAP(V/F/A)}X_y\text{-L-Z}$ (SEQ ID NO: 4); $X_yN^4N^3N^2N^1X_y\text{-Z}$ (SEQ ID NO: 5) or $X_yN^4N^3N^2N^1X_y\text{-L-Z}$ (SEQ ID NO: 6), wherein X, N1, N2, N3, N4, L and Z are each, as described above (including variant polypeptides) may be synthesized using solid-phase peptide synthesis (see, Merrifield et al., *J. Am. Chem. Soc.* 85 (14): 2149–2154).

[00396] Still further, the compound of Formula I having the structure A–I may be synthesized in a single reaction chamber or multiple reaction chambers.

[00397] Diagnostic and Therapeutic Methods:

[00398] In one embodiment, the compositions, dressing materials, articles, kits and systems described herein are useful in diagnosing or treating wounds, particularly chronic or infected

wounds. Although any type of wound may be diagnosed and/or treated, the embodiments are particularly suitable for diagnosing and treating wounds that exude wound fluid. For example, the wound may be a chronic or acute wound. Representative examples of chronic wounds include, *e.g.*, venous ulcers, pressure sores, decubitis ulcers, diabetic ulcers and chronic ulcers of unknown aetiology. Representative examples of acute wounds include, *e.g.*, acute traumatic laceration, perhaps resulting from an intentional operative incision.

[00399] As used herein, the term “a wound fluid” refers to any wound exudate or other fluid (suitably substantially not including blood) that is present at the surface of the wound, or that is removed from the wound surface by aspiration, absorption or washing. The determining, measuring or quantifying is suitably carried out on wound fluid that has been removed from the body of the patient, but can also be performed on wound fluid *in situ*. The term “wound fluid” does not normally refer to blood or tissue plasma remote from the wound site. The wound fluid is mammalian wound fluid, suitably human wound fluid.

[00400] In one embodiment, the diagnostic method comprises contacting a wound with at least one composition comprising a compound of Formula I or Formula II, a dressing material comprising such compounds, article comprising such materials or compounds, kits comprising such materials or compounds, or a system comprising such materials or compounds described herein; and measuring a parameter associated with the wound. In a specific embodiment, the parameter being measured is a level or activity of a wound-specific hydrolase. Particularly, the parameter being measured is the activity of the hydrolase.

[00401] In the aforementioned embodiments, the measurement may either be made *in situ* or *ex situ*. As used herein, the term “*in situ*” refers to processes, events, objects, or components that are present or take place within the context of the system or device, including, the surrounding environment, for example, the biological material with which the composition, article, system or device is in contact with. As an example, an *in situ* reaction may refer to the reaction of the various components present in the device (*e.g.*, compound of Formula I or Formula II), including, components provided by the human skin tissue (*e.g.*, wound exudate containing the enzyme). The term is contrasted with *ex situ*, which refers to outside of the environment.

[00402] In a second embodiment, the measurement is performed *ex situ*, *e.g.*, removing the fluid from the wound for analysis in the apparatus or device of the disclosed technology.

[00403] Suitably, the measurement is made *in situ*.

[00404] In one diagnostic embodiment, the method comprising determining a level of a reporter, *e.g.*, a product of a substrate acted upon by a wound-specific enzyme. More specifically, the method comprises determining a level of a hydrolase enzyme product. As used herein, the term “determining” includes measuring a numerical value of the activity or level of

said hydrolase; establishing if the activity or level falls above or below a predetermined range; and/or comparing the numerical value of activity or level with a control standard. The control standard may comprise determining a level or activity of the hydrolase in a biopsy material obtained from an unwounded site or from a healthy subject.

[00405] In one specific embodiment, the term “determining” comprises measuring the parameter (*e.g.*, activity or level) of at least one wound specific protease is selected from the group consisting of MMP-1 (collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), human neutrophil elastase (HNE), cathepsin G, urokinase-type plasminogen activator (uPA), and lysozyme, or a combination thereof; establishing if said parameter exceeds a first predetermined threshold; and/or comparing the numerical value of parameter with a control standard. The control standard may comprise determining a parameter of the protease in a biopsy material obtained from an unwounded site or from a healthy subject. In related embodiments, the term “determining” comprises establishing whether a weighted average (weighted sum) of the parameters associated with a plurality of the aforementioned proteases exceeds a predetermined threshold value for said weighted average.

[00406] In one particular embodiment, the parameter is activity level of the analyte (*e.g.* a protease) in a wound fluid. Typically, the activity of an individual analyte is expressed in terms units/mL.

[00407] In another embodiment, the parameter is the level of the analyte (*e.g.*, protease) in a wound fluid. Typically, the term amount is also indicative of the activity of a particular analyte.

[00408] When used herein, the term “combined amount” or “combined activity” refers to a single numerical value that results from the application of a mathematical function to a plurality of values, for example those amounts obtained for a number of individual analytes. For example, the term “combined amount” or “combined activity” may refer to the sum or product of a group of individual values. Typically, the term “combined amount” or “combined activity” relates to the sum of a group of individual values. For example, in suitable embodiments, the amount of elastase refers to elastase-like activity (*e.g.*, U/mL) and the amount of metalloproteinase (MMP) refers to total concentration of the respective analyte (*e.g.*, in ng/mL).

[00409] When used herein, the term “quantifying” refers to measuring an absolute numerical quantity of a particular analyte(s) or substrate(s) in a sample, within the margins of experimental error.

[00410] The term “marker” or “analyte” refers to any chemical entity that is identified or determined using the apparatus, devices, kits or methods defined herein. The markers or analytes determined or identified by the apparatus, devices, kits or methods of the disclosed technology are cleaved products of the aforementioned enzymes.

[00411] When used herein, the term “predetermined range” refers to a data range or profile that the skilled person would understand is indicative of a particular sub-class of patient. For instance, the predetermined range may be a data range or profile that is typical of a wound that would respond well to a particular wound treatment, such as antibiotic therapy. Alternatively, the predetermined range may suitably refer to a data range that is typical of a wound that would not respond well to a particular wound treatment, such as antibiotic therapy.

[00412] When used herein, the term “predetermined threshold” refers to a minimum level that the skilled person would determine is indicative of a non-healing wound based on statistical analysis of levels determined for known healing and non-healing wounds, for example as explained further above. For the test to be clinically useful, the threshold should be set at an appropriate level so that non-healing wounds with high protease activity are correctly identified. Increasing the threshold will increase the chance of only non-healing wounds being over the threshold. However, if the threshold is too high, wounds that are non-healing due to a high level of proteases would not be identified and clinically this would mean they would not receive the required protease modulating treatment.

[00413] When used herein, the term “control standard” or “control” refers to a data set or profile that can be used as a reference or comparison in order to define or normalize another data point or set of data. For instance, the term “control” or “control standard” may be data set or profile that is indicative of a particular sub-class of patient. Suitably, the control standard may be a data set or profile indicative of healing or non-healing wound status.

[00414] Suitably, in other aspects or embodiments of the disclosed technology, the “control” or “control standard” can be a data set or profile that can be used as a comparative tool to allow a skilled person to determine whether a wound is likely to be responsive or non-responsive to a wound treatment, such as antibiotic therapy. In one embodiment, the control standard is a data set or profile indicative of a patient that does not respond well to wound treatment. Typically, the control standard is a data set or profile indicative of a patient that responds well to wound treatment. Patients that tend to respond well to wound treatment as disclosed herein exhibit lower combined amount or activity of hydrolases than patients that tend not to respond well to the treatment. For example, patients that tend to respond well to wound treatment as disclosed herein exhibit lower combined amounts of at least one wound-specific hydrolase.

[00415] In one embodiment, the threshold human neutrophil elastase activity is about 5 U/mL to about 30 U/mL, including all values in between, *e.g.*, about 6 U/mL, about 7 U/mL, about 8 U/mL, about 9 U/mL, about 10 U/mL, about 11 U/mL, about 12 U/mL, about 13 U/mL, about 14 U/mL, about 15 U/mL, about 16 U/mL, about 17 U/mL, about 18 U/mL, about 19 U/mL,

about 20 U/mL, about 21 U/mL, about 22 U/mL, about 23 U/mL, about 24 U/mL, about 25 U/mL, or more, indicate chronic wound infection.

[00416] In one specific embodiment, the threshold human neutrophil elastase activity levels of at least 9.6 indicate chronic wound infection. In some embodiments, human neutrophil elastase activity levels of at least 22.9 U/mL indicate chronic wound infection.

[00417] In one embodiment, the threshold lysozyme activity levels of about 1000 U/mL to about 10000 U/mL, including all values in between, *e.g.*, about 1100 U/mL, about 1200 U/mL, about 1300 U/mL, about 1400 U/mL, about 1500 U/mL, about 1600 U/mL, about 1700 U/mL, about 1800 U/mL, about 1900 U/mL, about 2000 U/mL, about 2100 U/mL, about 2200 U/mL, about 2300 U/mL, about 2400 U/mL, about 2500 U/mL, about 2600 U/mL, about 2700 U/mL, about 2800 U/mL, about 2900 U/mL, about 3000 U/mL, about 3250 U/mL, about 3500 U/mL, about 3750 U/mL, about 4000 U/mL, about 4250 U/mL, about 4500 U/mL, about 4750 U/mL, about 5000 U/mL, about 5250 U/mL, about 5500 U/mL, about 5750 U/mL, about 6000 U/mL, or more, indicate chronic wound infection. In one specific embodiment, lysozyme activity levels of at least 4800 U/mL indicate chronic wound infection.

[00418] In one embodiment, the threshold cathepsin G activity levels of about 10 U/mL to about 100 U/mL, including all values in between, *e.g.*, about 15 U/mL, about 20 U/mL, about 25 U/mL, about 30 U/mL, about 35 U/mL, about 40 U/mL, about 45 U/mL, about 50 U/mL, about 55 U/mL, about 60 U/mL, about 65 U/mL, about 70 U/mL, about 75 U/mL, about 80 U/mL, about 85 U/mL, about 90 U/mL, about 95 U/mL, about 100 U/mL, about 110 U/mL, about 120 U/mL, or more, indicate chronic wound infection. In some embodiments, cathepsin G activity levels of at least 50 U/mL, at least 40 U/mL, at least 30 U/mL, at least 20 U/mL, at least 15 U/mL or at least 10 U/mL indicates chronic wound infection.

[00419] Embodiments disclosed herein further relate to treatment of chronic or infected wounds using the compositions, materials, articles, dressings, kits and/or systems described herein. The therapeutic embodiment includes, contacting a composition, material, article, dressing, kit, system or devices of the disclosed technology with a subject in need thereof. Optionally, the method may include determination of whether the subject is responding to the treatment.

[00420] The skilled person would be able to easily identify whether wounds are “responsive to treatment” or not. In particular, the skilled person will readily be able to determine the levels of the proteases identified in the present claims that are predictive or indicative of a good response or poor response to wound treatment, particularly to treatment with wound dressings comprising oxidized cellulose. The terms “responsive” and “responder(s)” as used herein refer to wounds that are considered to respond well to wound treatment, particularly to treatment with

a pharmacological agent, *e.g.*, antibiotics. Similarly, “non-responsive” and “non-responder(s)” refers to wounds that are not considered to respond well to wound treatment, particularly to treatment with the pharmacological agent, *e.g.*, antibiotics. For instance, patients who exhibit better than 50% wound closure after 4 weeks of wound treatment are considered to be responsive to said treatment.

[00421] In certain embodiments, a patient may be simultaneously diagnosed and treated with the compositions, articles, systems, or devices described herein. When used herein, the term “simultaneously” means performing the stated objectives, *e.g.*, diagnosis and treatment, together.

[00422] In certain embodiments, a patient may be sequentially diagnosed and treated with the compositions, articles, systems, or devices described herein. When used herein, the term “sequentially” means the stated objectives, *e.g.*, diagnosis and treatment, are temporally or spatially separated, *e.g.*, diagnosis prior to treatment or diagnosis following treatment or a combination thereof, *e.g.*, 1st diagnosis==>treatment==>2nd diagnosis.

[00423] Embodiments described herein further enable a care giver or a patient to determine quickly and reliably whether a wound is likely to be non-healing, and to select an appropriate therapy based on this determination. For example, non-healing wounds may require the application of special wound dressings such as wound dressings comprising specific therapeutic agents, to promote healing. Accordingly, embodiments described herein further provide methods of treatment of a wound, *e.g.*, chronic or infected wounds, comprising determining whether a wound is healing or non-healing, followed by applying a wound dressing comprising a therapeutic agent to the wound if it is non-healing.

[00424] Embodiments described herein provide methods and assays for diagnosis or detection of infected wounds. The methods are suitable for the detection of bacterial infectious agents. In one embodiment, the wounds are infected with gram-negative bacteria. Typical gram-negative bacteria include proteobacteria such as *E. coli*, *Salmonella*, *Pseudomonas*, and *Helicobacter*, and *cyanobacteria*. When classified in connection with medicine, they include *Pseudomonas aeruginosa* and *Hemophilus influenzae* causing the disturbance of the respiratory system, *Escherichia coli* and *Proteus mirabilis* causing the disturbance of the urinary system, and *Helicobacter pylori* and *Bacillus Gaertner* causing the disturbance of the alimentary system and micrococci such as *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Neisseria gonorrhea*.

[00425] In another embodiment, the wounds are infected with gram-positive bacteria. By “gram-positive bacteria” is meant a bacterium or bacteria that contain(s) teichoic acid (*e.g.*, lipoteichoic acid and/or wall teichoic acid), or a functionally equivalent glycopolymer (*e.g.*, a rhamnopolysaccharide, teichuronic acid, arabinogalactan, lipomannan, and lipoarabinomannan)

in its cell wall. Non-limiting examples of functionally equivalent glycopolymers are described in Weidenmaier *et al.*, *Nature*, 6:276-287, 2008.

[00426] The bacteria include pathogenic bacteria that infect mammalian hosts (*e.g.*, bovine, murine, equine, primate, feline, canine, and human hosts). Examples of such pathogenic bacteria include, *e.g.*, members of a bacterial species such as *Bacteroides*, *Clostridium*, *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Haemophilus*, *Legionella*, *Mycobacterium*, *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, or *Listeria*. Some clinically relevant examples of pathogenic bacteria that cause disease in a human host include, but are not limited to, *Bacillus anthracis*, *Bacillus cereus*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, vancomycin-resistant *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli*, *E. coli* O157:H7, *Francisella tularensis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Legionella pneumophila*, *Leptospira interrogans*, *Listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Proteus*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Staphylococcus saprophyticus*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VSA), *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum*, *Vibrio cholerae*, and *Yersinia pestis*.

[00427] In another embodiment, the infectious bacteria is selected from the group consisting of *Clostridium difficile*, Carbapenem-Resistant *Enterobacteriaceae* (CR-*Klebsiella* spp; CR-*E. coli*), and *Neisseria gonorrhoeae*. In another embodiment, the infectious bacteria is selected from the group consisting of multidrug-resistant *Acinetobacter*, drug-resistant *Campylobacter*, extended spectrum β -Lactamase (ESBL)-producing *enterobacteriaceae*, vancomycin-resistant *enterococcus*, multidrug-resistant *pseudomonas aeruginosa*, drug-resistant non-typhoidal *Salmonella*, drug-resistant *Salmonella enterica* serovar *Typhi*, drug-resistant *Shigella*, methicillin-resistant *Staphylococcus aureus* (MRSA), drug-resistant *Streptococcus pneumoniae*, and drug-resistant *Tuberculosis*. In another embodiment, the infectious bacteria is selected from the group consisting of vancomycin-resistant *Staphylococcus aureus*, erythromycin-resistant Group A *Streptococcus*, clindamycin-Resistant Group B *Streptococcus*.

[00428] In certain embodiments, the chronic or infected wounds are found in host subjects. Preferably, the hosts are mammals, *e.g.*, a rodent, a human, a livestock animal, a companion

animal, or a non-domesticated or wild animal. In one embodiment, the subject may be a rodent, *e.g.* a mouse, a rat, a guinea pig, etc. In another embodiment, the subject may be a livestock animal. Non-limiting examples of suitable livestock animals may include pigs, cows, horses, goats, sheep, llamas and alpacas. In still another embodiment, the subject may be a companion animal. Non-limiting examples of companion animals may include pets such as dogs, cats, rabbits, and birds. In yet another embodiment, the subject may be a zoo animal. As used herein, a “zoo animal” refers to an animal that may be found in a zoo. Such animals may include non-human primates, large cats, wolves, and bears. In an exemplary embodiment, the subject is a human.

[00429] In one aspect, provided herein are methods of detecting levels of one or more enzymes in a mammalian wound, the method comprising the steps of: (a) placing the wound dressing material described herein in contact with the mammalian wound; (b) visually comparing the wound dressing material in contact with the mammalian wound with one or more reference samples; and (c) obtaining a qualitative determination of the concentration of reporter molecules in the wound dressing material in contact with the mammalian wound.

[00430] Preferably, the diagnosis and treatment is conducted *in situ*. Embodiments described herein therefore allow diagnosis and treatment of wounds in an easy, non-invasive manner. For instance, the diagnosis may be made in real time and the treatment may be applied to the infected wound or to the patient (systemically) and the progress of wound treatment be monitored over real-time, *e.g.*, dissipation of the signal generated by the reporter molecule due to wound-healing.

[00431] In another aspect, provided herein are methods of detecting protease activity in wounds using a chemical entity, wherein the chemical entity comprises one or more components selected from the group consisting of: an anchor region, an enzyme-labile or enzyme-reactive region, and an indicator region. In another aspect, the method comprises placing substrates for MPO, elastase, lysozyme, phospholipase, and catalase on a solid surface such that any reaction is visible to the eye. In another aspect, the method serves to assess a variety of body fluids including wound, tear, vitreal, CSF, airway aspirates or sputum, synovial, blood, plasma, serum, urine, peritoneal, interstitial, subcutaneous, bile, intestinal or similar fluids, via contacting them with a material containing the substrates and assessing the change of the substrates thereafter.

EXAMPLES

[00432] The structures, materials, compositions, and methods described herein are intended to be representative examples, and it will be understood that the scope of the disclosure is not limited by the scope of the examples. Those skilled in the art will recognize that the

embodiments and disclosed technology may be practiced with variations on the disclosed structures, materials, compositions and methods, and such variations are regarded as within the ambit of the disclosure.

[00433] Example 1: Purification of chitosan

[00434] Chitosan (10 g, from shrimp shells) was dissolved in 2% acetic acid solution (1 L). The solution was stirred overnight at room temperature and afterwards filtered using a nylon filter (0.45 μm). Subsequently the pH was adjusted to 8 by the addition of 4M NaOH to precipitate chitosan. The obtained precipitate was isolated by centrifugation (10000 rpm, 10 min) and thoroughly washed with distilled water until the pH of the washing solution reached ~ 7 . Afterwards the precipitate was washed with 90% ethanol, the remaining ethanol was allowed to evaporate and the chitosan was freeze-dried. The purified product was analyzed by FTIR (1560 cm^{-1} , 1640 cm^{-1}) and material with an approximate DA of 48% was used further

[00435] Example 2: Selective *N*-acetylation of chitosan

[00436] Purified chitosan (example 1) was dissolved in 10% acetic acid solution to obtain a 1% chitosan solution. An equal volume of 96% Ethanol was added as well as acetic anhydride. The reaction was stirred for 1 h before the pH was adjusted to 7. The precipitate was isolated by centrifugation and freeze-dried. Subsequently, the obtained acetylated chitosan was several times washed with distilled water and again freeze-dried. The degree of *N*-acetylation (DA) was analyzed by ^1H -NMR and FTIR (1560 cm^{-1} , 1640 cm^{-1}) and found to be 40-60% as indicated in the graphic below. Chitosan derivatives with varying DA were produced, but only material with a DA of 48% was further used

[00437] Example 3: Conjugation of reactive black 5 onto acetyl-chitosan

[00438] Acetylated chitosan (DA = 48%, 100 mg; Example 2) was suspended in distilled water before a 0.5% (w/w) solution of reactive black 5 was added (0.5 mL). A solution consisting of 2.5% (w/v) Na_2SO_4 and 1% (w/v) Na_2CO_3 in distilled water was added, and the mixture was incubated at 25 $^\circ\text{C}$ for 10 min. After a subsequent incubation step at 65 $^\circ\text{C}$, the solid was isolated by centrifugation (7800 rpm, 5 min). The precipitate was washed with distilled water until the washing solution remained colorless and subsequently freeze dried. The dye content was determined measuring the unbound reactive black 5 after the reaction was completed. The dye was largely associated with the precipitate and high MW fraction. Degree of acylation was 48%.

Percent dye solution applied in the reaction	Dye content [% w/w]
0.4	4.58
0.2	2.55
0.1	1.33

[00439] Example 4: Hydrolysis of chitosan to obtain chitooligosaccharides

[00440] Purified chitosan (2.5 g; Example 1) was dissolved in acetate buffer (100 mM, pH 5) to obtain a 1% chitosan solution. Afterwards chitosanase from *Streptomyces griseus* (1 unit) was added and the reaction mixture was stirred for 5 d on a thermomixer (37 °C, 150 rpm). The solution was concentrated *in vacuo* and non-oligomeric chitosan was precipitated using 1 volumetric equivalent of 96% ethanol. The supernatant was concentrated again and subsequently oligosaccharides were precipitated by the addition of 9 volumetric equivalents of acetone. The oligosaccharides were isolated by centrifugation, several times washed with 50% acetone in water, and freeze-dried without prior washing. The degree of polymerization was determined by TLC and SEC (TSK gel G5000 PWXL, Pullulan as standard), indicating a mean MW of 5360 associated to a degree of polymerization of ca. 24.

Number molecular weight (Mn)	Polydispersity Index (PDI)	Degree of polymerization (DP) expected from SEC data
5360	2.51	~ 30

[00441] Example 5: Selective N-acetylation of chitooligosaccharides

[00442] A chitooligosaccharide mixture (7 g) (example 4) was dissolved in distilled water (200 mL) before 96% ethanol (400 mL) was added. The solution was stirred for 5 min and acetic anhydride (4.12 mL, 1 molar equiv. calc. to free amines) was added. The mixture was stirred for further 2 h at room temperature before the pH was adjusted to 7 with a 10% NaOH solution. The solvent was removed, and the remaining precipitate was freeze-dried. The degree of N-acetylation was analyzed by ¹H-NMR, FTIR (1560 cm⁻¹, 1640 cm⁻¹) and dye content (photometrically, 626 nm). The degree of N-acetylation was found to be 48 %.

[00443] Example 6: Conjugation of Toluidine O Blue onto chitooligosaccharides

[00444] Acetylated chitooligosaccharides (DA = 48 %, 7 g; Example 5) were dispersed in 1% acetic acid (500 mL). Afterwards toluidine O blue (3.9 g) was added and allowed to dissolve before 1ml of a solution of glutaraldehyde was added. The mixture was stirred for 2 h and the pH was adjusted to 7 using 10% NaOH. The solid fraction was isolated by filtration, several times washed with distilled water, and freeze-dried. The degree of N-acetylation was analyzed

by ^1H -NMR, FTIR and photometrically. Degree of acetylation was found to be 48%. Dye content was found to be 0.74.

[00445] Lysozyme digestion of dyed chitosan derivatives. The synthesized substrates were investigated in different media: potassium phosphate buffer (66 mM, pH 6.2) as well as artificial wound fluid containing 5000 U of lysozyme from hen egg white, and human wound fluid from infected wounds (see fig. 5 and 6).

[00446] Two milligrams of the lysozyme substrates was suspended in the respective test medium and incubated at 35 °C. For time measurements, the sample was briefly centrifuged, 200 μL of the supernatant was transferred to a 96-well plate and photometrically analyzed at the absorption maximum of the respective dye. After the analysis, the withdrawn sample was returned in the reaction vial and further incubated.

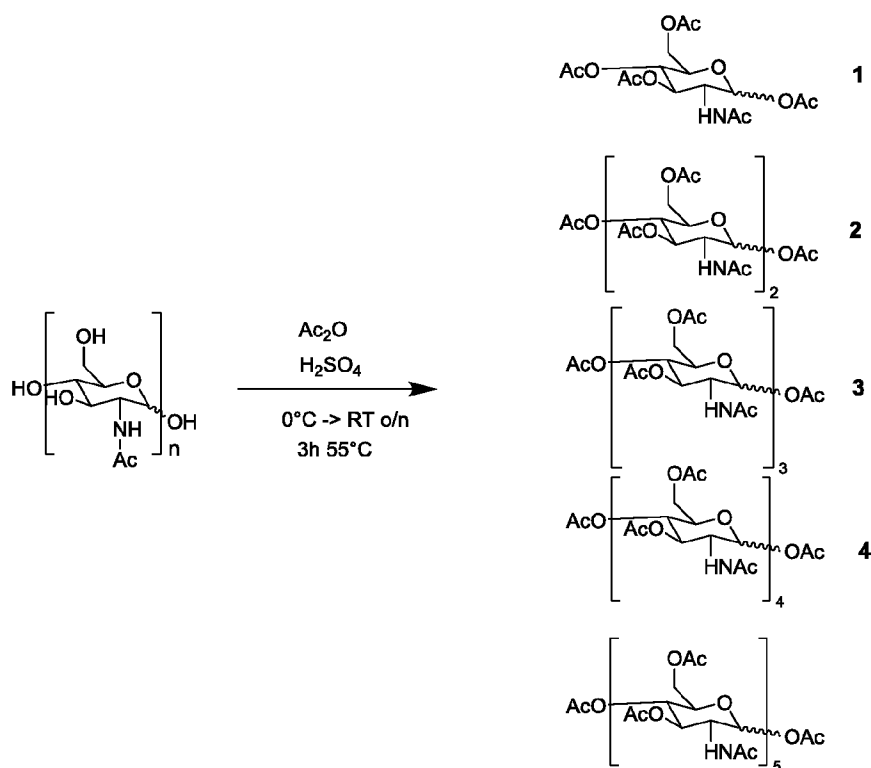
[00447] The composition of the artificial wound fluid contained human serum albumin (2%), sodium chloride (0.36%), sodium bicarbonate, (0.05%), sodium citrate (0.02%), sodium lactate (0.1%), glucose (0.1%), calcium chloride dihydrate (0.01%), magnesium chloride (0.02%), and urea (0.01%).

[00448] **Example 7: Synthesis of Indol/chitooligomer based substrates for the detection of wound infection.**

[00449] Synthesis of a $(\text{GlcNAc})_n$ – Indol ($n = 4-6$) Acetolysis of chitin

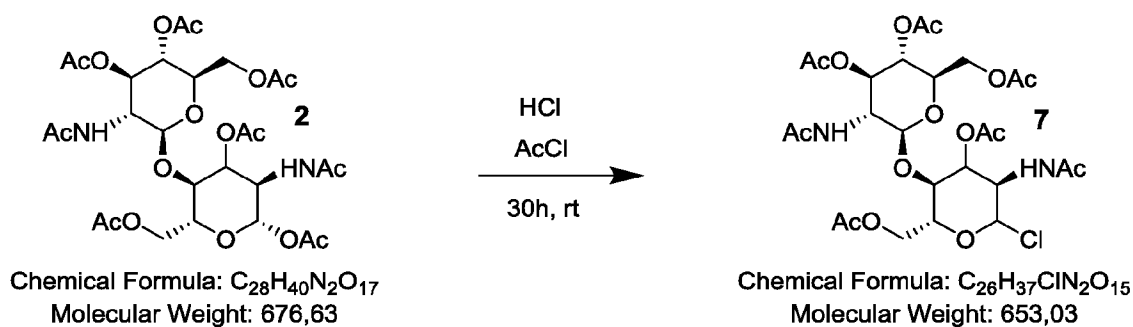
[00450] Chitin (2 g) was suspended in cooled acetic anhydride (20 mL) and conc. sulfuric acid (2,3 mL) is added dropwise. The suspension was stirred overnight and allowed to reach room temperature. The solution was neutralized with sodium acetate (12 g) after dilution with ice cold water. The solution was filtered and the filtrate was extracted with chloroform. The chloroform phase was washed with bicarbonate, dried over magnesium sulfate and concentrated *in vacuo*.

[00451] Resulting chito oligosaccharides (COSs) of varying degree of polymerization (DP) were fractionized by MPLC (SiO_2 , 40g) using a chloroform:ethanol gradient (98:2 $\rightarrow$ 90:10).



[00452] **Example 8: 1-chloro,3-O,6-O-Diacetyl-4-O-[3-O,4-O,6-O-triacetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranosyl]-2-(acetylamino)-2-deoxy- α -D-glucopyranoside (compound 7)**

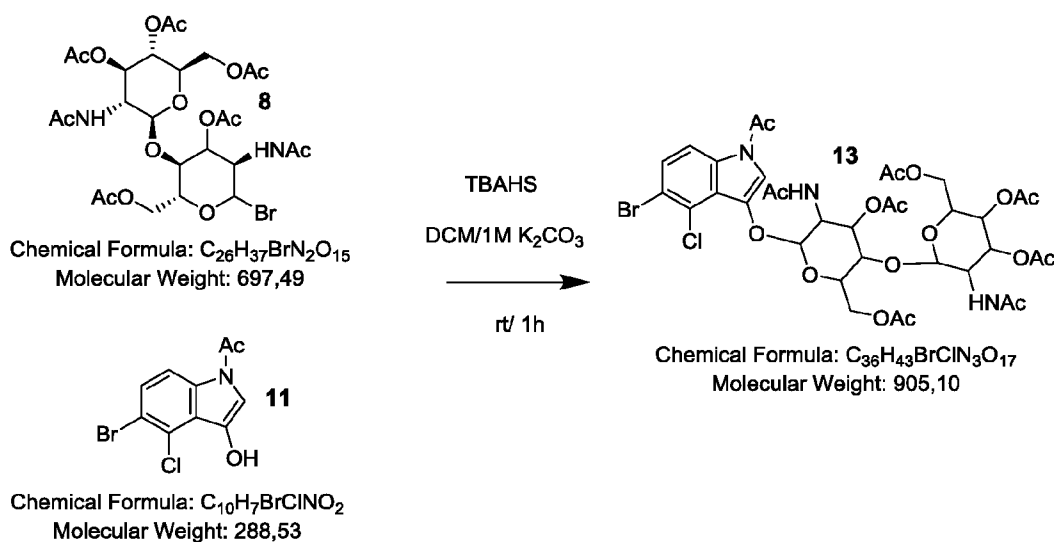
[00453] Chitobiose octaacetate 2 (20 mg) was suspended in acetyl chloride and the solution was saturated with HCl gas and stirred for 30 h. Afterwards the solution was evaporated and used without further purification.



[00454] **Example 9: 1-(5-Bromo-4-chloro-N-acetyl-3-indolyl),3-O,6-O-Diacetyl-4-O-[3-O,4-O,6-O-triacetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranosyl]-2-(acetylamino)-2-deoxy- α -D-glucopyranoside (compound 13)**

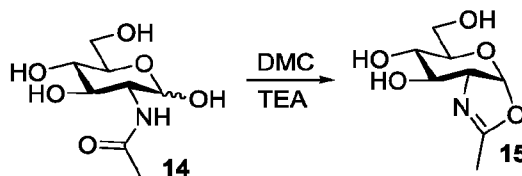
[00455] Compound 7 (0,028 mmol) was dissolved in DCM (2.5 mL), 11 (82.87 mg) was added and dissolved. Afterwards of tetrabutyl-ammonium hydrogen sulphate (9.7 mg) and 1 mL of 1 M potassium carbonate solution were added. Under vigorous stirring the reaction was left for 1 h at room temperature. The organic phase was separated and the solvent was removed under pressure.

[00456] A 12 g MPLC column was used for the separation with a gradient of pure chloroform to 15 parts chloroform to one part ethanol over 10 column volumes. Then 5 column volumes 15 parts chloroform to one part ethanol were used isocratic. The final flushing step was 10 parts chloroform to one part ethanol for 5 column volumes.



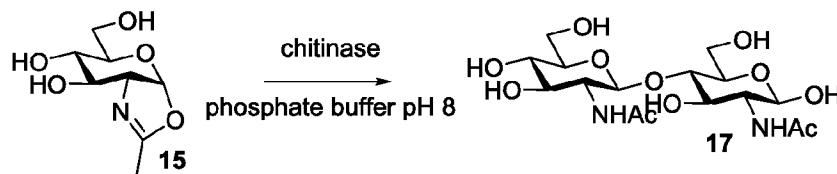
[00457] Example 10: N-Acetylglucosamine (GlcNAc) oxazoline 15

[00458] GlcNAc 14 (100 mg) was dissolved in H_2O (1,8 mL) and triethylamine (0,6 mL) was added. The solution was chilled in an ice bath prior to the addition of 2-chloro-1, 3-dimethylimidazolinium chloride (DMC, 230 mg). The solution was stirred for 30 min. DMC and TEA were removed by MPLC (C18) using H_2O as eluent. The product fractions were pooled and concentrated *in vacuo*.



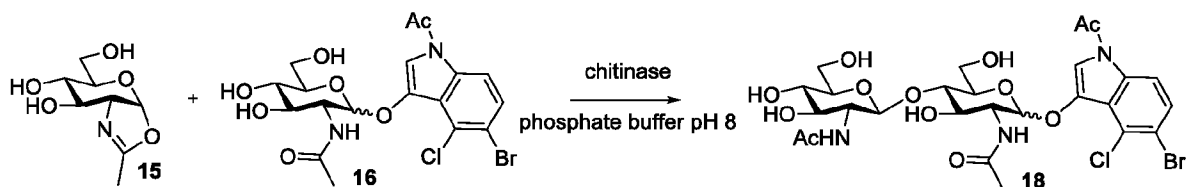
[00459] GlcNAc dimer (compound 17)

[00460] Compound **15** (27 mg) was dissolved in a sodium phosphate buffer (50 mM, pH 8,0) and chitinase (25 mU/mL) was added. The solution was incubated and the reaction progress was monitored by TLC (CHCL₃:MeOH 2:1).

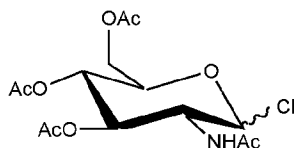


[00461] (GlcNAc)₂ – Indol (compound **18**)

[00462] Compound **15** (27 mg) and GlcNAc – Indol **16** were dissolved in a sodium phosphate buffer (50 mM, pH 8,0) and chitinase (25 mU/mL) was added. The solution was incubated and the reaction progress was monitored by TLC (CHCL₃:MeOH 2:1).

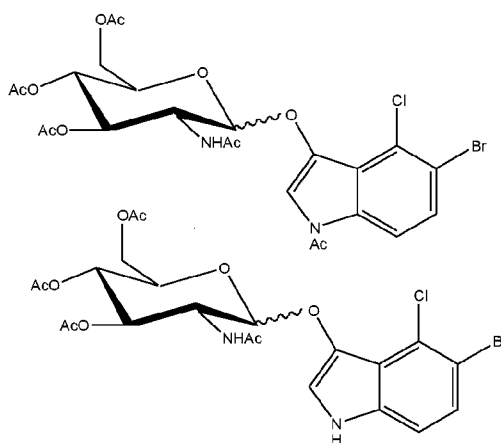


[00463] Example 11: 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-glucopyranosyl chloride



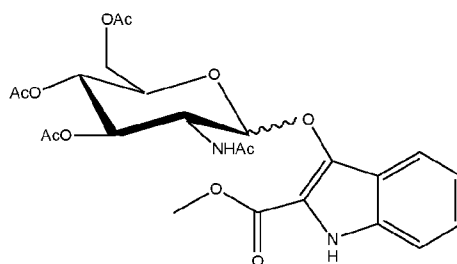
[00464] *N*-Acetyl- β -D-glucosamine tetraacetate (391 mg, 1.00 mmol) was suspended in acetyl chloride (7 mL) while cooling with an ice bath. The mixture was degassed with argon for 5 min. Afterwards MeOH (1.00 mL) was added dropwise over a period of two hours. During the first 15 min of MeOH addition the reaction mixture was degassed with argon; then it was kept under argon atmosphere (stirred in ice bath all the time). After complete addition of the MeOH the reaction mixture was stirred additional 10 min with cooling in an ice bath. Afterwards it was allowed to warm to RT and was stirred at RT overnight. The mixture was concentrated to dryness, taken up in DCM (10 mL), concentrated to dryness again, taken up in diisopropylether (15 mL) and concentrated to dryness once more to yield a yellow solid which was used without further purification.

[00465] **Example 12: (*N*-Acetyl-5-bromo-4-chloro-indol-3-yl) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*D*-glucopyranoside (12a) and (5-Bromo-4-chloro-indol-3-yl) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*D*-glucopyranoside (12b)**



[00466] DMF (dry, 4 mL) was degassed with argon for 5 min before 1-Acetyl-5-bromo-4-chloro-3-indolylacetate (100 mg, 0.30 mmol) was added. The mixture was degassed with argon for additional 5 min, then NaOMe (51 mg, 0.94 mmol) was added in one portion. Stirring at RT while degassing with argon was continued for 25 min, afterwards the 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*D*-glucopyranosyl chloride (110 mg, 0.30 mmol) was added in one portion. The reaction mixture was stirred at RT under protection from light (aluminium-foil) while degassing with argon for 1 h, then it was stirred under argon atmosphere at RT under protection from light overnight. The mixture was concentrated to dryness and co-evaporated with toluene (3 x 20 mL). Afterwards it was taken up in EtOAc (30 mL) and was filtered. The filtrate was concentrated to dryness to yield 150 mg of the crude products. ESI-MS (positive): $[M+Na]^+$: 597 (14b) and $[M+Na]^+$: 639 (12a).

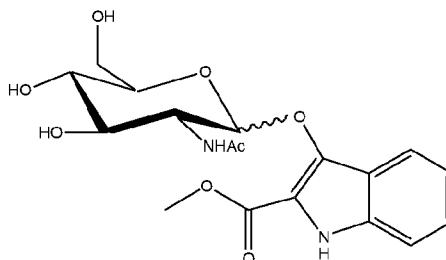
[00467] **Example 13: (Indox-3-yllic acid methyl ester) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*D*-glucopyranoside**



[00468] 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*D*-glucopyranosyl chloride (1.00 mmol, Example 13), TBAHS (339 mg, 1.00 mmol) and methyl-3-hydroxy-1 *H*-indole-2-carboxylate (210 mg, 1.10 mmol) were dissolved in DCM (dry, 10 mL) at RT. A K_2CO_3 -solution (12 mL) was then added in one portion and the mixture was stirred at RT for 2.5 h. DCM (20 mL) and

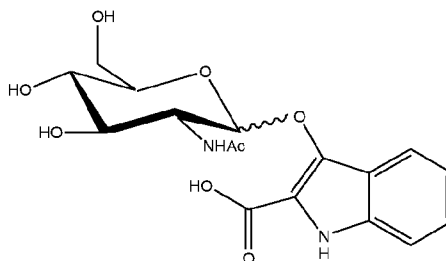
water (20 mL) were added. After extraction the organic phase was dried (Na_2SO_4) and concentrated to dryness. The crude product was purified by column chromatography (30.0 g silica gel, eluent: 5 % MeOH in DCM) 250 mg of the pure product and 92 mg of the product with impurities were isolated. ESI-MS (positive): $[\text{M}+\text{Na}]^+$: 543, $[\text{M}+\text{K}]^+$: 559.

[00469] Example 14: (Indox-3-yllic acid methyl ester) 2-acetamido-2-deoxy-D-glucopyranoside



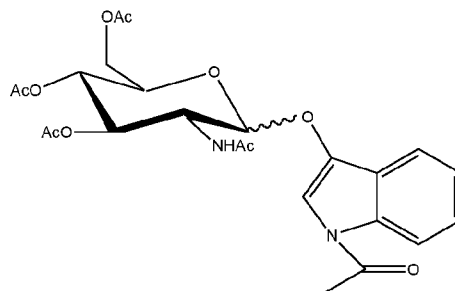
(Indox-3-yllic acid methyl ester) 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-glucopyranoside (250 mg, 0.48 mmol) was suspended in MeOH (7 mL) at RT. NaOMe (catalytic amount) was added and the mixture was stirred at RT for 1 h 45 min. The precipitate was filtered off. Since MS of precipitate and filtrate were identical they were combined again and concentrated to dryness. The product was suspended in DCM/MeOH (9:1, 10mL) and kept in the fridge for 3h. The colorless solid was filtered off to yield 90 mg of a colorless solid. The filtrate was concentrated to dryness again to yield 105 mg of a brownish solid. Both, solid and filtrate were used without further purification. ESI-MS (positive): $[\text{M}+\text{Na}]^+$: 417.

[00470] Example 15: (Indox-3-yllic acid) 2-acetamido-2-deoxy-D-glucopyranoside



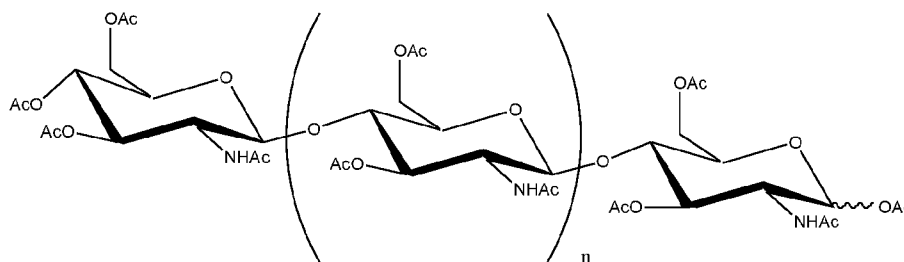
[00471] (Indox-3-yllic acid methyl ester) 2-acetamido-2-deoxy-D-glucopyranoside (105 mg, 0.25 mmol) was suspended in a NaOH-solution (0.1 M in water, 10 mL) and stirred at RT for 3.5 h. Reaction mixture was concentrated to dryness and the product was used without further purification. ESI-MS (positive): $[\text{M}+\text{Na}]^+$: 403.

[00472] Example 16: (N-Acetyl-indol-3-yl) 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside

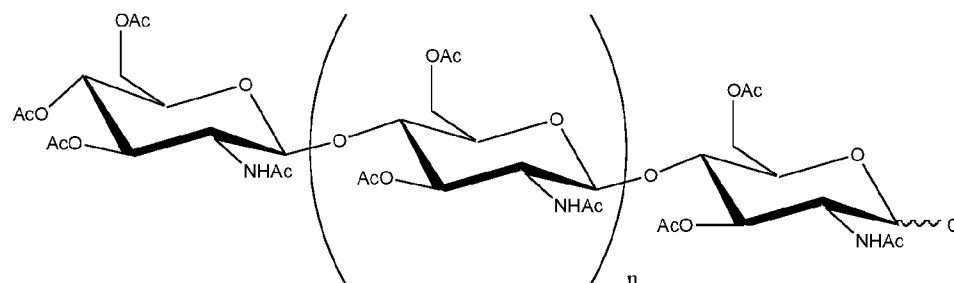


[00473] (Indox-3-yllic acid) 2-acetamido-2-deoxy-D-glucopyranoside (105 mg, 0.25 mmol), AgOAc (125 mg, 0.75 mmol), K₂CO₃ (207 mg, 1.50 mmol), and Ac₂O (6 mL) were heated to 118 °C (oil bath temperature) for 1 h. Then the reaction mixture was allowed to cool down to RT. DCM (40 mL) and water (30 mL) were added. After extraction the organic phase was washed with sat. NaHCO₃-sol. (3 x 30 mL), dried (Na₂SO₄), and concentrated to dryness. ESI-MS (positive): [M+Na]⁺: 527.

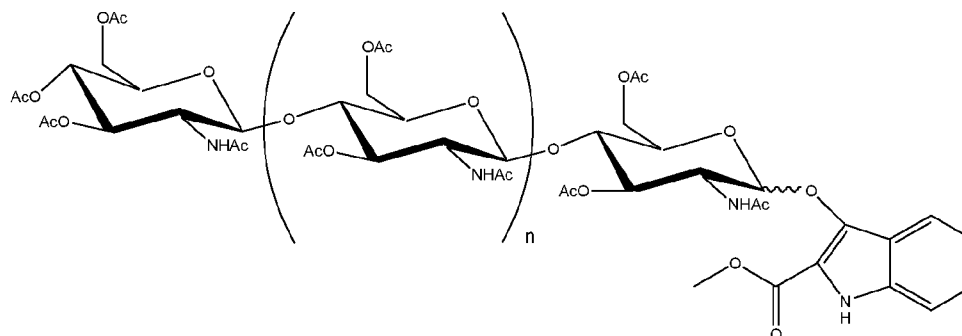
[00474] Example 17: Peracetyl-oligo-β-D-1,4-glucosamine



[00475] Pyridine (500 μL) and acetic anhydride (500 μL) were added to chitosan oligomers (mixture of the oligomers and sodium acetate in a ratio of 5 to 4.1) (101 mg, chain length between 1 and 4) and the mixture was shaken at RT overnight. Since not all material was dissolved the mixture was shaken overnight again. Afterwards it was kept in the fridge for ten days. DCM (20 mL) was added and the mixture was poured on ice cold citric acid (10 % in water, 20 mL). After extraction the organic phase was washed with ice cold citric acid (10 % in water, 20 mL), followed by brine (15 mL). The organic phase was dried (Na₂SO₄) and concentrated to dryness (64 mg crude product). The crude product was used without further purification. MS data: . ESI-MS (positive): [M+Na]⁺ (dimer): 699, [M+Na]⁺ (trimer): 986.

[00476] Example 18: 1-Chloro-peracetyl-oligo-β-D-1,4-glucosamine

[00477] The crude product of Example 19 was dissolved in acetyl chloride (1 mL) under argon atmosphere and cooled in an ice bath. MeOH (55 μ L) was added dropwise within approximately 1 min. After complete addition the mixture was stirred in the ice bath for additional 10 min, then the solution was allowed to warm to RT; additional acetyl chloride (05 mL) was added. The mixture was stirred at RT overnight. Afterwards it was concentrated to dryness, taken up in dichloromethane (5 mL), concentrated to dryness again, suspended in diisopropyl ether and concentrated to dryness another time to yield 55 mg crude product. The crude product was used for the next step without further purification.

[00478] Example 19: O-(2-Carboxymethyl-3-indoxyl)-peracetyl-oligo-β-D-1,4-glucosamine

[00479] The crude product of example 20 (55 mg crude product), tetrabutylammonium hydrogen sulfate (20 mg), and methyl 3-hydroxy-1H-indole-2-carboxylate (13 mg) were dissolved in DCM (dry, 1.5 mL) at RT.; K_2CO_3 (1 M in water, 1 mL) was added and the mixture was stirred at RT overnight. DCM (20 mL) and water (15 mL) were added. After extraction the organic phase was dried (Na_2SO_4) and concentrated to dryness (39 mg crude product). The crude product was purified by column chromatography. Eluent: 5% MeOH in DCM. Isolated: 1.8 mg product with one sugar moiety, 1.2 mg product with two sugar moieties. ESI-MS (positive): $[M+Na]^+$ (monomer): 543, $[M+Na]^+$ (dimer): 830.

[00480]

[00481] Example 20. Synthesis of phenol/chitosan/laccases based substrates for the detection of wound infection.

[00482] N-acetyl chitosan grafted with sinapic acid (SA).

[00483] N-acetyl chitosan was dissolved in sodium acetate buffer (100mM, pH 5,0) to obtain a 1% solution (w/v). 20 mL of this solution were mixed with 20 mL of a sinapic acid solution in ethanol (20 mM). EDC and NHS were added (1,3 g each) and the solution was stirred for 2 h. The reaction was stopped by adding NaOH (1M) drop-wise, the resulting precipitate was washed with water until no phenol could be detected anymore in the washing solution. Afterwards the product was freeze dried.

[00484] N-acetyl chitosan / aminomethoxyphenol nanoparticles

[00485] N-acetyl chitosan was dissolved in sodium acetate buffer (100 mM, pH 5,0) and aminomethoxyphenol (dissolved in ethanol) was added to obtain a total concentration of 20 mM. 2,3 mL of this solution were mixed with 1,6 mL of dodecane and applied to a sonicator to produce an emulsion.

[00486] Hydrolysis of SA grafted N-acetyl chitosan by lysozyme

[00487] SA grafted N-acetyl chitosan (5 mg) was suspended in 500 μ L potassium phosphate buffer (62 mM, pH 6,2) containing lysozyme (0,1 mg/mL). The solution was incubated for one hour. The reaction supernatant was incubated with laccase (1 U/mL) and an immediate color change was observed.

[00488] Hydrolysis of N-acetyl chitosan / aminomethoxyphenol nanoparticles by lysozyme

[00489] An N-acetyl chitosan / aminomethoxyphenol nanoparticle emulsion (50 μ L) was mixed with 450 μ L potassium phosphate buffer (62 mM, pH 6,2) containing lysozyme (0,1 mg/mL) and laccase (1 U/mL). The nanoparticles were destructed after 15 min (yielding a clear solution) developing strong colour.

[00490] **Example 21. Dying of peptidoglycan for the detection of wound infection**

[00491] Different reactive dyes can be used for the dying of peptidoglycan (Table 1).

Preferable are reactive dyes containing a sulfonylethyl-hydrogensulphate-reactive-group such as reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16. Alternatively dyes containing a dichlortriazine reactive-group such as reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10 can be used. Dyes were evaluated by consideration of their degree of reaction with peptidoglycan and the speed with which the dye was released when the dyed peptidoglycan was incubated with lysozyme (see earlier section of Example 7 for dying and Example 6 for digestion assay).

[00492] **Table 1.** Evaluation of reactive dyes

Dyes	Evaluation
Reactive black 5	+++
Remazol brilliant blue	++
Reactive green	+
Reactive brown	-

[00493] Peptidoglycan dying procedure 1:

[00494] Preparation and sterilization of peptidoglycan for staining with reactive dyes:

[00495] Micrococcus lysodeicticus cell broth from an animal free fermentation was centrifuged at 4000 g and 4 °C for 15 min to gain a wet cell pellet. The cell pellet (20 g) was washed twice with ddH₂O (800 ml) to get rid of any remaining media components (constant centrifugation conditions).

[00496] The wet Micrococcus lysodeicticus cell pellet (20 g, wet:dry ration 4:1, 5 g dry M. lysodeicticus cells) were suspended in 1 M HCl (80 g) and incubated for 1 h at 60 °C and 720 rpm. The sterilized and interrupted cells were then centrifuged at 4000 g and 4 °C for 15 min. The peptidoglycan pellet was resuspended and washed with Na-Phosphate buffer (400 ml, 100 mM, pH 7.0) to adjust the pH of the suspension to a neutral pH and to get rid of cell components of the disruption process (constant centrifugation conditions). Another washing step with 500 ml of ddH₂O was carried out to get rid of disrupted cell components and buffer salts. The gained pellet after the last washing step was ready to use for the staining procedure.

[00497] Peptidoglycan (50 mg), reactive black 5 (5 mg), Na₂CO₃ (10 mg), and Na₂SO₄ (25 mg) were dissolved/suspended in double-distilled water (ddH₂O) (2 mL). After shaking for 1 min, the reaction mixture was incubated at 25 °C and 750 rpm for 10 min followed by a second incubation step at 65 °C and 750 rpm-shaking for another 30 min.

[00498] The dyed peptidoglycan was centrifuged at 10000g for 10 min. The supernatant was collected, and the pellet was re-suspended in ddH₂O and centrifuged again at 10000g for 10 min. The washing procedure was repeated until the supernatant was clear. Different buffers or organic solvents like ethanol could be used for the washing procedure. The absorbance levels of all supernatants were measured at 597 nm. The supernatant was defined as clear, if the absorbance was below a value of 0.05. The amount of unbound dye in the supernatant was calculated using a calibration curve and the measured absorbance levels of the supernatants. Consequentially, the amount of peptidoglycan-bound dye was calculated. The dyed peptidoglycan was stored at 4-8 °C for a maximum of 2 wks or dried via lyophilization for a longer storage period.

[00499] Peptidoglycan dying procedure 2:

[00500] Reactive black 5 (200 mg) and peptidoglycan (300 mg) were dissolved in ddH₂O (40 mL). The reaction solution was stirred at 50 °C for 30 min. During the 30 min, Na₂SO₄ (1 g) was added every 6 min (total amount: 4 g). After the first 30 min incubation, Na₃PO₄ (200 mg) was added to the reaction solution, which was stirred again for 30 min at 50 °C.

[00501] The dyed peptidoglycan was centrifuged at 10000g for 10 min. The supernatant was collected, and the pellet was re-suspended in ddH₂O and was again centrifuged at 10000g for 10 min. The washing procedure was repeated until the supernatant was clear. Different buffers or organic solvents like ethanol could be used for the washing procedure. The absorbance levels of all supernatants were measured at 597nm. The supernatant was defined as clear, if the absorbance was below a value of 0.05. The amount of unbound dye in the supernatant was calculated using a calibration curve and the measured absorbance levels of the supernatants. Consequentially, the amount of peptidoglycan-bound dye was calculated. The dyed peptidoglycan was stored at 4-8 °C where it appears to be stable or dried via lyophilisation.

[00502] Peptidoglycan dying procedure 3:

[00503] Peptidoglycan (150 mg, dry weight) was suspended in ddH₂O (20 ml) and heated to 50 °C. The reaction was started by the addition of reactive black 5 (7 different variants with different amounts of reactive dye according to table X). The reaction was stirred (210 rpm) at 50 °C for 1 h. After the first 10 min Na₂CO₃ was added periodically every 10 minutes (5 x 100 mg, after 10, 20, 30, 40 and 50 min reaction time). The reaction mixture was stirred for another 10 min after the last addition of Na₂CO₃. The reaction solution was centrifuged at 4000 g at 4°C for 15 min. The pellet was resuspended and washed in ddH₂O (40 g) 3 times and always centrifuged as before. Different buffers or organic solvents like ethanol could be used for the washing procedure. All the supernatants were weight out and were used for the determination of the unbound dye concentration and in further consequence for the determination of the staining degree. Therefore, 1 ml of each supernatant was transferred into a 1.5 ml Eppendorf tube and centrifuged for 5 min at 10000 g and ambient temperature. 3 times 100 µl of each supernatant were transferred into a 96-well plate and the absorbance levels were measured at 597 nm. The supernatant was defined as clear, if the absorbance was below a value of 0.05. The amount of unbound dye in the supernatant was calculated using a calibration curve and the measured absorbance levels of the supernatants. The percentages of bound dye are listed in table 2. The percentage of reactive black 5 of the whole stained peptidoglycan construct (LPG-RB5) is also listed in Table 2.

[00504] Table 2: Different variants of stained peptidoglycan

Variants	Amount Reactive Black 5 (mg)	% Reactive Black 5 bound	% RB5 of LPG-RB5
LPG-RB5-05	150	74	45
LPG-RB5-06	200	64	50
LPG-RB5-07	75	89	34
LPG-RB5-08	50	92	22
LPG-RB5-09	25	92	12
LPG-RB5-10	10	88	5

[00505] Example 22. Lysozyme Activity measurement for the detection of wound infection**[00506]** Dyed peptidoglycan:

[00507] One suspension of every LPG-RB5 variant (Example 21) (1.5 mg/ml) was prepared (NaCl-solution, 0.9%) and divided into 6 1 ml samples each (3 positive, 3 negative). The positive controls were mixed with a Lysozyme stock solution (10 µl, 1,000,000 U/ml) to a final Lysozyme activity of 10,000 U/ml. All samples were incubated at 37°C for 60 min and centrifuged afterwards (5 min, 10000 g). A development of blue colour was detected in the supernatants. 100 µl of each supernatant was transferred into a 96-well plate and the absorbance was measured at 597 nm. The absorbances of the supernatants of the different variants are shown in Figure 8. The influence of the staining degree is shown in Figure 9.

[00508] Calculation of dye : PG ratio:**[00509]** Amount of unbound dye:

$$\text{[00510]} \quad x = \left(\frac{\text{AbsWL} - d}{k} \right) * z * y$$

[00511] AbsWL... measured absorbance of different washing solutions at 597nm**[00512]** d... intercept of linear regression of dye calibration curve**[00513]** k... slope of linear regression of dye calibration curve**[00514]** z... Dilution factor of measured washing solution**[00515]** y... Volume of washing solution (ml)**[00516]** x... amount of RB5 in washing solution (mg)**[00517]** Amount of bound dye:

$$\text{[00518]} \quad a = (\text{amount used dye}) - (\text{amount unbound dye})$$

[00519] a... amount of bound dye**[00520]** Consideration of dye content in dye container:

$$\text{[00521]} \quad c = a * 0.85$$

[00522] c... amount of bound dye (mg) (dye content considered)

[00523] 0.85... 85% dye content

a... amount of bound dye (mg)

[00524] Consideration of weight loss due to leaving groups of reactive dye (Reactive Black 5):

[00525] $f = c * 0.76$

[00526] f... final amount of bound dye (mg) (-reactive groups)

[00527] c... amount of bound dye (mg) (dye content considered)

[00528] Ratio between PG and RB5:

[00529] $R = \frac{L-f}{f}$

[00530] R... ratio between pure PG and RB5

[00531] L... Yield of dry PG-RB5 (mg)

[00532] f... final amount of bound dye (mg)

[00533] **Example 23: Colorimetric enzyme assay for detection of myeloperoxidase activity**

[00534] Reagents like 3,4-diamino benzoic acid, 3-amino-4-hydroxy benzoic acid, 4-amino-3-hydroxy benzoic acid, 2,3-diamino benzoic acid, 3,4-dihydroxy benzoic acid, 2-amino phenol, 2-amino-3-methoxy benzoic acid, Methyl-3,4-diaminibenzoate and 2-amino-4-methoxy phenol can be used for the detection of MPO activity (Table 2). Assay conditions: 1 mg/mL DABA or equivalent was dissolved in 100 mM sodium-phosphate-buffer (pH 6.4). H₂O₂ was added to a final concentration of 5 mM. The substrate/H₂O₂ solution (95 µL) was added to a 96-well microtiter plate along with 5 µL of a MPO-containing sample. The solution turned brownish upon MPO oxidation. The reaction was monitored at 450 nm using a standard photometric plate reader.

[00535] **Table 3. Evaluation of novel MPO substrates**

Substrates	Evaluation
3,4-Diamino benzoic acid	+++
3-Amino-4-hydroxy benzoic acid	++
4-Amino-3-hydroxy benzoic acid	+++
2,3-Diamino benzoic acid	++
3,4-Dihydroxy benzoic acid	+
2-Amino phenol	++
2-Amino-3-methoxy benzoic acid	+
Methyl-3,4-diaminibenzoate	+++
2-Amino-4-methylphenol	+

[00536] Example 24. Construction of human leukocyte elastase (HLE) and human cathepsin G (CatG) substrates with polymer binding sequences

[00537] Chimeric gene variants were synthesized encoding one to four concatemers of the hydrophilic carbohydrate binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*), or the hydrophobic binding module (PDB) from Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*). Chimeric variants with the hydrophilic binding module (CBM) enable the attachment onto cellulose based filter papers/fabrics, in contrast, chimeric variants with the hydrophobic binding module (PDM) enable the attachment onto PET (Polyethylene terephthalate) based strips.

[00538] In order to confer a proper recombinant protein expression, variants of TrxA-ElaSub1_CBM_His fusion proteins were designed. The construct consists of the *trx4* (thioredoxin) gene, a short spacer sequence that encodes a 6xHis-Tag and the enterokinase cleavage site (Asp Asp Asp Lys) for separating the TrxA fusion tag from the protein of interest. Subsequently to the enterokinase site, the newly designed *elasub1_cbm* coding sequence was introduced into the construct. The *elasub1* sequence encodes for amino acids that expose a lot of functional groups like thiol-, hydroxyl-, amino- or carboxy groups. These amino acids (cysteine, lysine, arginine, glutamine, asparagine, glutamic acid, aspartic acid, serine, threonine or tyrosine) should facilitate the coupling of dyes or pro-dyes to the chimeric peptides. The coding sequence for the natural hydrophilic binding module (CBM) was located directly downstream to the *elasub1* sequence, termed *elasub1_cbm*. Additionally, two or more recognition/cleavage sites for HLE (Ala-Ala-Pro-Val) or for CatG (Ala-Ala-Pro-Phe) were introduced into the *elasub1* coding sequence. To visualize the action of HLE or CatG, certain dye molecules (e.g. Remazol Brilliant Blue), comprising positive or negative charged groups were attached to the functional groups of the amino acids listed above. Enzymatic cleavage of the chimeric peptides through HLE or CatG leads to the release of peptide fragments that carry the coupled dyes or pro-dyes. Through ionic exchange, the enzyme reactions (HLE or CatG) can be visualized by binding the released peptide fragments through the charged groups of the dye. For the purification of the chimeric peptide, an additional repetitive sequence stretch encoding a His-tag (6xHis) was attached to the C-terminal end of to the *elasub1_cbm* fusion construct.

[00539] DNA sequence of the fusion construct *trx4__elasub1_cbm_his*; sequences from the expression vector pET32b(+) are undelined:

[00540] ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGT
ACTCAAAGCGGACGGGGCGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGGTCCGT
GCAAAATGATCGCCCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACGTG

ACCGTTGCAAACTGAACATCGATCAAAACCCTGGCACTGCGCCGAAATATGGCAT
 CCGTGGTATCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAG
 TGGGTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCCTCGACGCTAACCTGGCCGGTT
 CTGGTTCTGGCCATATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCG
 GTTCTGGTATGAAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGC
 CCAGATCTGGGTACCGACGACGACGACAAGGCCATGGGTGGTAGCTGCGGTGGTGG
 TGGTAGCGCAGCACCGGTTGGTGGTGGCGGTTACAGCTGCTCCTGTGGGTGGCGGTG
 GTTACCGCCTGGTGGTAATCGTGGTACAACCACCACCCGTCGTCGGCAACCACA
 ACCGGTAGCAGTCCGGGTCCGACCCAGAGCCATTATGGTCAGTGTGGTGGTATTGG
 TTATAGCGGTCCGACCGTTTGTGCAAGCGGCACCACCTGTCAGGTTCTGAATCCGTA
 TTATAGCCAGTGTCTGCTCGAGCACCACCACCACCACCCTGA

[00541] Protein sequence of the TrxA_ElaSub1_CBM-His fusion protein:

[00542] MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQG
 KLTVAKLNIDQNPQTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGS
 GSGHMHSHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMGGSCGGG
 GSAAPVGGGGSAAPVGGGGSPPGNRRGTTTTTRRPATTTGSSPGPTQSHYGQCGGIGYS
 GPTVCASGTTTCQVLNPYYSQCLLEHHHHHHH*

[00543] Protein sequence of the native ElaSub1_CBM_His fusion protein:

[00544] MGGSCGGGGSAAPVGGGGSAAPVGGGGSPPGNRRGTTTTTRRPATTTGSSPG
 PTQSHYGQCGGIGYSPTVCASGTTTCQVLNPYYSQCLLEHHHHHHH*

[00545] The following variants with the hydrophilic binding module were designed as shown in Table 4.

[00546] **Table 4: Variants with the hydrophilic binding module (CBM) as well as with possible hydrophobic binding modules**

variant	construct (name)	binding unit	cutting unit	purification	reaction site	color
1	ElaSub1_CBM	CBM	HLE		thiol group (n=1)	blue
2	CatGSub1_CBM	CBM	CatG		thiol group (n=1)	blue
3	ElaSub1_CBM His	CBM	HLE	His-Tag	thiol group (n=1)	blue
4	CatGSub1_CBM His	CBM	CatG	His-Tag	thiol group (n=1)	blue

variant	construct (name)	binding unit	cutting unit	purification	reaction site	color
5	ElaSub2_CBM	CBM	HLE		thiol groups (n=3)	blue
6	CatGSub2_CBM	CBM	CatG		thiol groups (n=3)	blue
7	ElaSub2_CBM His	CBM	HLE	His-Tag	thiol groups (n=3)	blue
8	CatGSub2_CBM His	CBM	CatG	His-Tag	thiol groups (n=3)	blue
9	ElaSub1_PDM	PDM	HLE		thiol group (n=1)	blue
10	CatGSub1_PDM	PDM	CatG		thiol group (n=1)	blue
11	ElaSub1_PDM His	PDM	HLE	His-Tag	thiol group (n=1)	blue
12	CatGSub1_PDM His	PDM	CatG	His-Tag	thiol group (n=1)	blue
13	ElaSub2_PDM	PDM	HLE		thiol groups (n=3)	blue
14	CatGSub2_PDM	PDM	CatG		thiol groups (n=3)	blue
15	ElaSub2_PDM His	PDM	HLE	His-Tag	thiol groups (n=3)	blue
16	CatGSub2_PDM His	PDM	CatG	His-Tag	thiol groups (n=3)	blue

[00547] Variant 1: ElaSub1_CBM.

[00548] The ElaSub1 construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the ElaSub1 protein sequence, termed ElaSub1_CBM. Within this region a codon for cysteine is inserted. The thiol group of the cysteine should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, two recognition/cleavage sites for HLE (Ala-Ala-Pro-Val) are introduced into the hydrophilic spacer region. To visualize the action of HLE certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of

the cysteine. Enzymatic cleavage of the chimeric peptide through HLE should lead to the release of a peptide fragment that carries the coupled dye or pro-dye. Through ionic exchange, the HLE enzyme reaction should be visualized by binding the released peptide fragment through the charged groups of the dye. The amino sequence of this variant is shown in table 4.

[00549] Variant 2: CatGSub1_CBM.

[00550] The CatGSub1 construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the ElaSub1 protein sequence, termed CatGSub1_CBM. Within this region a codon for cysteine is inserted. The thiol group of the cysteine should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, two recognition/cleavage sites for CatG (Ala-Ala-Pro-Phe) are introduced into the hydrophilic spacer region. To visualize the action of CatG certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteine. Enzymatic cleavage of the chimeric peptide through CatG should lead to the release of a peptide fragment that carries the coupled dye or pro-dye. Through ionic exchange, the CatG enzyme reaction should be visualized by binding the released peptide fragment through the charged groups of the dye. The amino sequence of this variant is shown in table 4.

[00551] Variant 3: ElaSub1_CBM_His.

[00552] The ElaSub1 construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the ElaSub1 protein sequence, termed ElaSub1_CBM. Within this region a codon for cysteine is inserted. The thiol group of the cysteine should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, two recognition/cleavage sites for HLE (Ala-Ala-Pro-Val) are introduced into the hydrophilic spacer region. To visualize the action of HLE certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteine. Enzymatic cleavage of the chimeric peptide through HLE should lead to the release of a peptide fragment that carries the coupled dye or pro-dye. Through ionic exchange, the HLE enzyme reaction should be visualized by binding the released peptide fragment through the charged groups of the dye. For the purification of this chimeric variant, a repetitive sequence stretch encoding a His-tag is attached subsequently to the sequence encoding the carbohydrate binding module. The amino sequence of this variant is shown in table 4.

[00553] Variant 4: CatGSub1_CBM_His.

[00554] The CatGSub1 construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the ElaSub1 protein sequence, termed CatGSub1_CBM. Within this region a codon for cysteine is inserted. The thiol group of the cysteine should facilitate the coupling of dyes or pro-dyes to

the chimeric peptide. Additionally, two recognition/cleavage sites for CatG (Ala-Ala-Pro-Phe) are introduced into the hydrophilic spacer region. To visualize the action of CatG certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteine. Enzymatic cleavage of the chimeric peptide through CatG should lead to the release of a peptide fragment that carries the coupled dye or pro-dye. Through ionic exchange, the CatG enzyme reaction should be visualized by binding the released peptide fragment through the charged groups of the dye. For the purification of this chimeric variant, a repetitive sequence stretch encoding a His-tag is attached subsequently to the sequence encoding the carbohydrate binding module. The amino sequence of this variant is shown in table 4.

[00555] Variant 5: ElaSub2_CBM.

[00556] The ElaSub2 construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the ElaSub2 protein sequence, termed ElaSub2_CBM. Within this region three codons for cysteine are inserted. The thiol group of the cysteines should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, three recognition/cleavage sites for HLE (Ala-Ala-Pro-Val) are introduced into the hydrophilic spacer region. To visualize the action of HLE certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteines. Enzymatic cleavage of the chimeric peptide through HLE should lead to the release of a peptide/peptides fragment/fragments that carry the coupled dye or pro-dye. Through ionic exchange, the HLE enzyme reaction should be visualized by binding the released peptide fragment/fragments through the charged groups of the dye. The amino sequence of this variant is shown in Table 4.

[00557] Variant 6: CatGSub2_CBM.

[00558] The CatGSub2_CBM construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the CatGSub2 protein sequence, termed CatGSub2_CBM. Within this region three codons for cysteine are inserted. The thiol group of the cysteines should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, three recognition/cleavage sites for CatG (Ala-Ala-Pro-Phe) are introduced into the hydrophilic spacer region. To visualize the action of CatG certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteines. Enzymatic cleavage of the chimeric peptide through CatG should lead to the release of a peptide/peptides fragment/fragments that carry the coupled dye or pro-dye. Through ionic exchange, the CatG enzyme reaction should be visualized by binding the released peptide fragment/fragments through the charged groups of the dye. The amino sequence of this variant is shown in Table 4.

[00559] Variant 7: ElaSub2_CBM_His.

[00560] The ElaSub2 construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the ElaSub2 protein sequence, termed ElaSub2_CBM. Within this region three codons for cysteine are inserted. The thiol group of the cysteines should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, three recognition/cleavage sites for HLE (Ala-Ala-Pro-Val) are introduced into the hydrophilic spacer region. To visualize the action of HLE certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteines. Enzymatic cleavage of the chimeric peptide through HLE should lead to the release of a peptide/peptides fragment/fragments that carry the coupled dye or pro-dye. Through ionic exchange, the HLE enzyme reaction should be visualized by binding the released peptide fragment/fragments through the charged groups of the dye. For the purification of this chimeric variant, a repetitive sequence stretch encoding a His-tag is attached subsequently to the sequence encoding the carbohydrate binding module. The amino sequence of this variant is shown in Table 4.

[00561] Variant 8: CatGSub2_CBM_His

[00562] The CatGSub2_CBM construct encodes a stretch of mainly hydrophilic amino acids. The sequence for the natural hydrophilic binding module (CBM) is located directly downstream to the CatGSub2 protein sequence, termed CatGSub2_CBM. Within this region three codons for cysteine are inserted. The thiol group of the cysteines should facilitate the coupling of dyes or pro-dyes to the chimeric peptide. Additionally, three recognition/cleavage sites for CatG (Ala-Ala-Pro-Phe) are introduced into the hydrophilic spacer region. To visualize the action of CatG certain dye molecules comprising positive or negative charged groups can be attached to the thiol group of the cysteines. Enzymatic cleavage of the chimeric peptide through CatG should lead to the release of a peptide/peptides fragment/fragments that carry the coupled dye or pro-dye. Through ionic exchange, the CatG enzyme reaction should be visualized by binding the released peptide fragment/fragments through the charged groups of the dye. For the purification of this chimeric variant, a repetitive sequence stretch encoding a His-tag is attached subsequently to the sequence encoding the carbohydrate binding module. The amino sequence of this variant is shown in Table 5.

[00563] Variant 9 to variant 16 could be designed identically as variant 1 to variant 8 with the exception of changing the hydrophilic carbohydrate binding module (CBM) against the hydrophobic binding module (PDB).

[00564] Table 5A: Amino acid sequence of the chimeric protein variants with the hydrophilic carbohydrate binding module.

Construct	Sequence
ElaSub1_CBM	MGGSCGGGGSAAPVGGGGSAAPVGGGGSPGGNRGTTTTRRP ATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQ CL
CatGSub1_CBM	MGGSCGGGGSAAPFGGGGSAAPFGGGGSPGGNRGTTTTRRPA TTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQ L
ElaSub1_CBM_His	MGGSCGGGGSAAPVGGGGSAAPVGGGGSPGGNRGTTTTRRP ATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQ CLLEHHHHHH
CatGSub1_CBM_His	MGGSCGGGGSAAPFGGGGSAAPFGGGGSPGGNRGTTTTRRPA TTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQ LLEHHHHHH
ElaSub2_CBM	MCGGGGSAAPVEQLEWLQGGGGSCGGGGSAAPVSEALEQWG GGGSCGGGGSAAPVAGAGAGTQSHYGQCGGIGYSGPTVCASG TTCQVLNPYYSQCL
CatGSub2_CBM	MCGGGGSAAPFEQLEWLQGGGGSCGGGGSAAPVSEALEQWGG GGSCGGGGSAAPFAGAGAGTQSHYGQCGGIGYSGPTVCASGT CQVLNPYYSQCL
ElaSub2_CBM_His	MCGGGGSAAPVEQLEWLQGGGGSCGGGGSAAPVSEALEQWG GGGSCGGGGSAAPVAGAGAGTQSHYGQCGGIGYSGPTVCASG TTCQVLNPYYSQCLLEHHHHHH
CatGSub2_CBM_His	MCGGGGSAAPFEQLEWLQGGGGSCGGGGSAAPVSEALEQWGG GGSCGGGGSAAPFAGAGAGTQSHYGQCGGIGYSGPTVCASGT CQVLNPYYSQCLLEHHHHHH

Table 5B: Nucleotide sequence of the chimeric gene variants with the hydrophile carbohydrate binding module. All eight constructs were cloned into a pET-32b(+) expression vector (Novagen) using the *Nco* I and *Xho* I restriction sites located in the MCS of the cloning vector.

ElaSub1_CBM
 ATGGGTGGTAGCTGCGGTGGTGGTGGTAGCGCAGCACCGGTTGGTGGTGGCGGTTTCAGCTGCTC
 CTGTGGGTGGCGGTGGTTACCGCCTGGTGGTAATCGTGGTACAACCACCCGTCGTCCGGC
 AACCACAACCGGTAGCAGTCCGGGTCCGACCCAGAGCCATTATGGTCAGTGTGGTGGTATTGGT
 TATAGCGGTCCGACCGTTTGTGCAAGCGGCACCACCTGTCAGGTTCTGAATCCGTATTATAGCC
 AGTGTCTG

CatGSub1_CBM ATGGGTGGTAGCTGCGGTGGTGGTGGTAGCGCAGCACCGTTTGGTGGTGGCGGTTTCAGCTGCTC CTTTTGGTGGCGGTGGTTACCGCCTGGTGGTAATCGTGGTACAACCACCACCGTCGTCCGGC AACCACAACCGGTAGCAGTCCGGGTCCGACCCAGAGCCATTATGGTCAGTGTGGTGGTATTGGT TATAGCGGTCCGACCGTTTGTGCAAGCGGCACCACCTGTCAGGTTCTGAATCCGTATTATAGCC AGTGTCTG
ElaSub1_CBM_His ATGGGTGGTAGCTGCGGTGGTGGTGGTAGCGCAGCACCGTTTGGTGGTGGCGGTTTCAGCTGCTC CTGTGGGTGGCGGTGGTTACCGCCTGGTGGTAATCGTGGTACAACCACCACCGTCGTCCGGC AACCACAACCGGTAGCAGTCCGGGTCCGACCCAGAGCCATTATGGTCAGTGTGGTGGTATTGGT TATAGCGGTCCGACCGTTTGTGCAAGCGGCACCACCTGTCAGGTTCTGAATCCGTATTATAGCC AGTGTCTGCTCGAGCACCACCACCACCACCTGA
CatGSub1_CBM_His ATGGGTGGTAGCTGCGGTGGTGGTGGTAGCGCAGCACCGTTTGGTGGTGGCGGTTTCAGCTGCTC CTTTTGGTGGCGGTGGTTACCGCCTGGTGGTAATCGTGGTACAACCACCACCGTCGTCCGGC AACCACAACCGGTAGCAGTCCGGGTCCGACCCAGAGCCATTATGGTCAGTGTGGTGGTATTGGT TATAGCGGTCCGACCGTTTGTGCAAGCGGCACCACCTGTCAGGTTCTGAATCCGTATTATAGCC AGTGTCTGCTCGAGCACCACCACCACCACCTGA
ElaSub2_CBM ATGTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGAACAGCTGGAATGGCTGCAGGGCGGCGGGC GCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTAGCGAAGCGCTGGAACAGTGGGGCGGCGG CGGCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGCGGGCGCGGGCGCGGGCACCCAGAGC CATTATGGCCAGTGC GGCGGCATTGGCTATAGCGGCCCGACCGTGTGCGCGAGCGGCACCACCT GCCAGGTGCTGAACCCGTATTATAGCCAGTGCCTG
CatGSub2_CBM ATGTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGAACAGCTGGAATGGCTGCAGGGCGGCGGGC GCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTAGCGAAGCGCTGGAACAGTGGGGCGGCGG CGGCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGCGGGCGCGGGCGCGGGCACCCAGAGC CATTATGGCCAGTGC GGCGGCATTGGCTATAGCGGCCCGACCGTGTGCGCGAGCGGCACCACCT GCCAGGTGCTGAACCCGTATTATAGCCAGTGCCTG
ElaSub2_CBM_His ATGTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGAACAGCTGGAATGGCTGCAGGGCGGCGGGC GCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTAGCGAAGCGCTGGAACAGTGGGGCGGCGG CGGCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGCGGGCGCGGGCGCGGGCACCCAGAGC CATTATGGCCAGTGC GGCGGCATTGGCTATAGCGGCCCGACCGTGTGCGCGAGCGGCACCACCT GCCAGGTGCTGAACCCGTATTATAGCCAGTGCCTGCTCGAGCACCACCACCACCACCTGA
CatGSub2_CBM_His ATGTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGAACAGCTGGAATGGCTGCAGGGCGGCGGGC GCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTAGCGAAGCGCTGGAACAGTGGGGCGGCGG CGGCAGCTGCGGCGGCGGGCGGCAGCGCAGCACCGTTTGCGGGCGCGGGCGCGGGCACCCAGAGC CATTATGGCCAGTGC GGCGGCATTGGCTATAGCGGCCCGACCGTGTGCGCGAGCGGCACCACCT GCCAGGTGCTGAACCCGTATTATAGCCAGTGCCTGCTCGAGCACCACCACCACCACCTGA

[00565] Example 25: Expression of elastase (HLE) substrates with polymer binding sequences.

[00566] Variant 3 (example 9): BL21 Gold (*DE3*) [pET32b(+)*ela sub1_cbm his*]

[00567] The chimeric gene was cloned into a pET32b(+) expression system using *NcoI* and *XhoI* restriction sites/enzymes. Final protein expression of the chimeric peptide was carried out in *E. coli* BL21 Gold (*DE3*), a protease deficient expression host, which enables a proper protein expression based on the T7 promoter of the pET32b(+) expression vector. Fermentation of the BL21 Gold (*DE3*) host strain, harbouring the recombinant [pET32b(+) *ela sub1_cbm*] construct, was done with 2xTY media supplemented with 100 µg ml⁻¹ ampicillin for maintaining the plasmid. Overnight cultures grown at 30°C with shaking were used to inoculate the main cultures. Cells were grown at 37°C (fast induction protocol) and at 30°C (slow induction protocol), with shaking, until the optical density at 600 nm reached approximately 0.6. Expression of the recombinant constructs were induced by the addition of 0.5 mM IPTG (final concentration), whereas protein expression was carried out 4 hours at 37°C (fast induction) and 20 hours at 18°C (slow induction). Both protocols (fast and slow induction) displayed the same results concerning the yield of soluble recombinant protein. After protein expression, cells were harvested by centrifugation and cells were disrupted by lysozyme treatment followed by sonification. The proper protein expression of the recombinant protein was monitored with SDS-PAGE, compared to the identically expressed empty pET32b(+) vector. Purification of the chimeric construct from crude lysate was done using the IBA Ni-NTA sepharose gravity flow column (1ml) according to the IBA protocol. Fractions containing the purified protein were examined using SDS-PAGE. In order to prevent protein exposure to the high imidazole concentration after elution, buffer exchange was carried out using PD 10 desalting columns from GE Healthcare. The yield of the purified chimeric fusion construct could be calculated with 30 mg protein per 2 g initial cell pellet. To separate the chimeric construct from the TrxA fusion tag, the recombinant expressed bovine enterokinase from Merck Millipore was used. The separation of the desired chimeric construct from the TrxA-Tag was verified by SDS-PAGE. The isolated chimeric protein, containing the HLE recognition/cleavage site twice, was used for the coupling procedures with varying dyes or pro dyes that exhibit different properties concerning their ability to expose positive and negative charged groups.

[00568] The finished chimeric construct was adsorbed onto a cellulose based filter paper (or fabrics) through the hydrophilic carbohydrate binding module. The filter strip containing the chimeric peptide was incubated for 30 min with a 0.1 M sodium phosphate buffer solution (pH 7.4), containing 0.05 U/ml HLE, as well as with human wound fluids. Initially non coloured samples (clear supernatant) that exhibit elastase activity developed a blue colour, depending on the attached dye. Due to enzymatic cleavage at one or both of the internal HLE restriction sites (Ala-Ala-Pro-Val), peptide fragments with the attached Remazol Brilliant Blue dye molecules get uncoupled, resulting in a blue coloured supernatant. Alternatively, chimeric constructs with

the hydrophobic binding module (PDB) can be used for adsorption of PET (Polyethylene terephthalate) based carrier materials. The production of chimeric variants harbouring the CatG cleavage sites instead of the HLE cleavage sites, allow the detection of human CatG enzyme under the same requirements as denoted for constructs possessing the HLE restriction sites.

[00569] In constructs containing the PDM domains, the PDM group can be interchanged with the CBD in a manner described before. The polyhydroxybutyrate depolymerase from *A. faecalis* (PBM or PDM) sequences are described in Ribitsch et al., “Fusion of Binding Domains to *Thermobifida cellulosilytica* Cutinase to Tune Sorption Characteristics and Enhancing PET Hydrolysis,” *Biomacromolecules*, 14 (6), pp 1769–1776, 2013. The disclosure in Ribitsch is incorporated by reference herein in its entirety. Analogously, any solid phase binding domain can be used in this manner. Other types of CBD peptides may be employed interchangeably to achieve the desired functionality.

[00570] As demonstrated in the aforementioned Example and the functional assays, the constructs disclosed herein comprise a non-soluble anchor like CBD, this can be interchanged with other anchors, notably other CBDs or PDMs. Smaller anchors are preferred for stoichiometric reasons.

[00571] With regard to the enzyme recognition site contained in the construct, elastase, as exemplified herein, has broad preferences, wherein AAPV sequence is most preferred, however, AAPF is almost the same in terms of turnover and AAAA is similar. Thus, anyone of these sequences could be exchanged for the other without loss of function.

[00572] With regard to the C-terminus of the constructs and amino acids appended thereto, many variations are possible but the consensus is that lack of sidechains and lack of charge are important to un-hinder the cutting site. Cyteines are particularly employed as a means to anchor dyes and particularly not more than one per construct, preferably one per cutting site. They should be distal from the cutting sites to ensure that the sulfonated dye does not interact with the enzyme more than necessary.

[00573] With respect to the dyes that are employed in peptide substrates, it is important to consider the particular enzymes which are target the recognition sites. To this end, elastase is a basic enzyme and it is inhibited by strong negatively charged groups. This means that more than one sulfonated dye per cutting site is not required. It is desirable that the number of sulfonate groups is lower than 4 per cutting site. With greater number of such sulfonate groups, there is a risk that they will mask access to the cutting site.

[00574] Example 26: Staining of purified Trx-Ela-Sub1 with vinyl sulfone dyes as elastase substrate

[00575] Purified and lyophilised Trx-Ela-Sub1 was dissolved in H₂O (0.01g/2.5ml) (protein concentration ~0.2mg/ml). The buffer was exchanged from Tris-HCl to a Na₂PO₄/Na₂SO₄ solution using a PD-10 column.

[00576] Staining with a vinyl sulfone dye (remazol brilliant blue R, RBB): 210 µl RBB (7.7 mg dissolved in 1.5 ml Na₂PO₄/Na₂SO₄) solution was added to 1900 µl protein solution and was shaken (750 rpm) at 37 °C for 80 min.

[00577] Purification was carried out via His-Tag purification.

[00578] Reaction of Trx-Ela-Sub1-RBB with Elastase:

[00579] The stained and purified peptide construct was applied on a cellulose surface and dried at 37 °C for 2 h. Unbound Trx-Ela-Sub1-RBB was removed with H₂O. The stripes with the applied Elastase substrate were incubated with and without Elastase containing buffer, respectively. The Elastase responsive dye release can be seen in Figure 10.

[00580] Example 27: Immobilization of nitrazine yellow and bromocresol purple

[00581] Immobilization of nitrazine yellow and bromocresol purple onto cellulose via (3-Glycidyloxypropyl)trimethoxysilane (GPTMS). A 15 mM solution of the above dyes is reacted with GPTMS (300 mM) overnight at 25° C. The complete reaction mixture (5 µL) is pipetted onto a filter paper strip and dried for 30 minutes at 80° C and 5 min at 170° C. Thereafter the dyed strips are washed exhaustively in water. The pH response of the strips can be observed by incubating them in solutions of different pH. Color response after incubating in pH 4.5, 7.2 or 8.5 is indicated in Figure 7. Both dye mixtures show no color response except in the pH 8.5 buffer showing that their pH transition lies between pH 7.2 and 8.5.

[00582] Example 28: 3-step-Immobilisation of bromocresol purple onto OH-rich surfaces like cellulose as pH indicator.

[00583] 1. Step: Reaction of GPTMS (9 mM – 450 mM) in acetic acid (57 µM) for 10 - 180 min at ambient temperature under stirring conditions.

[00584] 2. Step: Soaking of OH-rich surface (e.g. cellulose) in reaction solution. Incubation of soaked material at 80 - 120 °C for 5 - 20 min.

[00585] 3. Step: Bromocresol purple (0.1 - 3.3 mg/ml) applied on pretreated material and dried at 120 °C for 20 min.

[00586] The different colours at different pH values are listed in Table 6.

[00587] Table 6: different pH values and the corresponding colours of immobilised bromocresol purple

pH value	colour
4.5	Yellow
5.0	Yellow/green
5.5	green
6.0	Green
6.2	green
6.5	Dark green
7.0	Petrol
7.5	Blue
8.0	Blue

[00588] Example 29: Phospholipase C based test - liquid system

[00589] Preparation of diagnostic system

[00590] For this diagnostic tool, p-Nitrophenylphosphorylcholine as substrate for Phospholipase C was used. p-Nitrophenylphosphorylcholine is dissolved in water (50-100 mM). A 250 mM Tris/HCl buffer with 70% Sorbitol pH 7.2 was used as assay buffer. 230 µl of the buffer and 100 µl of the substrate are pipetted into a microtiterplate.

[00591] Diagnosis: A volume between 5 and 12 µL of wound fluid sample is added to the test system, preferentially between 8 and 10 µl and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 30 minutes. Thereafter, infection will be indicated by a colour change from colourless to yellow.

[00592] Test protocol and results:

[00593] Infected wound fluid samples (A, B, C) and non-infected samples (D, E, F) are incubated with the diagnostic system described in IA. Visual inspection of the samples after 30 minutes of incubation indicated a colour change to yellow only for infected samples A, B, and C.

Wound fluid sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00594] Example 30: Phospholipase A2 based test - liquid system

[00595] Preparation of diagnostic system. For this diagnostic tool, 4-Nitro-3-octanoylbenzoic acid as Phospholipase A2 - substrate was used. 4-Nitro-3-octanoylbenzoic acid (1,7 mM) is dissolved in assay buffer containing 50 mM Tris/HCl buffer pH 7,2, 150 mM KCL and 10 mM CaCl₂. 190 µl of the buffer are pipetted into a microtiterplate.

[00596] Diagnosis: A volume between 5 and 12 µL of wound fluid sample is added to the test system, preferentially between 8 and 10 µl and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 30 minutes. Thereafter, infection will be indicated by a colour change from colourless to yellow.

[00597] Test protocol and results: Infected wound fluid samples (A, B, C) and non infected samples (D, E, F) are incubated with the diagnostic system described in IA. Visual inspection of the samples after 30 minutes of incubation indicated a colour change to yellow only for infected samples A, B, and C.

Wound fluid sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00598] Example 31: Catalase based test - liquid system

[00599] Preparation of diagnostic system:

[00600] For this diagnostic tool: Purpald as substrate for Catalase was used. Purpald is dissolved in water (50-100 mM). 200 µl of substrate solution are pipetted into a microtiterplate.

[00601] Diagnosis: A volume between 5 and 12 µL of wound fluid sample is added to the test system, preferentially between 8 and 10 µl and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 30 minutes. Thereafter, infection will be indicated by a colour change from colourless to dark violet.

[00602] Test protocol and results: Infected wound fluid samples (A, B, C) and non infected samples (D, E, F) are incubated with the diagnostic system described in IA. Visual inspection of the samples after 30 minutes of incubation indicated a colour change to violet only for infected samples A, B, and C.

Wound fluid sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00603] Example 32: Testing body fluids

[00604] A material containing substrates for one or more of MPO, elastase, lysozyme, phospholipase, and catalase and optionally a pH indicator, is contacted with a body fluids such as wound, tear, vitreal, CSF, airway aspirates or sputum, synovial, blood, plasma, serum, urine, peritoneal, interstitial, subcutaneous, bile, intestinal or similar fluids. Samples from infected organisms or tissues tend to show a higher degree of reaction. A combination of one or more of the reactions is used to detect the infection and its degree.

[00605] Example 33: Elastase based test - liquid system

[00606] 100 μ L of a solution of N-methoxysuccinyl-ala-ala-pro-val-p- nitroanilide dissolved at a concentration of 20 mM in DMSO in 0.1 M HEPES buffer (pH 7.4, containing 0.5 M NaCl) is pipetted into a transparent eppendorf tube. The final concentration of N- methoxysuccinyl-ala-ala-pro-val-p-nitroanilide can be between 0.05 to 2.50 mM, and is preferentially between 0.80 and 1.20 mM. A volume between 1 and 15 μ L of sputum sample is added to the test system, preferentially between 2-5 μ L and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 5 minutes. Thereafter, wound infection will be indicated by a colour change from light pink to yellow. Mixtures containing non infected sputum samples will not change colour. 2 μ L of infected (A, B, C) and non-infected sputum samples (D, E, F) were incubated with the diagnostic system described in IA containing a substrate concentration of 1.0 mM N- methoxysuccinyl-ala-ala-pro-val-p-nitroanilide . Visual inspection of the samples after 10 minutes of incubation indicated a colour change to yellow only for infected sputum samples A, B, and C.

Sputum Sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour	no

		change	
E	no	No colour change	no
F	no	No colour change	no

[00607] Example 34: Elastase based test – cellulose paper based system

[00608] Preparation of diagnostic system.

[00609] N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide is dissolved at a concentration of 281 mM in dimethoxysulfoxide. 2-5 µl of this solution, preferentially 2 µl were pipetted onto Whatman-Filterpapier.

[00610] The test system of Whatman-Filterpapier incubated with N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide is then incubated with 1-5 µl, preferentially 2 µl sputum sample and 2 µl 2µl NaCl (50mM) and incubated at room temperature for 5 minutes. Thereafter, infection will be indicated by a colour change of the filterpaper to yellow. Non infected sputum samples will not change colour .

[00611] Using small swabs of Whatman-Filterpapier, infected samples (A, B) and non infected samples (C, D) were taken and placed into the diagnostic system described in 1C containing the substrate mM N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide . Visual inspection of the samples after 10 minutes of incubation indicated a colour change to yellow only for infected samples A, B.

Sputum Sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	no	No colour change	no
D	no	No colour change	no

[00612] Example 35: Cathepsin based test - liquid system

[00613] N-succinyl-ala-ala-pro-phe-p-nitroanilide is dissolved at a concentration of 20 mM in dimethoxysulfoxide and diluted in 0.1 M HEPES buffer (pH 7.4, containing 0.5 M NaCl). The final concentration of N-methoxysuccinyl-ala-ala-pro-phe p-nitroanilide can be between 0.5 to 5 mM, and is preferentially 3 mM.

[00614] A volume between 1 and 5 µL of sputum sample is added to the test system, preferentially 2-4 µL and mixed by manual shaking. This mixture is incubated at 37°C for 20

minutes. Thereafter, infection will be indicated by a colour change to yellow. Mixtures containing non infected samples will not change colour.

[00615] 5 μ L of infected (A, B, C) and non infected sputum samples (D, E, F) were incubated with the diagnostic system described in 1A comprising a substrate concentration of 3.0 mM N-

[00616] ala-ala-pro-phe-p-nitroanilide. Visual inspection of the samples after 20 to 30 minutes of incubation indicated a colour change to yellow only for infected samples A, B, and C.

Sputum Sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00617] **Example 36: Cathepsin based test – Cellulose based system**

[00618] N-methoxysuccinyl-ala-ala-pro-phe-p-nitroanilide is dissolved at a concentration of 20 mM in dimethoxysulfoxide and diluted in 0.1 M HEPES buffer (pH 7.4, containing 0.5 M NaCl). The final concentration of N-methoxysuccinyl-ala-ala-pro-phe-p-nitroanilide can be between 0.5 to 10 mM, and is preferentially 5.00 mM. 10 μ L of this solution were pipetted onto Whatman filter paper.

[00619] The test system of Whatman-Filterpapier incubated with N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide is then incubated with 1-5 μ l, preferentially 2 μ l sputum sample and 2 μ l 2 μ l NaCl (50mM) and incubated at room temperature for 20 minutes. Thereafter, infection will be indicated by a colour change of the filterpaper to yellow. Non infected sputum samples will not change colour,

[00620] Using small swabs of Whatman-Filterpapier, infected samples (A, B) and non-infected samples (C, D) were taken and placed into the diagnostic system described in 1C containing the substrate mM N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide. Visual inspection of the samples after 210 minutes of incubation indicated a colour change to yellow only for infected samples A, B.

Sputum Sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	no	No colour change	no
D	no	No colour change	no

[00621] Example 37: Myeloperoxidase based test - liquid system

[00622] For this diagnostic tool, six different substrates can be used, namely TMB, ABTS, Guaiacol, 4-Amino-3-methoxy benzoic acid and Fast Blue RR. 3350 µl of succinate buffer (pH 5.4) comprising 0.3 M sucrose were used, 15 µl 1% H₂O₂ and 7 µl of the substrates (5-40 mM ABTS, 20-150 mM TMB, 0.025 mM of Fast Blue RR, 10-50 mM 4-Amino-3-methoxy benzoic acid and 10-20 mM Guaiacol) were added. TMB (3, 3', 5, 5'-Tetramethylbenzidine) is firstly dissolved in N, N- Methylformamide, Fast Blue RR is dissolved in ethanol, while ABTS can be dissolved in water and 4-Amino-3-methoxy benzoic acid is dissolved in DMSO. Guaiacol. 100 µl of the solutions are pipetted into a microtiterplate.

[00623] A volume between 1 and 7 µL of sputum sample is added to the test system, preferentially between 4-6 µL and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 10 minutes. Thereafter, infection will be indicated by a colour change from colourless to blue (TMB or green (ABTS), respectively and to brown to red (4-Amino-3-methoxy benzoic acid and Guaiacol) Mixtures containing non infected samples will not change colour

[00624] Infected Sputum samples of (A, B, C) and non infected sputum samples (D, E, F) are incubated with the diagnostic system described in IA containing the different substrates. Visual inspection of the samples after 5 minutes of incubation indicated a colour change to blue in case of TMB only for infected samples A, B, and C.

Sputum Sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	blue	yes
B	yes	blue	yes
C	yes	blue	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00625] Example 38: Myeloperoxidase based test – Cellulose based system

[00626] 1,5 µl of H₂O₂ (50 mM) and 1,5 µl of the substrates (109 mM ABTS, 454 mM TMB, 230 mM Fast Blue, x mM Guaiacol, x mM 4-Amino-3-methoxy benzoic acid) were incubated on small Whatmann Filterpaper discs.

[00627] The test system of Whatman-Filterpapier incubated with the different MPO substrates is then incubated with 1-5 µl, preferentially 2 µl sputum sample and incubated at room temperature for 5 minutes. Thereafter, infection will be indicated by a colour change of the filterpaper to blue, green or brown-red. Non infected sputum samples will not change colour.

[00628] Using small swabs of Whatman-Filterpapier, infected samples (A, B) and non infected samples (C, D) were taken and placed into the diagnostic system described in 1C containing the substrates TMB, ABTS, Fast Blue RR, Guaiacol and 4A3Mba. Visual inspection of the samples after 5 minutes of incubation indicated a colour change to brown (4A3Mba) in case of for infected samples A, B.

Sputum Sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	brown	yes
B	yes	brown	yes
C	no	No colour change	no
D	no	No colour change	no

[00629] Example 39: Phospholipase A2 based test - liquid system

[00630] For this diagnostic tool, 4-Nitro-3-octanoylbenzoic acid as Phospholipase A2 - substrate was used. 4-Nitro-3-octanoylbenzoic acid (1,7 mM) is dissolved in assay buffer containing 50 mM Tris/HCl buffer pH 7,2, 150 mM KCL and 10 mM CaCl₂. 190 µl of the buffer are pipetted into a microtiterplate.

[00631] A volume between 5 and 12 µL of sputum wound fluid sample is added to the test system, preferentially between 8 and 10 µl and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 30 minutes. Thereafter, infection will be indicated by a colour change from colourless to yellow. Infected sputum samples (A, B) and non infected samples (E, F) are incubated with the diagnostic system described in IA. Visual inspection of the samples after 30 minutes of incubation indicated a colour change to yellow only for infected samples A, B, and C.

Sputum sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00632] Example 40: Catalase based test - liquid system

[00633] For this diagnostic tool: Purpald as substrate for catalase was used. Purpald is dissolved in water (50-100 mM?). 200 µl of substrate solution are pipetted into a microtiterplate.

[00634] A volume between 5 and 12 µL of sputum sample is added to the test system, preferentially between 8 and 10 µl and mixed by manual shaking for 10 seconds. This mixture is incubated at room temperature for 30 minutes. Thereafter, infection will be indicated by a colour change from colourless to dark violet.

[00635] Infected wound fluid samples (A, B, C) and non-infected samples (D, E, F) are incubated with the diagnostic system described in IA. Visual inspection of the samples after 30 minutes of incubation indicated a colour change to violet only for infected sputum samples A, B, and C.

Sputum sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	yellow	yes
B	yes	yellow	yes
C	yes	yellow	yes
D	no	No colour change	no
E	no	No colour change	no
F	no	No colour change	no

[00636] Example 41: Lysozyme based test – liquid system

[00637] An amount between 1 and 15 mg *Micrococcus lysodeikticus* pep- tidoglycan is suspended in 15 ml 0.1 M KH₂PO₄ buffer (pH 7.0) . Preferentially, an amount between 8 and 10 mg is suspended. 290 µL of this solution is pipetted into a transparent microtiterplate.

[00638] A volume of 10 µL of sputum is added to the test system and mixed by manual shaking for 10 seconds. This mixture is incubated at 37°C for 15 minutes. Thereafter, infection will be indicated by a decrease in turbidity. Mixtures containing non infected samples will not change.

[00639] 10 μ L of infected (A, B, C) and non-infected samples (D, E, F) were incubated with the diagnostic system described in 2A containing *Micrococcus lysodeikticus* as a substrate for lysozyme. Visual inspection of the samples after 20 minutes of incubation indicated a change of turbidity only for infected samples A, B, and C.

Sputum sample	Infection according to diagnosis of medical doctors	Test response	Infection according to test
A	yes	clear	yes
B	yes	clear	yes
C	yes	clear	yes
D	no	No turbidity loss	no
E	no	No turbidity loss	no
F	no	No turbidity loss	no

[00640] **Lysozyme Activity measurement for the detection of infection**

[00641] Dyed peptidoglycan.

[00642] One suspension of every LPG-RB5 variant (1.5 mg/ml) was prepared (NaCl-solution, 0.9%) and divided into 6 1 ml samples each (3 positive, 3 negative). The positive controls were mixed with a Lysozyme stock solution (10 μ l, 1,000,000 U/ml) to a final Lysozyme activity of 10,000 U/ml. All samples were incubated at 37°C for 60 min and centrifuged afterwards (5 min, 10000 g). A development of blue colour was detected in the supernatants. 100 μ l of each supernatant was transferred into a 96-well plate and the absorbance was measured at 597 nm.

[00643] Other embodiments:

[00644] The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this disclosed technology for those used in the preceding examples.

[00645] From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this disclosed technology and, without departing from the spirit and scope thereof, can make various changes and modifications of the disclosed technology to adapt it to various usages and conditions.

[00646] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosed technology belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the disclosed technology, suitable methods and materials are described in the foregoing paragraphs. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. In case of conflict, the present specification, including definitions, will control.

[00647] All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All published references, documents, manuscripts, scientific literature cited herein are hereby incorporated by reference. All identifier and accession numbers pertaining to NCBI, GENBANK, EBI, PUBMED databases that are cited herein are hereby incorporated by reference.

[00648] While preferred embodiments of the present disclosed technology have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the disclosed technology. It should be understood that various alternatives to the embodiments of the disclosed technology described herein may be employed in practicing the disclosed technology. It is intended that the following claims define the scope of the disclosed technology and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

What is claimed is:

1. A chemical entity comprising a compound of Formula I:



Formula I

wherein

A is an anchor;

I is an indicator region.

2. The chemical entity of claim 1, wherein the anchor A is covalently associated with the indicator I via a covalent interaction to form a recognition site S.
3. The chemical entity of claim 2, wherein the S is specific for a wound-specific hydrolase.
4. The chemical entity of claim 1, wherein A comprises a polysaccharide, a cellulose, a polyacrylate, a polyethyleneimine, a polyacrylamide, a peptidoglycan, or a chitosan, or a monomer thereof, an oligomer thereof, a derivative thereof, a mixture or a combination thereof.
5. The chemical entity of claim 4, wherein the anchor A comprises chitosan or a monomer thereof, an oligomer thereof, a derivative thereof, a mixture or a combination thereof.
6. The chemical entity of claim 5, wherein the monomer of chitosan comprises D-glucosamine and N-acetyl-D-glucosamine, an oligomer thereof, or a combination thereof.
7. The chemical entity of claim 5, wherein the chitosan comprises at least two units of D-glucosamine, N-acetyl-D-glucosamine or a combination thereof.
8. The chemical entity of claim 5, wherein the chitosan derivative comprises a randomly substituted partial N-, partial O-acetylated chitosan, chitosan oligosaccharide, carboxymethyl chitosan, or hydroxyalkyl chitosan.
9. The chemical entity of claim 8, wherein the chitosan derivative comprises randomly substituted partial N-, partial O-acetylated chitosan.
10. The chemical entity of claim 9, wherein the chitosan derivative is an acetylated chitosan comprising a degree of acetylation (DA) between about 40% to about 90%.
11. The chemical entity of claim 9, wherein the chitosan derivative is an acetylated chitosan comprising a degree of acetylation (DA) of greater than 50%.
12. The chemical entity of claim 9, wherein the chitosan derivative is halogenated.
13. The chemical entity of claim 5, wherein the anchor A comprises a chitosan compound or a derivative thereof selected from the group consisting of chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitooligosaccharide; 2-acetamido-2-deoxy-D-

glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; an oligomer thereof; or a combination thereof.

14. The chemical entity of claim 1, wherein the indicator (I) or a motif therein attached to the anchor is a substrate for a glycosidase.

15. The chemical entity of claim 12, wherein the indicator (I) or a motif therein attached to the anchor is a substrate for a glycosidase which is lysozyme.

16. The chemical entity of claim 15, wherein the indicator (I) or a motif therein is attached to an 1 α -carbon of chitosan or a monomer thereof, an oligomer thereof, or a derivative thereof.

17. The chemical entity of claim 1, wherein the indicator comprises a dye containing a sulfonylethyl-hydrogensulphate-reactive-group or a dye containing a dichlortriazine reactive-group.

18. The chemical entity of claims 17, wherein the dye containing a sulfonylethyl-hydrogensulphate-reactive-group is reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16 or a combination thereof.

19. The chemical entity of claims 17, wherein the dye containing a dichlortriazine reactive-group is reactive blue 4, reactive red 120, reactive blue 2, reactive green 19, or reactive brown 10, or a combination thereof.

20. The chemical entity of claim 1, wherein the indicator comprises a detectable label selected from the group consisting of a luminescent molecule, a chemiluminescent molecule, a fluorochrome, a fluorescent quenching agent, a lipid, a colored molecule, a radioisotope, a scintillant, biotin, avidin, streptavidin, protein A, protein G, an antibody or a fragment thereof, a polyhistidine, Ni²⁺, a Flag tag, a myc tag, a heavy metal, and an enzyme.

21. The chemical entity of claim 1, wherein the anchor A is conjugated with the indicator I directly via a glycosidic linkage.

22. The chemical entity of claim 21, wherein the anchor comprises a compound selected from the group consisting of chitosan, N-acetyl chitosan; oligo- β -D-1,4-glucosamine; acetyl-D-glucopyranoside; N-Acetylglucosamine (GlcNAc); glucosamine dimer (GlcNAc)₂; acetyl-chitosan; chitobiose octaacetate; a chitoooligomer comprising the structure (GlcNAc)_n wherein n=4, 5, or 6; a chitoooligosaccharide; 2-acetamido-2-deoxy-D-glucopyranoside; 2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside; or a combination thereof.

23. The chemical entity of claim 21, wherein the indicator is selected from the group consisting of reactive black 5, remazol brilliant blue, reactive violet 5 or reactive orange 16, reactive blue 4, reactive red 120, reactive blue 2, reactive green 19 and reactive brown 10, or a combination thereof.

24. The chemical entity of claim 1, wherein the indicator comprises a hydrophilic binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*) or the hydrophobic binding module (PDB) from Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*) or a chimeric variant thereof.
25. The chemical entity of claim 24, wherein the anchor comprises cellulose or a derivative thereof or polyethylene terephthalate or a derivative thereof.
26. The chemical entity of claim 25, wherein the indicator comprises a hydrophilic binding module (CBM) from Cellobiohydrolase I (*Trichoderma reesei*) and the anchor comprises cellulose or a derivative thereof, wherein the indicator is hydrophilically associated with the anchor.
27. The chemical entity of claim 24, wherein the indicator comprises Polyhydroxyalkanoate depolymerase (*Alcaligenes faecalis*) and the anchor comprises polyethylene terephthalate or a derivative thereof, wherein the indicator is hydrophobically associated with the anchor.
28. A chimeric construct comprising polynucleotide sequences encoding a *trxA* (thioredoxin) or a portion thereof, a spacer sequence encoding a histidine-tag and an enterokinase cleavage site, or a portion thereof.
29. The chimeric construct of claim 28, wherein the polynucleotide comprises *trxA* gene.
30. The chimeric construct of claim 28, wherein the spacer comprises a polyhistidine tag.
31. The chimeric construct of claim 28, wherein the spacer comprises a hexahistidine (His6) tag.
32. The chimeric construct of claim 28, which comprises the enterokinase cleavage site (Asp Asp Lys).
33. The chimeric construct of claim 28, which further comprises an *elasub1* sequence.
34. The chimeric construct of claim 28, wherein the *elasub1* sequence is located subsequent to the enterokinase cleavage site.
35. The chimeric construct of claim 28, wherein the *elasub1* sequence encodes for functional amino acids selected from the group consisting of cysteine, lysine, arginine, glutamine, asparagine, glutamic acid, aspartic acid, serine, threonine or tyrosine.
36. The chimeric construct of claim 33, further comprising nucleic acids encoding a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence.
37. The chimeric construct of claim 33, further comprising nucleic acids encoding a hydrophobic *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) directly downstream to the *elasub1* sequence.

38. The chimeric construct of claim 36, further comprising nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition in the *elasub1* sequence.

39. The chimeric construct of claim 37, further comprising nucleic acids encoding an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition in the *elasub1* sequence.

40. A vector comprising an expression control sequence and the chimeric construct of claim 37.

41. A vector comprising an expression control sequence and the chimeric construct of claim 38.

42. A host cell comprising the vector of claim 40.

43. A host cell comprising the vector of claim 41.

44. The host cell of claim 42, which is a bacterial cell or an insect cell.

45. The host cell of claim 43, which is a bacterial cell or an insect cell.

46. A method of making the protein encoded by a chimeric construct comprising polynucleotide sequences encoding:

- a. a *trxA* (thioredoxin) or a portion thereof;
- b. a spacer sequence encoding a histidine-tag;
- c. an enterokinase cleavage site or a portion thereof;
- d. an *elasub1* sequence subsequent to the enterokinase cleavage site;
- e. a hydrophilic binding module (CBM) directly downstream to the *elasub1* sequence; and
- f. an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites in the *elasub1* sequence,

comprising culturing the host cell of claim 42 under conditions sufficient to induce expression of the chimeric construct and obtaining the chimeric protein from the cell culture; and optionally purifying the construct by His-tag affinity purification.

47. A method of making the protein encoded by a chimeric construct comprising polynucleotide sequences encoding:

- a. a *trxA* (thioredoxin) or a portion thereof;
- b. a spacer sequence encoding a histidine-tag;
- c. an enterokinase cleavage site or a portion thereof;
- d. an *elasub1* sequence subsequent to the enterokinase cleavage site;

- e. a hydrophobic *Alcaligenes faecalis* Polyhydroxyalkanoate depolymerase (PDM) sequence directly downstream to the *elasub1* sequence; and
- f. an HLE recognition site (Ala-Ala-Pro-Val) or CatG recognition site (Ala-Ala-Pro-Phe) or both HLE and CatG recognition sites in the *elasub1* sequence,

comprising culturing the host cell of claim 43 under conditions sufficient to induce expression of the chimeric construct and obtaining the chimeric protein from the cell culture; and optionally purifying the construct by His-tag affinity purification.

- 48. A polypeptide encoded by the chimeric construct of claim 37.
- 49. A polypeptide encoded by the chimeric construct of claim 38.
- 50. A composition comprising the polypeptide of claim 48 and a dye or a label.
- 51. A composition comprising the polypeptide of claim 49 and a dye or a label.
- 52. A chemical entity comprising an anchor and the polypeptide of claim 48.
- 53. A chemical entity comprising an anchor and the polypeptide of claim 49.
- 54. The chemical entity of claim 52, which comprises a hydrophilic anchor comprising cellulose or a derivative thereof.
- 55. The chemical entity of claim 53, which comprises a hydrophobic anchor comprising polyethylene terephthalate or a derivative thereof.
- 56. A method for the determining the presence or absence of an enzyme selected from the group consisting of HLE and CatG in a biological sample, comprising contacting the sample with the composition of claim 50 or a chemical entity of claim 52; and detecting the label.
- 57. A method for the determining the presence or absence of an enzyme selected from the group consisting of HLE and CatG in a biological sample, comprising contacting the sample with the composition of claim 51 or a chemical entity of claim 53; and detecting the label.
- 58. A method for diagnosing an infected or a chronic wound, comprising, contacting the wound with the composition of claim 50 or a chemical entity of claim 52; and detecting the label.
- 59. A method for diagnosing an infected or a chronic wound, comprising, contacting the wound with the composition of claim 51 or a chemical entity of claim 53; and detecting the label.
- 60. The method of claim 58, comprising *in situ* detection of the wound via visual inspection.
- 61. The method of claim 59, comprising *in situ* detection of the wound via visual inspection.
- 62. A polypeptide comprising the sequence set forth in
 - (a) X_yAAPX_y-Z (SEQ ID NO: 1),
 - (b) X_yAAPX_y-L-Z (SEQ ID NO: 2),
 - (c) X_yAAP(V/F/A)X_y-Z (SEQ ID NO: 3), or
 - (d) X_yAAP(V/F/A)X_y-L-Z (SEQ ID NO: 4),

wherein each X is independently any amino acid,
 y is each, independently, an integer between 0 and 200,
 L is a linking moiety, and
 Z comprises a detectable label.

63. The polypeptide of claim 62, wherein y is, each, independently an integer from 1 to 50.
64. The polypeptide of claim 62, wherein y is, each, independently an integer from 1 to 10.
65. The polypeptide of claim 62, wherein y is, each, independently an integer from 1 to 6.
66. The polypeptide of claim 62, wherein each of the peptides comprising the sequence X_yAAPX_y-Z (SEQ ID NO: 1), X_yAAPX_y-L-Z (SEQ ID NO: 2), $X_yAAP(V/F/A)X_y-Z$ (SEQ ID NO: 3) or $X_yAAP(V/F/A)X_y-L-Z$ (SEQ ID NO: 4), are each, individually, labile to elastase.
67. The polypeptide of claim 62 further comprising an amine protection group.
68. The polypeptide of claim 62 further comprising an amine protection group which is fluorenylmethyloxycarbonyl (Fmoc).
69. A composition comprising the polypeptide of claim 62 and a carrier.
70. A chemical entity comprising an anchor region (A) and an indicator region (I) comprising a peptide of claim 62.
71. A polypeptide sequence comprising the amino acid sequence set forth in
 - (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or
 - (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6),
 wherein

X is each, independently any amino acid;
 y is each, independently, a number selected from 0 to 6;
 N^4 is selected from alanine, glycine, valine, and glutamine;
 N^3 is selected from alanine, glycine, proline, lysine, and serine;
 N^2 is selected from proline, alanine, and glycine;
 N^1 is selected from serine, lysine, phenylalanine, arginine, leucine, and methionine; and
 L is a linking moiety; and
 Z comprises a detectable label.
72. The polypeptide of claim 71, wherein y is, each, independently an integer from 1 to 3.
73. The polypeptide of claim 71, wherein each of the peptides comprising the sequence (a) $X_yN^4N^3N^2N^1X_y-Z$ (SEQ ID NO: 5), or (b) $X_yN^4N^3N^2N^1X_y-L-Z$ (SEQ ID NO: 6), are each, individually, labile to cathepsin G.
74. The polypeptide of claim 71 further comprising an amine protection group.

75. The polypeptide of claim 71 further comprising an amine protection group which is fluorenylmethyloxycarbonyl (Fmoc).
76. A composition comprising the polypeptide of claim 71 and a carrier.
77. A chemical entity comprising an anchor region (A) and an indicator region (I) comprising a peptide of claim 71.

1/10

FIG. 1

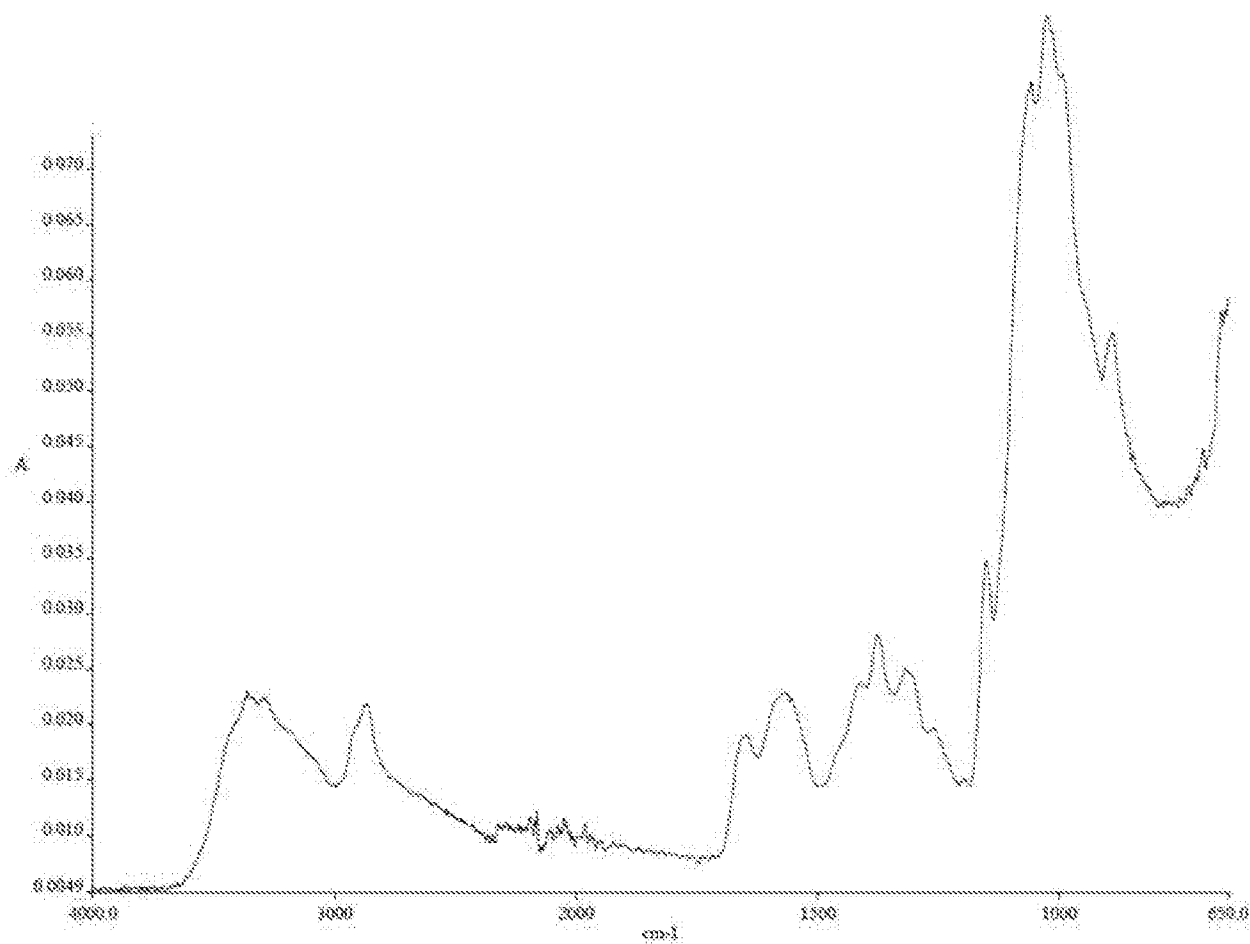


FIG. 2

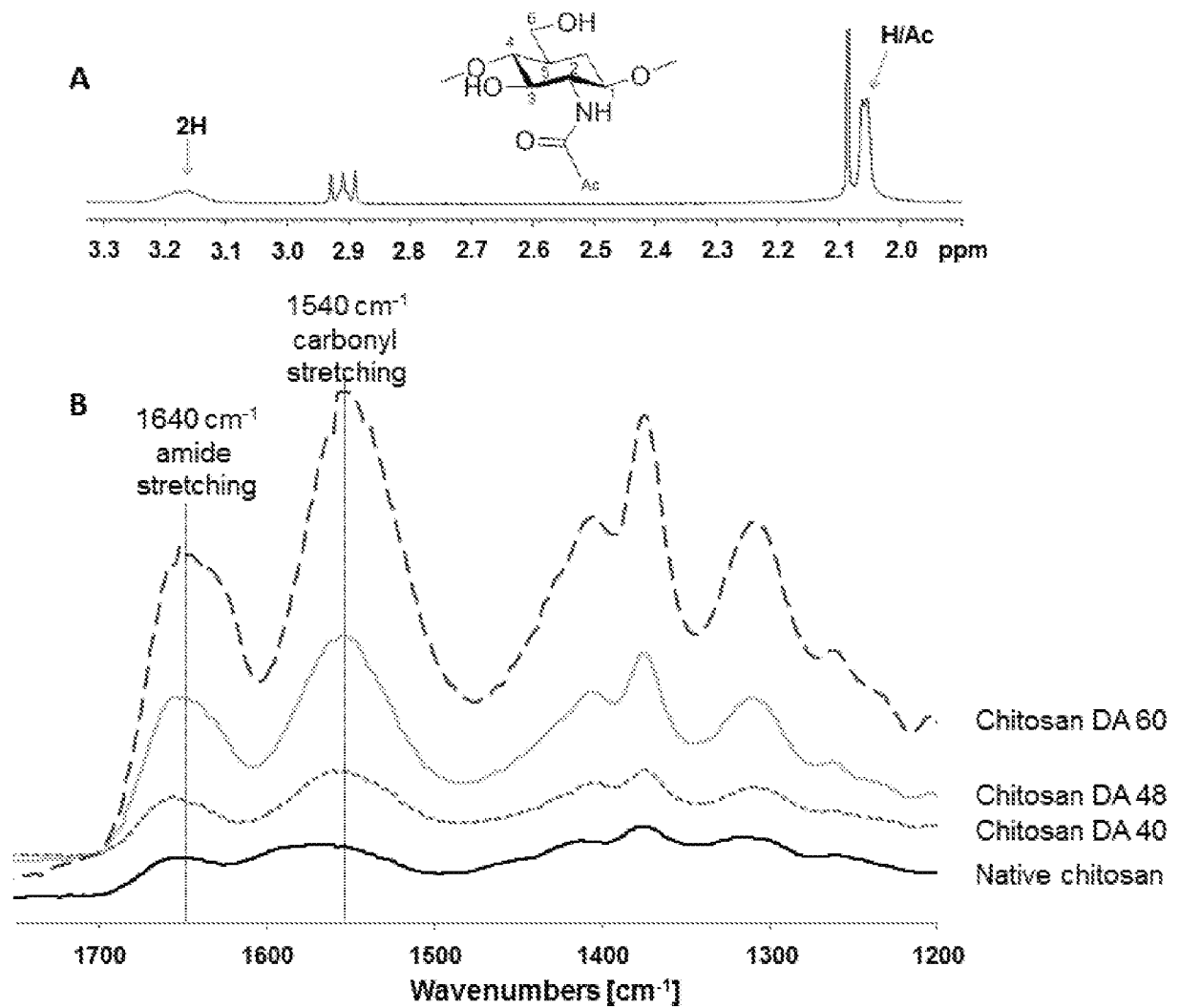


FIG. 3

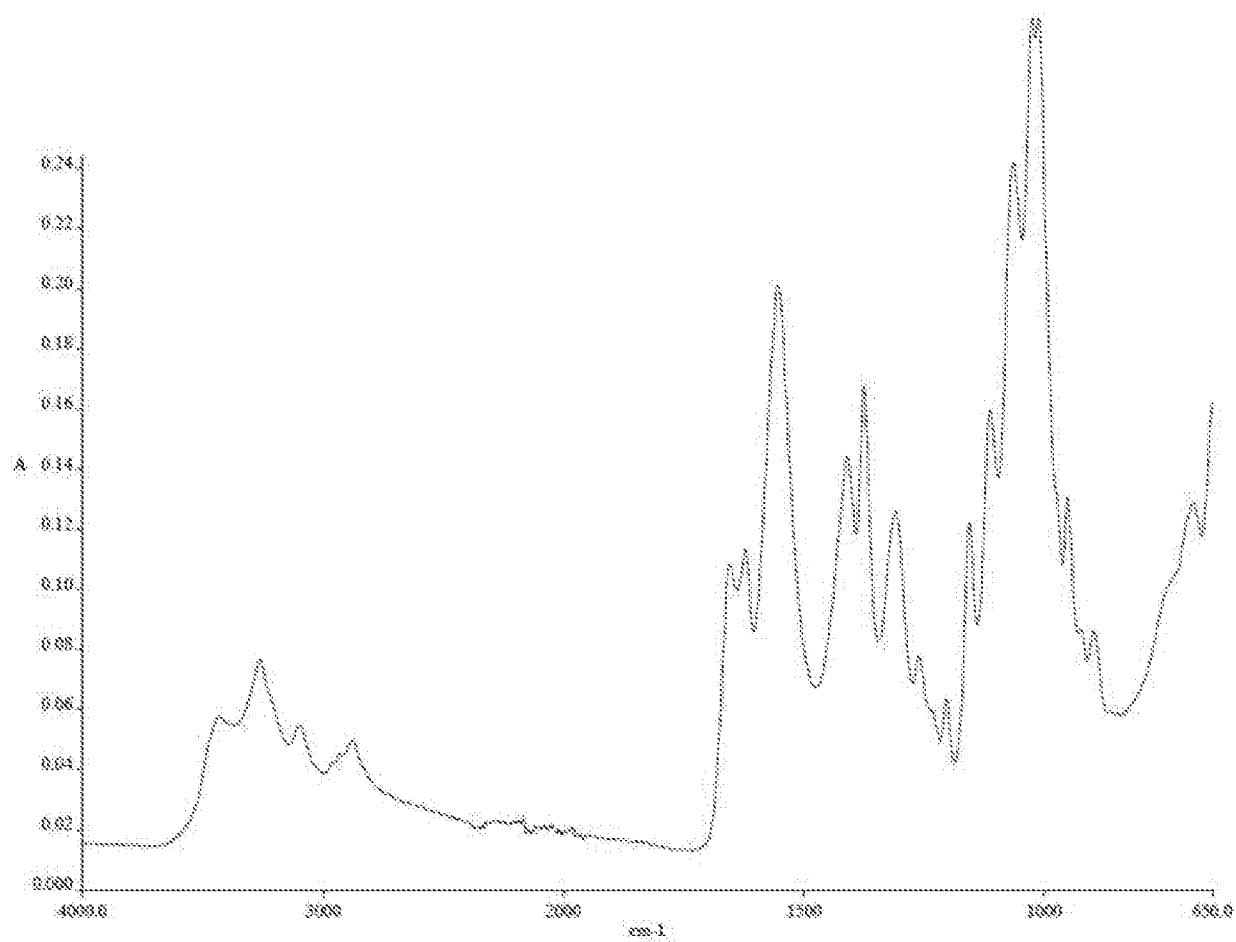


FIG. 4

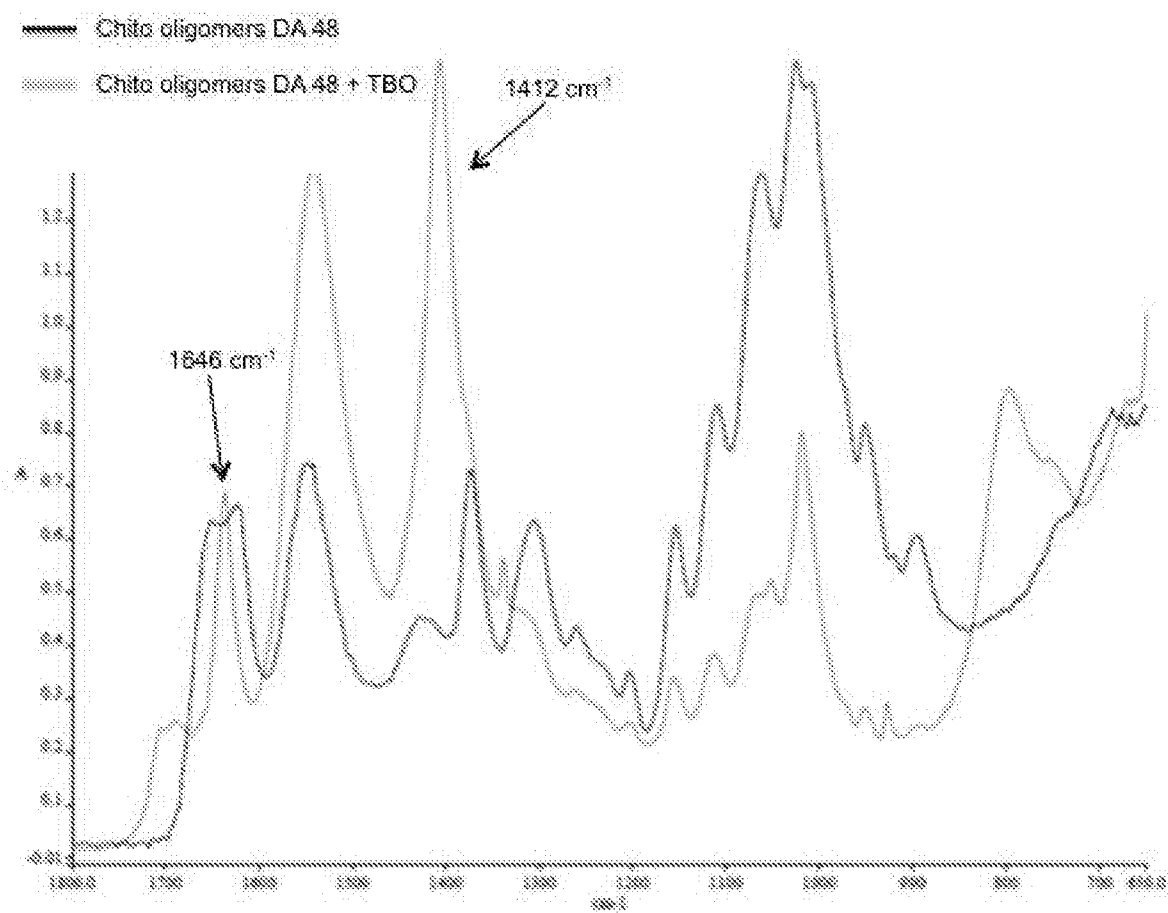


FIG. 5

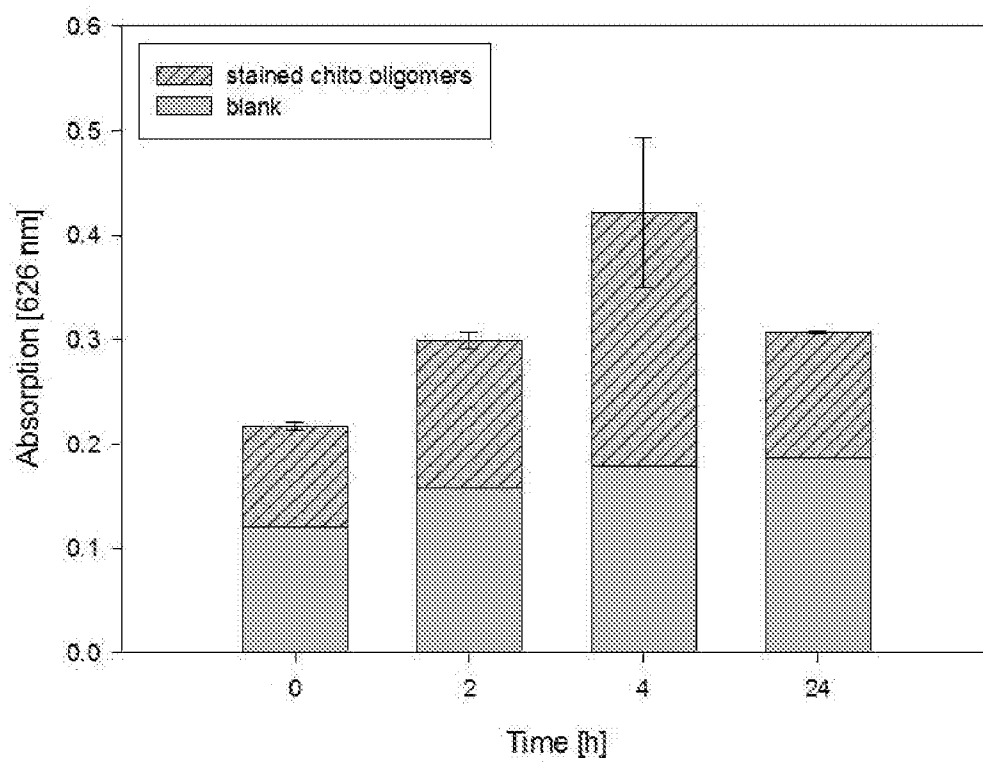


FIG. 6

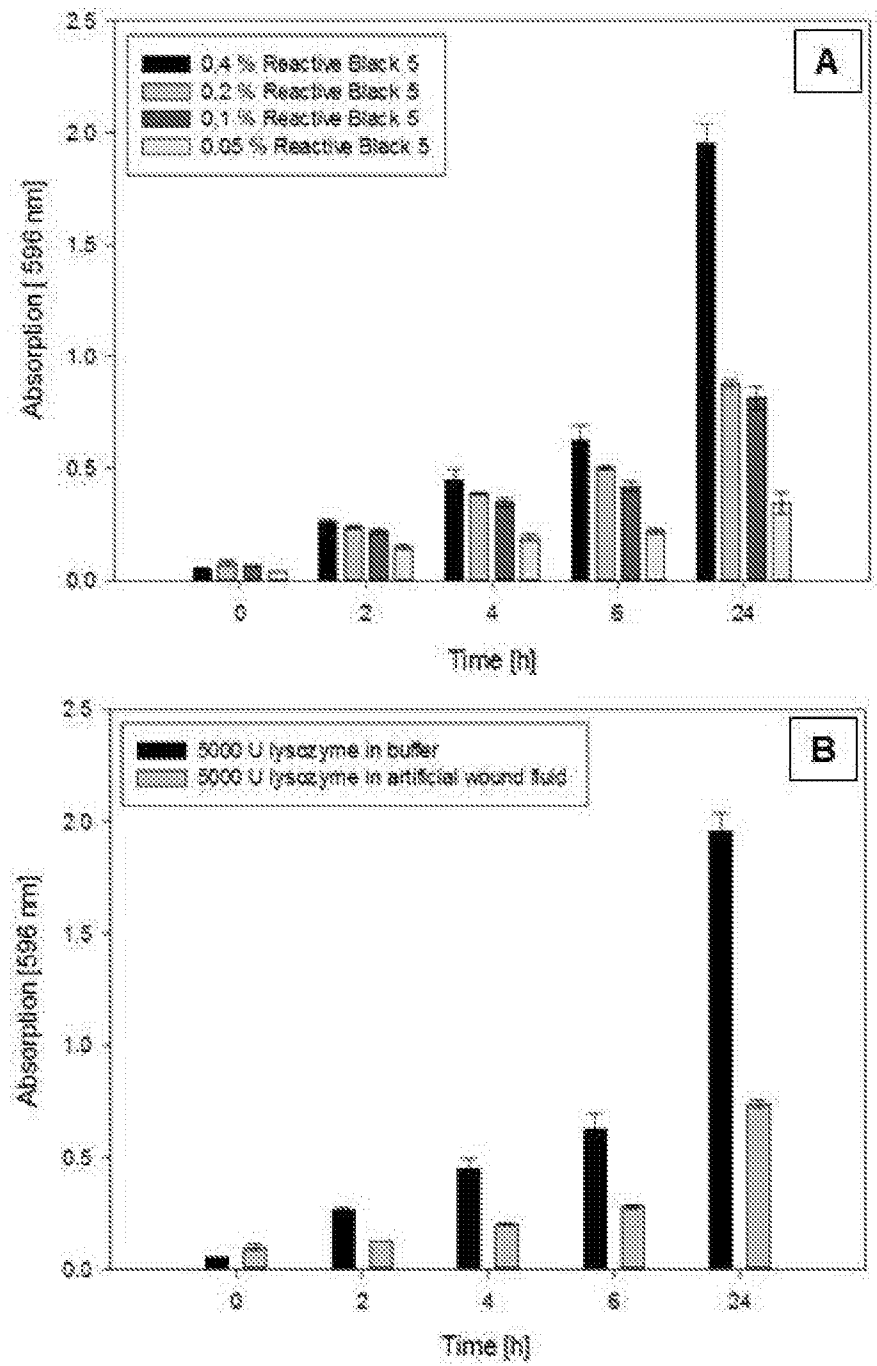


FIG. 7

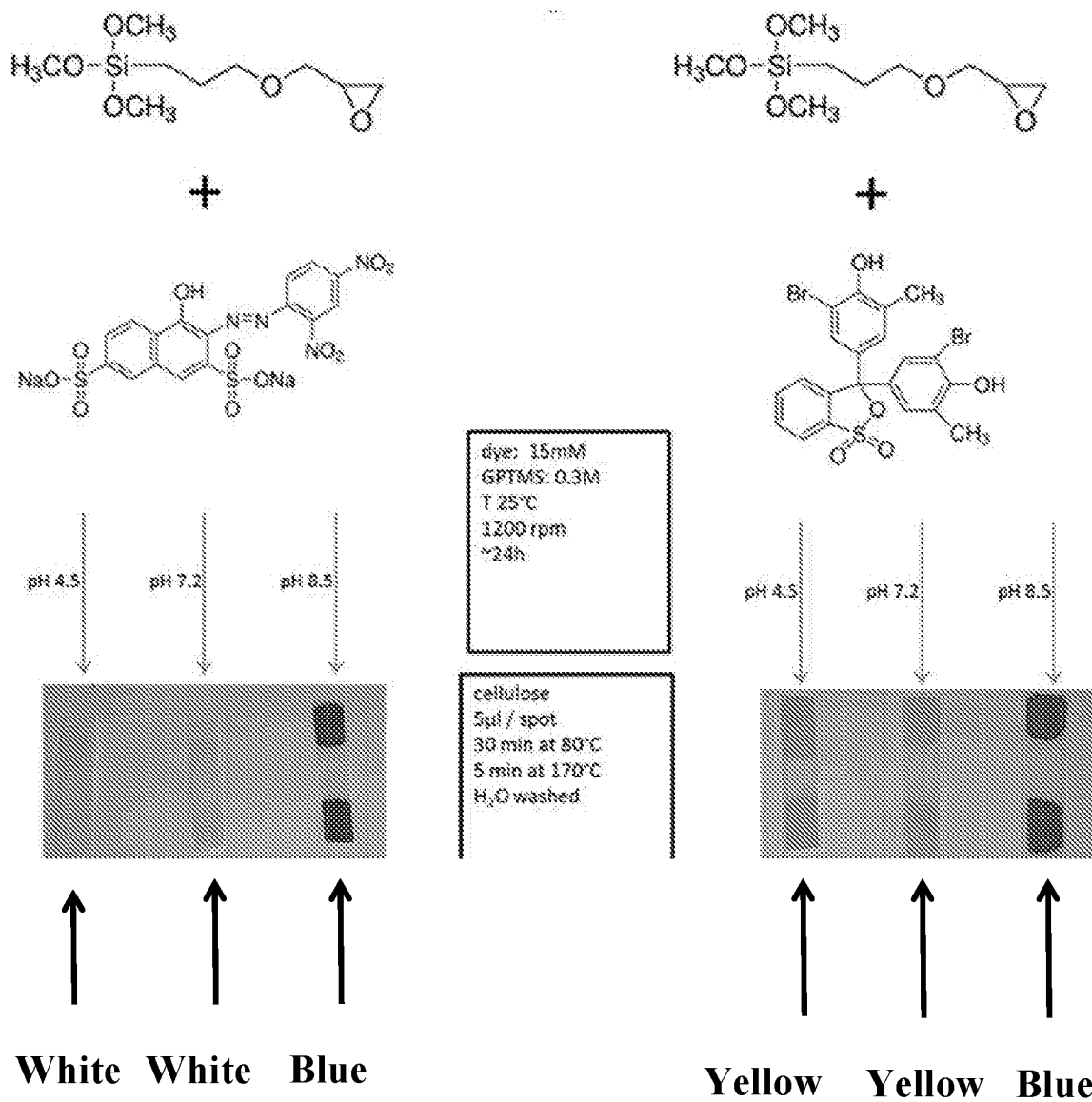


FIG. 8

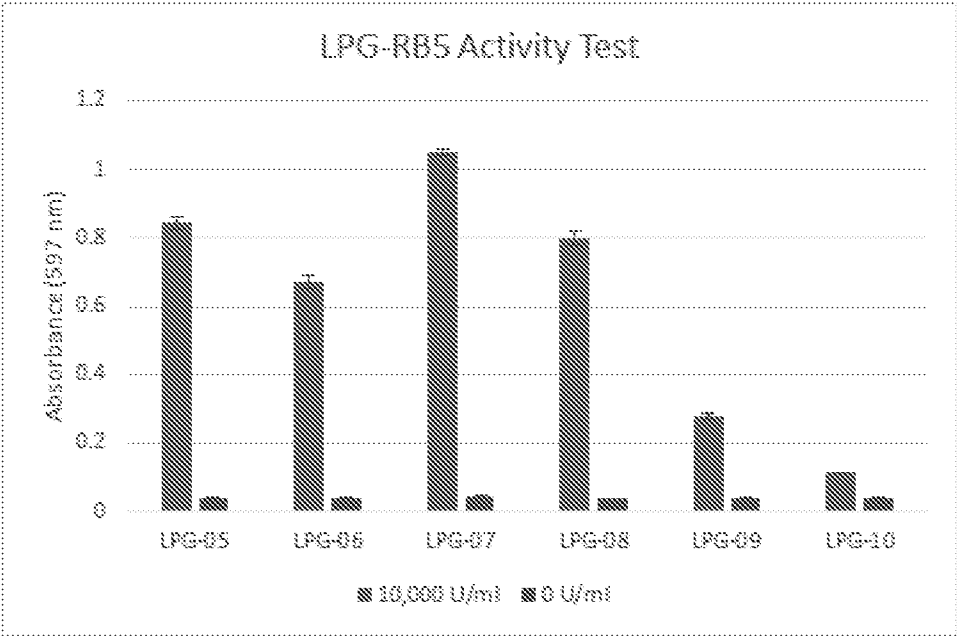


FIG. 9

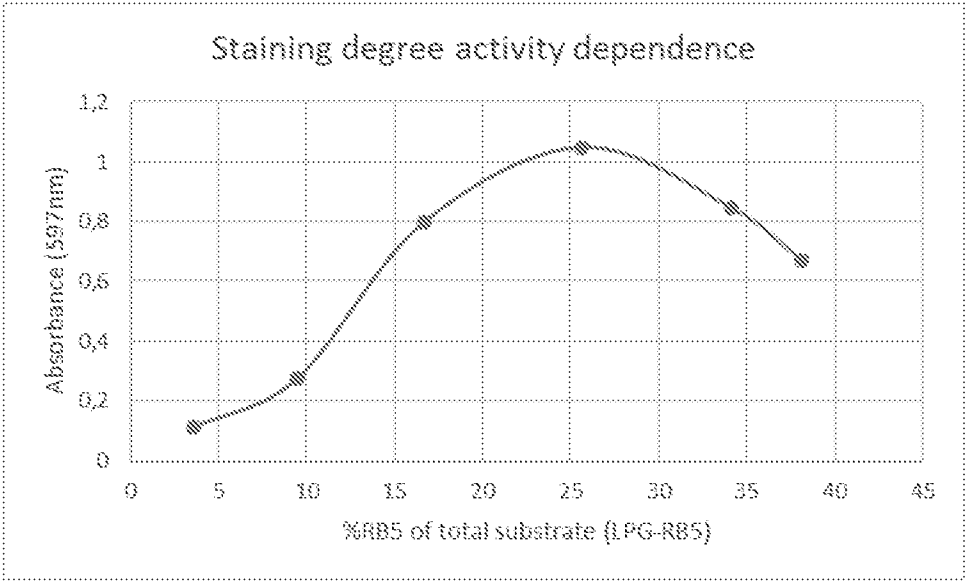


FIG. 10

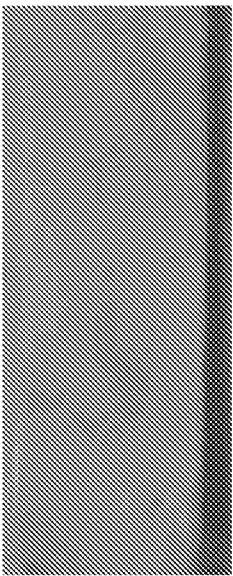
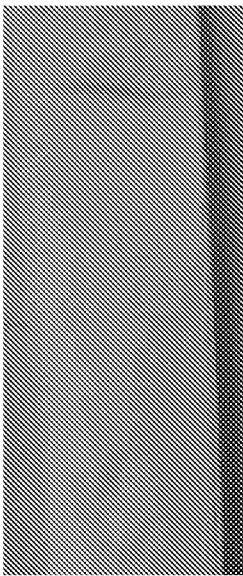
0 U/ml Elastase	1 U/ml Elastase
	

FIG. 11

Multiple Sequence Alignment of the Various Constructs

```

CatGSub2CBM      MCGGGGSAAPFEQLEWLQGGGGSCGGGSAAPFSEALE--QWGGGGSCGGGGG-----
ElaSub2CBM       MCGGGGSAAPVEQLEWLQGGGGSCGGGSAAPVSEALE--QWGGGGSCGGGGG-----
CatGSub2CBMHis   MCGGGGSAAPFEQLEWLQGGGGSCGGGSAAPFSEALE--QWGGGGSCGGGGG-----
ElaSub2CBMHis    MCGGGGSAAPVEQLEWLQGGGGSCGGGSAAPVSEALE--QWGGGGSCGGGGG-----
ElaSub1CBM       -----MGGSCGGGSAAPVGGGSAAPVGGGSPPGNNGTTTTRR
ElaSub1CBMHis    -----MGGSCGGGSAAPVGGGSAAPVGGGSPPGNNGTTTTRR
CatGSub1CBM      -----MGGSCGGGSAAPFGGGSAAPFGGGSPPGNNGTTTTRR
CatGSub1CBMHis   -----MGGSCGGGSAAPFGGGSAAPFGGGSPPGNNGTTTTRR

                *****.. . .      *****  **,

CatGSub2CBM      -AAPFAGAGAGTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCL-----
ElaSub2CBM       -AAPVAGAGAGTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCL-----
CatGSub2CBMHis   -AAPFAGAGAGTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCLLEHHHHHH
ElaSub2CBMHis    -AAPVAGAGAGTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCLLEHHHHHH
ElaSub1CBM       PATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCL-----
ElaSub1CBMHis    PATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCLLEHHHHHH
CatGSub1CBM      PATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCL-----
CatGSub1CBMHis   PATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTTCQVLNPYYSQCLLEHHHHHH

                *:  ..: .  *****

```