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(54) Title: ENGINEERED CYCLIC PEPTIDES

(57) Abrégé/Abstract:
An engineered cyclic peptide provides structural constraints to resist non-specific degradation in the human body and includes environment- specific cleavage sites to allow release of a linearized peptide upon reaching a target environment. The linearized peptide can include a reporter molecule or a bioactive therapeutic such that the cyclic peptide is essentially inactive at administration and in circulation but becomes reactive only upon exposure to target- specific environmental factors such as a specific combination of differentially-expressed proteases associated with a target tissue or disease state. The peptides can include tuning that modulate distribution by targeting the particle to specific tissue, bodily fluids, or cell types.

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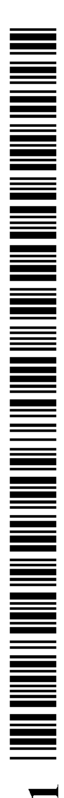

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(54) **Title:** ENGINEERED CYCLIC PEPTIDES

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# ENGINEERED CYCLIC PEPTIDES

## Cross-Reference to Related Applications

This application claims priority to and the benefit of U.S. Provisional Application No. 62/682,492, filed June 8, 2018, the content of which is incorporated by reference.

## Technical Field

The invention relates to engineered cyclic peptides with target or protease-specific cleavage sites.

## Background

New mechanisms for diagnosing, monitoring, and treating serious diseases, such as cancer, continue to evolve and offer new hope for patients. Some promising methods rely on uptake and residence in target areas (e.g., specific tissues or a tumor microenvironment) in order to achieve a therapeutic or diagnostic result. However, a major problem with those types of approaches is that the diagnostic or therapeutic entities are typically degraded, at least in part, before they arrive at the tissue environment in which they are intended to act. Peptides are particularly susceptible to degradation.

Additionally, some diagnostic and therapeutic molecules have the potential for undesirable reactions with off-target tissues or environments or have functionalities that depend on reacting only with a specific target tissue or environment.

Accordingly, many promising diagnostic and treatment methods remain impractical to apply due to the inability to reach a target environment in the body without degrading or otherwise having off-target reactions.

## Summary

The invention provides cyclic peptides that are resistant to degradation in the body while in transit to a target environment. Cyclic peptides provide structural constraints that resist degradation by, for example, proteases in the blood. The cyclic peptides are engineered with one

or more target-specific cleavage sites such that, upon arriving in the target environment, they are cleaved to release a molecule that is reactive in the target environment.

In one example, the target environment may be a tumor microenvironment in which a specific enzyme or set of enzymes are differentially-expressed. According to the invention, a cyclic peptide is engineered with cleavage sites specific to enzymes in the tumor (e.g., unique enzymes expressed preferentially in the tumor). The engineered peptide, in its cyclic form, can travel through the blood and other potentially harsh environments protected against degradation by common non-specific proteases and without interacting in a meaningful way with off-target tissues. Only upon arrival within the specific target tissue and exposure to the required enzyme or combination of enzymes, the cyclic peptide is cleaved to produce a linear molecule that is capable of carrying out a diagnostic or therapeutic function. For purposes of the application and as will be apparent upon consideration of the detailed description thereof, a linear peptide is any peptide that is not cyclic. Thus, for example, a linearized peptide may have various branch chains.

In various embodiments, a linearized peptide of the invention comprises a reporter molecule or a therapeutic molecule such that reaction with the environment includes releasing a detectable reporter to diagnose or monitor a disease state or releasing a bioactive compound such as a therapeutic peptide operable to treat the target tissue.

Cyclic peptides can be engineered with other cleavable linkages, such as ester bonds in the form of cyclic depsipeptides in which the degradation of the ester bond releases a linearized peptide ready to react with its target environment. Thioesters and other tunable bonds can be included in the cyclic peptide to create a timed-release in plasma or other environments. See Lin and Anseth, 2013 Biomaterials Science (Third Edition), pages 716-728, incorporated herein by reference.

According to the invention, cyclic peptides may release a reporter molecule upon linearization and cleavage of a specific cleavage site that is the target of a unique enzyme in the disease microenvironment. For example, a cyclic peptide may include a polyethylene glycol (PEG) scaffold and one or more polypeptide reporters. The cleavable links in the cyclic peptide are specific for different enzymes whose activity is characteristic of a condition of tissue. When administered to a patient, the cyclic peptide can locate to a target tissue, where it is linearized by

the enzymes to release the detectable analytes. The analytes are then detected in a patient sample, such as urine. The detected analytes serve as a report of which enzymes are active in the tissue.

Because enzymes are differentially expressed under the physiological state of interest, such as a disease stage or degree of disease progression, analysis of the sample provides a non-invasive test for the physiological state (e.g., disease stage or condition) of the organ, bodily compartment, bodily fluid, or tissue. Due to the protection against off-target degradation afforded by cyclic peptides of the invention, false-positives are reduced as reporter release and detection is more likely to have occurred due to differential-expression of enzymes specific to a certain disease or physiologic state. False-negatives are also reduced as the cyclic peptide is more likely to remain intact until arriving at the target tissue in order to perform its diagnostic role in the presence of the target-specific enzymes.

Macrocyclic peptides may contain two or more protease-specific cleavage sequences and can require two or more protease-dependent hydrolytic events to release a reporter peptide or a bioactive compound. The protease-specific sequences can be different in various embodiments. In cases where cleavage of multiple sites is required to release the linearized peptide, different protease-specific sequences can increase specificity for the release as the presence of at least two different target-specific enzymes will be required. In other embodiments, multiple different cleavage sites may be provided where cleavage of any single site will release the linearized peptide. In such instances, a single peptide can be tuned to linearize and interact with a variety of environments for two or more different targets. The specific and non-specific proteolysis susceptibility and rate can be tuned through manipulation of peptide sequence content, length, and cyclization chemistry.

Cyclic peptides of the invention may include a carrier molecule. Carrier structure can include multiple molecular subunits and may be, for example, a multi-arm polyethylene glycol (PEG) polymer, a lipid nanoparticle, or a dendrimer or a peptide sequence. The detectable analytes or reporters in diagnostic embodiments may be, for example, polypeptides that are cleaved by proteases that are differentially expressed in tissue or organs under a specified physiological state, e.g., affected by disease. Because the carrier structure and the detectable analytes are biocompatible molecular structures that locate to a target tissue and are cleaved by disease-associated enzymes to release analytes detectable in a sample, compositions of the disclosure provide non-invasive methods for detecting and characterizing a disease state or stage

of an organ or tissue. Because the compositions provide substrates that are released as detectable analytes by enzymatic activity, quantitative detection of the analytes in the sample provide a measure of rate of activity of the enzymes in the organ or tissue. Thus methods and compositions of the disclosure provide non-invasive techniques for measuring both stage and rate of progression of a disease or condition in a target organ or tissue.

Additionally, the cyclic peptides may include additional molecular structures to influence trafficking of the peptides within the body, or timing of the enzymatic cleavage or other metabolic degradation of the particles. The molecular structures may function as tuning domains, additional molecular subunits or linkers that are acted upon by the body to locate the cyclic peptide to the target tissue under controlled timing. For example, the tuning domain may target the particle to specific tissue or cell types. Trafficking may be influenced by the addition of molecular structures in the carrier polymer by, for example, increasing the size of a PEG scaffold to slow degradation in the body.

In certain embodiments, the invention provides a tunable cyclic peptide that reveals enzymatic activity associated with a physiological state, such as disease. When the activity reporter is administered to a patient, it is trafficked through the body to specific cells or specific tissues. Alternatively, the sensor may be designed or tuned so that it remains in circulation, e.g., in blood, or lymph, or both. If enzymes that are differentially expressed under conditions of a particular disease are present, those enzymes cleave the reporter and release a detectable analyte. The cyclic nature of the peptides may be used to resist non-specific degradation of the peptide in circulation while still providing an accessible substrate for cleavage by the target proteases.

Where the cleavage sites are specific to enzymes known to be active in tissue affected by a disease, detection of the analyte is indicative of the disease condition. For example, when the peptide includes cleavage targets of proteases expressed in liver fibrosis, the cyclic peptide is cleaved in the liver to release the detectable analyte into circulation after which renal filtration excretes the detectable analyte in urine. Presence of the analyte in a urine sample from the patient is a signature of liver fibrosis in the patient.

Molecular structures can be included in the cyclic peptide as tuning domains, to tune or modify a distribution or residence time of the cyclic peptide within the subject. The tuning domains may be linked any portion of the cyclic peptide and may be modified in numerous ways. Through the use of tuning domains, one may modify the cyclic peptide’s distribution

within the body depending on in vivo trafficking pathways to a specific tissue, or its residence time within systemic circulation or within a specific tissue. Additionally, the tuning domains may promote effective cleavage of the reporter by tissue-specific enzymes or prevent premature cleavage or hydrolysis.

Cyclic peptides according to the invention provide sensitive, specific, and non-invasive method for detecting disease-associated activity that are able to persist until reaching a target environment and minimize off-target cleavage and associated false positive indications. The cyclic peptides are acted upon in the body of the patient so that the detectable analyte is released in such a manner as to indicate critical disease states at a very early stage. The cyclic peptides may include additional molecular structures as tuning domains that employ the body for sample preparation by presenting a molecular complex that only releases the detectable analyte into a collectable sample when the body processes the cyclic peptide in a detectable manner. The tuning domains, which may be included within the peptide structure to modulate protease cleavage, allow for precise tuning of the biological fate of the cyclic peptide. Additionally, because the detectable analytes are the product of enzymatic activity and the cyclic peptides can be provided in excess, the signal given by the analyte is effectively amplified, and the presence of even very small quantity of active enzyme may be detected. Because the tuning domains can target the cyclic peptide to specific tissue of the body and because the reporter is known to be cleaved by enzymes associated with a disease, the cyclic peptides can provide for very rapid and sensitive disease detection.

Aspects of the invention include an engineered cyclic peptide with one or more cleavage sites cleavable within a target environment, wherein cleavage of the one or more cleavage sites releases a linearized peptide reactive with the target environment. In various embodiments, the target environment may be a tumor or a biological fluid such as blood. Any environment with a distinguishable characteristic (e.g. a specific pH or combination of proteases) that a cleavage site can be tuned to can be targeted using cyclic peptides of the invention.

The cleavage site may be cleaved by an enzyme present in the target environment. The enzyme may be known to be expressed with a certain disease or medical condition and the linearized peptide can be a therapeutic peptide operable to treat the disease or medical condition. The linearized peptide may be bioactive within the target environment. In various embodiments, the cyclic peptide may be a cyclic depsipeptide and the one or more cleavage sites can comprise

an ester bond. In some embodiments, the cyclic peptide can be a macrocyclic peptide with a cyclization chemistry other than ester bonds.

Cyclic peptides of the invention can include a carrier. The carrier can include a poly ethylene glycol (PEG) scaffold of covalently linked PEG subunits. As noted above, the linearized peptide can include a detectable reporter. The detectable reporter may include a volatile organic compound, an elemental mass tag, a peptide comprising one or more D-amino acids, a nucleic acid, or a neoantigen. The detectable reporter may include an elemental mass tag comprising an element of atomic number greater than 20.

In certain embodiments, the reporter can include an antigen detectable by a hybridization assay. The reporter can include a fluorescent donor such as a carboxyfluorescein (FAM) and the cyclic peptide may include a quencher such as $CPQ_2$ (available from CPC Scientific, Sunnyvale, CA) such that target-specific cleavage of the cyclic peptide results in dequenching of the fluorescent donor for subsequent detection. The one or more cleavage sites may comprise a plurality of different cleavage sites which may be cleaved by different enzymes. Cleavage of two or more of the plurality of different cleavage sites may be required to release the linearized peptide. The two or more of the plurality of different cleavage sites may be required to be cleaved in a specific order to release the linearized peptide.

In various embodiments, cyclic peptides may include a tuning domain that modifies a distribution or residence time of the engineered cyclic peptide within a subject when administered to the subject. Tuning domains can include ligands for receptors of a specific cell or a specific tissue type to target the cyclic peptide to the target cell or tissue type. The ligands may promote accumulation of the engineered cyclic peptide in the specific tissue type or body compartment and can include a small molecule, a peptide, an antibody, a fragment of an antibody, a nucleic acid, or an aptamer. The tuning domains can include hydrophobic chains that facilitate diffusion of the engineered cyclic peptide across a cell membrane. Aspects of the invention include methods of preparing or administering cyclic peptides as described herein to a subject for the diagnosis, monitoring, or treatment of a disease.

## Brief Description of the Drawings

FIG. 1 diagrams steps of a method for designing a cyclic peptide.

FIG. 2 shows an engineered macrocyclic peptide.

FIG. 3 shows protease-specific of an engineered macrocyclic peptide

FIG. 4 shows protease-specific cleavage of a double gated engineered macrocyclic peptide.

FIG. 5 shows a controlled-release cyclic peptide.

FIG. 6 shows of linearization of a cyclic depsipeptide through hydrolysis of the ester bond followed by protease-specific cleavage.

FIG. 7 shows activity detection according to certain embodiments.

FIG. 8 shows a bicyclic peptide with 3 cleavage substrates.

FIG. 9 shows an exemplary cleavage of a bicyclic peptide.

FIG. 10 shows a tricyclic peptide with 4 cleavage substrates.

FIG. 11 shows a polycyclic peptide with 4 cyclic structures and 5 cleavage substrates.

FIG. 12 shows a polycyclic peptide with an odd number (n) of cleavage substrates.

FIG. 13 shows a polycyclic peptide with an even number (n) of cleavage substrates.

## Detailed Description

The invention provides cyclic peptides that are structurally resistant to non-specific proteolysis and degradation in the body. Cyclic peptides of the invention include environment-specific cleavage sites such as protease-specific substrates or pH-sensitive bonds that allow the otherwise non-reactive cyclic peptide to release a reactive linearized peptide. Those elements allow the peptides, carrying diagnostic reporter or therapeutic molecules to reach their intended target tissue or environment intact to then carry out their intended purpose at the target. The inclusion of target-environment-specific cleavage sites allows for highly selective targeting for diagnostic reporting and therapeutic delivery. Cyclic peptides can include tuning domains to modify distribution and residence times in various tissues or environments within the body.

Cyclic peptides can require cleavage at a plurality of cleavage sites to increase specificity. The plurality of sites can be specific for the same or different proteases. Polycyclic peptides can be used comprising 2, 3, 4, or more cyclic peptide structures with various combinations of enzymes or environmental conditions required to linearize or release the functional peptide or other molecule. Cyclic peptides can include depsipeptides wherein hydrolysis of one or more ester bonds release the linearized peptide. Such embodiments can be used to tune the timing of peptide release in environments such as plasma.

Macrocyclic peptides occur naturally and have been studied and prized for their resistance to degradation by proteases generally present in blood. See Gang, et al., 2018, Cyclic Peptides: Promising Scaffolds for Biopharmaceuticals, Genes, 9:557, incorporated herein by reference. Cyclization of peptides has also been shown to facilitate passage through cell membrane allowing access to both extra and intracellular targets and, due to their promising attributes, several approaches for designing and producing synthetic cyclic peptides are known. Id.

Cyclic peptides of the invention may include a carrier, a therapeutic peptide, a reporter and one or more tuning domains that modifies a distribution or residence time of the cyclic peptide (or a released linearized peptide reporter or therapeutic) within a subject when administered to the subject. The cyclic peptide may be designed to detect and linearize in response to any enzymatic activity in the body, for example, enzymes that are differentially expressed under a physiological state of interest such as dysregulated protease activity indicative of a disease state. Dysregulated proteases have important consequences in the progression of diseases such as cancer in that they may alter cell signaling, help drive cancer cell proliferation, invasion, angiogenesis, avoidance of apoptosis, and metastasis.

The cyclic peptide may be tuned via the tuning domains in numerous ways to facilitate responsiveness to enzymatic activity within the body in specific cells or in a specific tissue. For example, the cyclic peptide may be tuned to promote distribution of the cyclic peptide to the specific tissue or to improve a residence time of the cyclic peptide in the subject or in the specific tissue.

When administered to a subject, the cyclic peptide is trafficked through the body and may diffuse from the systemic circulation to a specific tissue, where the peptide may be cleaved via enzymes indicative of the disease to release a detectable analyte or a therapeutic compound. In the case of a reporter molecule, the detectable analyte may then diffuse back into circulation where it may pass renal filtration and be excreted into urine, whereby detection of the detectable analyte in the urine sample indicates enzymatic activity upon the reporter.

The carrier may be any suitable platform for trafficking the molecules through the body of a subject, when administered to the subject. The carrier may be any material or size suitable to serve as a carrier or platform. Preferably the carrier is biocompatible, non-toxic, and non-immunogenic and does not provoke an immune response in the body of the subject to which it is

administered. The carrier may also function as a targeting means to target the cyclic peptide to a tissue, cell or molecule. In some embodiments the carrier domain is a particle such as a polymer scaffold. The carrier may, for example, result in passive targeting to tumors or other specific tissues by circulation. Other types of carriers include, for example, compounds that facilitate active targeting to tissue, cells or molecules. Examples of carriers include, but are not limited to, nanoparticles such as iron oxide or gold nanoparticles, aptamers, peptides, proteins, nucleic acids, polysaccharides, polymers, antibodies or antibody fragments and small molecules.

The carrier may include a variety of materials such as iron, ceramic, metallic, natural polymer materials such as hyaluronic acid, synthetic polymer materials such as poly-glycerol sebacate, and non-polymer materials, or combinations thereof. The carrier may be composed in whole or in part of polymers or non-polymer materials, such as alumina, calcium carbonate, calcium sulfate, calcium phosphosilicate, sodium phosphate, calcium aluminate, and silicates. Polymers include, but are not limited to: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, and hydroxypropyl cellulose. Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth) acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of lactic acid and glycolic acid, poly-anhydrides, polyurethanes, and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, albumin and other proteins, copolymers and mixtures thereof. In general, these biodegradable polymers degrade either by enzymatic hydrolysis or exposure to water in vivo, by surface or bulk erosion. These biodegradable polymers may be used alone, as physical mixtures (blends), or as co-polymers.

In preferred embodiments, the carrier includes biodegradable polymers so that whether the reporter is cleaved from the carrier, the carrier will be degraded in the body. By providing a biodegradable carrier, accumulation and any associated immune response or unintended effects of intact cyclic peptides remaining in the body may be minimized.

Other biocompatible polymers include PEG, PVA and PVP, which are all commercially available. PVP is a non ionogenic, hydrophilic polymer having a mean molecular weight ranging from approximately 10,000 to 700,000 and has the chemical formula (C6H9NO)[n]. PVP is also

known as poly[1 (2 oxo 1 pyrrolidinyl)ethylene]. PVP is nontoxic, highly hygroscopic and readily dissolves in water or organic solvents.

Polyvinyl alcohol (PVA) is a polymer prepared from polyvinyl acetates by replacement of the acetate groups with hydroxyl groups and has the chemical formula (CH2CHOH)[n]. Most polyvinyl alcohols are soluble in water.

Polyethylene glycol (PEG), also known as poly(oxyethylene) glycol, is a condensation polymer of ethylene oxide and water. PEG refers to a compound that includes repeating ethylene glycol units. The structure of PEG may be expressed as H–(O–CH2–CH2)n–OH. PEG is a hydrophilic compound that is biologically inert (i.e., non-immunogenic) and generally considered safe for administration to humans.

When PEG is linked to a particle, it provides advantageous properties, such as improved solubility, increased circulating life, stability, protection from proteolytic degradation, reduced cellular uptake by macrophages, and a lack of immunogenicity and antigenicity. PEG is also highly flexible and provides bio-conjugation and surface treatment of a particle without steric hindrance. PEG may be used for chemical modification of biologically active compounds, such as peptides, proteins, antibody fragments, aptamers, enzymes, and small molecules to tailor molecular properties of the compounds to particular applications. Moreover, PEG molecules may be functionalized by the chemical addition of various functional groups to the ends of the PEG molecule, for example, amine-reactive PEG (BS (PEG)n) or sulfhydryl-reactive PEG (BM (PEG)n).

In certain embodiments, the carrier is a biocompatible scaffold, such as a scaffold including polyethylene glycol (PEG). In a preferred embodiment, the carrier is a biocompatible scaffold that includes multiple subunits of covalently linked poly(ethylene glycol) maleimide (PEG-MAL), for example, an 8-arm PEG-MAL scaffold. A PEG-containing scaffold may be selected because it is biocompatible, inexpensive, easily obtained commercially, has minimal uptake by the reticuloendothelial system (RES), and exhibits many advantageous behaviors. For example, PEG scaffolds inhibit cellular uptake of particles by numerous cell types, such as macrophages, which facilitates proper distribution to a specific tissues and increases residence time in the tissue.

Cleavage of the cyclic peptide is preferably dependent on enzymes that are active in a specific disease state. For example, tumors are associated with a specific set of enzymes. For a

tumor, the cyclic peptide may be designed with one or more enzyme susceptible sites that match the enzymes expressed by the tumor or other diseased tissue.

In various embodiments, the cyclic peptide may include a reporter comprising a naturally occurring molecule such as a peptide, nucleic acid, a small molecule, a volatile organic compound, an elemental mass tag, or a neoantigen. In other embodiments, the reporter includes a non-naturally occurring molecule such as D-amino acids, synthetic elements, or synthetic compounds. The reporter may be a mass-encoded reporter, for example, a reporter with a known and individually-identifiable mass, such as a polypeptide with a known mass or an isotope.

An enzyme may be any of the various proteins produced in living cells that accelerate or catalyze the metabolic processes of an organism. Enzymes act on substrates. The substrate binds to the enzyme at a location called the active site before the reaction catalyzed by the enzyme takes place. Generally, enzymes include but are not limited to proteases, glycosidases, lipases, heparinases, phosphatases. Examples of enzymes that are associated with disease in a subject include but are not limited to MMP, MMP-2, MMP-7, MMP-9, kallikreins, cathepsins, seprase, glucose-6-phosphate dehydrogenase (G6PD), glucocerebrosidase, pyruvate kinase, tissue plasminogen activator (tPA), a disintegrin and metalloproteinase (ADAM), ADAM9, ADAM15, and matriptase.

Examples of substrates for disease-associated enzymes include but are not limited to Interleukin 1 beta, IGFBP-3, TGF-beta, TNF, FASL, HB-EGF, FGFR1, Decorin, VEGF, EGF, IL2, IL6, PDGF, fibroblast growth factor (FGF), and tissue inhibitors of MMPs (TIMPs).

The disease or condition targeted by the cyclic peptide may be any disease or condition that is associated with an enzymatic activity. For example, cancer progression and metastasis, cardiovascular disease, liver fibrosis, nonalcoholic fatty liver disease (NAFLD), arthritis, viral, bacterial, parasitic or fungal infection, Alzheimer’s disease emphysema, thrombosis, hemophilia, stroke, organ dysfunction, any inflammatory condition, vascular disease, parenchymal disease, or a pharmacologically-induced state are all known to be associated with enzymatic activity.

The tuning domains may include any suitable material that modifies a distribution or residence time of the cyclic peptide within a subject when the cyclic peptide is administered to the subject. For example, the tuning domains may include PEG, PVA, or PVP. In another example, the tuning domains may include a polypeptide, a peptide, a nucleic acid, a polysaccharide, volatile organic compound, hydrophobic chains, or a small molecule.

FIG. 1 diagrams steps of a method 100 for designing a target-specific cyclic peptide. At step 105, gene expression in subjects with a known disease may be determined, for example, by performing RNA sequencing (RNA-Seq) on gene transcripts using a next-generation sequencing platform, and determining fold-change in expression level of a transcript associated with the disease by normalizing read counts from the measured transcripts against healthy control read counts.

At step 110, for example, gene expression that is upregulated in relation to a non-diseased state may be determined, for example, to identify candidate proteases indicative of a disease. By identifying candidate proteases indicative of the disease, associated protease substrates may also be identified and incorporated into the reporter of the cyclic peptide.

At step 115, a cyclic peptide is assembled which may include a carrier, one or more tuning domains and a reporter and/or a therapeutic peptide.

The cyclic peptide can include one or more cleavage sites susceptible to the protease activity as identified in step 110. Tuning domains may be present in or linked to the cyclic peptide and may be based on the in vivo pathway through which the cyclic peptide is to be trafficked or based on the intended method of detection in the case of reporter-linked tuning domains. For example, the tuning domains may include PEG and can be linked to the peptide to facilitate distribution to the liver to detect protease activity in the liver, and the reporter may be detected via a ligand binding assay, such as an ELISA assay. The cyclic peptide may include any reporter or therapeutic peptide. The therapeutic peptide may be one operable to treat the disease associated with the upregulated genes identified in step 110.

At step 120, the cyclic peptide may be administered to a subject having the disease for targeted delivery of a therapeutic to diseased tissue or to detect enzymatic activity indicative of the disease, such as dysregulated protease activity.

The cyclic peptides may be administered by any suitable method of delivery. In preferred embodiments, the cyclic peptide is delivered intravenously or aerosolized and delivered to the lungs, for example, via a nebulizer. In other examples, the cyclic peptide may be administered to a subject transdermally, intradermally, intraarterially, intralesionally, intratumorally, intracranially, intraarticularly, intratumorally, intramuscularly, subcutaneously, orally, topically, locally, inhalation, injection, infusion, or by other method or any combination known in the art (see, for example, Remington's Pharmaceutical Sciences (1990), incorporated by reference).

At step 125, in the case of the cyclic peptide including a reporter molecule released by its cleavage, the target enzymatic activity may be detected in any biological sample. In preferred embodiments, the biological sample is non-invasively obtained and is preferably a bodily fluid or other substance that is naturally excreted from the body.

FIG. 2 shows an exemplary cyclic peptide 201 having a protease-specific substrate 209 and a stable cyclization linker 203. The N-terminus and C-terminus, coupled to the cyclization linker 203 comprise cyclization residues 205. The peptide may be engineered to address considerations such as protease stability, steric hindrance around cleavage site, macrocycle structure, and rigidity/flexibility of peptide chain. The type and number of spacer residues 207 can be chosen to address and alter many of those properties by changing the spacing between the various functional sites of the cyclic peptide. The cyclization linker and the positioning and choice of cyclization residues can also impact the considerations discussed above. Tuning domains such as PEG and/or reporters such as FAM can be included in the cyclic peptide.

FIG. 3 shows protease-specific cleavage of a macrocyclic peptide of the invention. The macrocyclic peptide is resistant to degradation during general circulation and in the presence of non-specific proteases it may encounter in off-target tissue. The macrocyclic peptide shown includes two protease-specific substrates and cleavage of both is required to release the linearized reporter molecule. In FIG. 3, both cleavage sites are the same and, accordingly, exposure to the protease in the target environment results in cleavage of both substrates which fully separates the quencher ($CPQ_2$) from a fluorescent reporter (FAM) producing a detectable signal indicative of the presence of the specific protease which, in turn, can be indicative of the presence of a disease. The unquenched fluorescent reporter or other reporter molecule may be linked to a tuning domain operable to promote concentration of the linked reporter molecule in the biological sample (e.g., urine) for detection.

In other embodiments, as depicted in FIG. 4, the substrates may be specific for different proteases creating a double-gated substrate wherein exposure to both proteases is required for the release of the linearized peptide (in the case of FIG. 4 a dequenched carboxy fluorescein reporter). The requirement of two or more different proteases can help further tune the cyclic peptide specificity which can be especially useful in instances where the upregulated proteases indicative of disease are individually common but present a more unique combination.

FIG. 5 shows a cyclic peptide 501 according to certain embodiments. The C-terminus and N-terminus of the peptide include cyclization residues 505 that are linked together by a cyclization linker 503. The peptide 501 is made as stable as possible to proteolysis but the cyclization linker 503 may be unstable overtime or otherwise responsive to the microenvironment of a targeted tissue (e.g., via redox state, pH, or presence of other enzymes). For example, as shown in FIG. 6, the cyclization linker 503 may comprise an ester bond having a known hydrolysis rate in plasma for a controlled degradation and release of the linearized, reactive peptide. The cyclic peptide 501 includes a protease-specific substrate 509 such that both a time-specific cleavage event (e.g., hydrolysis of the ester bond) as well as an protease-specific cleavage event (e.g., by a disease or environment-specific upregulated enzyme) are required to release the functional linearized peptide. Various cross linking within the peptide sequence to modify performance and response in the subject is possible in certain embodiments.

In certain embodiments, the substrate may be conformationally blocked from proteolytic interactions while in a cyclic form such that protease-specific cleavage of the substrate is impossible until the unstable cyclization linker has degraded. In such a manner, reporter or therapeutic release reactions can be delayed until the cyclic peptide has had time to localize in the target tissue (e.g., via targeting tuning domains). For example, where the disease indicative protease is known to be present in off-target sites but its presence in the target tissue is unexpected or indicative of disease, a delayed release of that substrate might be desirable.

In various embodiments, polycyclic peptides may be used to increase sensitivity through the inclusion of 2, 3, 4, or more cyclic structures which may require the presence of different environmental conditions for linearization or release of a functionalized reporter or therapeutic, or other molecule at a target location. For example, a bicyclic peptide according to certain embodiments is depicted in FIG. 8. The peptide includes three engineered cleavage sites which must be cleaved in order to release the functionalized molecule (in this case a glufib/K($CPQ_2$) reporter) into the target environment. Two cyclization linkers are attached to cyclization residues on the peptide and such that three environmentally sensitive cleavage substrates (in this case including a protease-sensitive substrate cleavable at an aspartic acid residue) hold the bicyclic peptide together and are required to be cleaved for release of the functionalized molecule. Any number of spacer residues may be used between the cyclization residues and the cleavage substrate in order to achieve the desired peptide conformation and allow access to the

cleavage site by the target-specific protease or other cleavage mechanism. The substrates can be the same with the redundant cleavage requirement further protecting from off-target or incidental release of the functionalized molecule. In certain embodiments, the various substrates can be sensitive to different combinations of environmental factors or enzymes, serving as an and gate and thereby increasing specificity.

Such sensitivity can be useful, for example, in instances where overexpression of a particular enzyme is indicative of disease only in a certain tissue but is normally present in other healthy tissues. Second, third, or more other cleavage substrates with different sensitivities can be included that are specific to the target tissue. Accordingly, only the presence of both the target-specific environmental cue and the tissue-specific, disease-specific environmental cue will release the reporter, reducing false positives.

FIG. 9 illustrates an exemplary cleavage of the bicyclic peptide in FIG. 8. The bicyclic peptide is exposed to a protease specific to the three engineered cleavage substrates and, after 3 cleavages, results in two separate molecules including separation of a fluorescent reporter from a quenching agent and mass tag. The polycyclic peptide concept can be expanded to include 3, 4, or more cyclic structures with correspondingly greater numbers of cleavages required for release of the reporter or other functionalized molecule. FIG. 10 shows a tricyclic peptide requiring 4 cleavages for release of its reporter molecule. Of note, the reporter molecule or other functional molecule to be released in polycyclic peptides having an odd number of cyclic structures (e.g., the tricyclic peptide shown in FIG. 10) is not located at a peptide terminus but instead is positioned inside of the second cyclization residue from a terminus (e.g., the N-terminus in FIGS. 10 and 13). FIG. 11 shows a polycyclic peptide with 4 cyclic structures and 5 cleavage substrates requiring cleavage to release its reporter molecule, which, having an even number of cyclic structures and a corresponding odd number of cleavage substrates, is located at the N-terminus. FIGS. 12 and 13 show polycyclic peptides having n cleavage structures and n+1 corresponding cyclic structures.

FIG. 12 provides a structure for polycylic peptides with the number of desired cleavage substrates (n) being an odd number. For such instances, the reporter molecule is positioned as shown and the bracketed cyclic section is repeated (n-3)/2 times to achieve the desired number of cleavage substrates (n).

FIG. 13 provides a structure for polycylic peptides with the number of desired cleavage substrates (n) being an even number. For such instances, the reporter molecule is positioned as shown and the bracketed cyclic section is repeated (n-4)/2 times to achieve the desired number of cleavage substrates (n).

FIG. 6 shows hydrolysis of the ester bond of a cyclic depsipeptide followed by cleavage of a protease-specific substrate to first linearize the peptide and then release and activate a reporter molecule by separating the quenching agent ($CPQ_2$) from the fluorescent reporter (FAM). The ester bond may be used to extend the half-life of the reporter molecule in blood.

When the cyclic peptide enters the diseased microenvironment, for example tissues of a diseased liver or kidney, proteases with activity specific to the linking substrates cleave the cyclic polypeptide, linearizing the peptide and liberating the reporter or therapeutic peptide from the carrier.

Therapeutic peptides have been or are being developed to treat a wide range of conditions including applications in metabolic disease, oncology, and cardiovascular disease. See Lau and Dunn, 2018, Therapeutic peptides: Historical perspectives, current development trends, and future directions, Bioorganic & Medicinal Chemistry 26:2700–2707, incorporated herein by reference. Cyclization of such therapeutic peptides to block off-target reactions and protect against degradation during circulation and targeting can enhance the opportunities and applications of such therapeutic peptides where engineered with a target-environment-specific cleavage site as described herein.

Where the cyclic peptide harbors a reporter molecule, linearization and release of the reporter can occur when exposed to target environment-specific protease or combination or proteases. The liberated reporter may then re-enter circulation and pass through renal filtration to urine or otherwise be excreted in any manner from the tissue and from the subject having the disease. The reporter may then be detected from the excreted sample in any suitable manner, for example, by mass spectrometry or a ligand binding assay, such as an ELISA-based assay. By detecting the liberated reporter in the sample, the presence of enzymatic activity upon the cyclic peptide is shown, thereby detecting the target enzymatic activity.

The detected enzymatic activity may be activity of any type of enzyme, for example, proteases, kinases, esterases, peptidases, amidases, oxidoreductases, transferases, hydrolases, lysases, isomerases, or ligases.

The biological sample may be any sample from a subject in which the reporter may be detected. For example, the sample may be a tissue sample (such as a blood sample, a hard tissue sample, a soft tissue sample, etc.), a urine sample, saliva sample, mucus sample, fecal sample, seminal fluid sample, or cerebrospinal fluid sample.

Reporter Detection

Where cleavage of the cyclic peptide releases a linearized reporter molecule, the reporter may be detected by any suitable detection method able to detect the presence of quantity of molecules within the detectable analyte, directly or indirectly. For example, reporters may be detected via a ligand binding assay, which is a test that involves binding of the capture ligand to an affinity agent. Reporters may be directly detected, following capture, through optical density, radioactive emissions, nonradiative energy transfers. Alternatively, reporters may be indirectly detected with antibody conjugates, affinity columns, streptavidin-biotin conjugates, PCR analysis, DNA microarray, or fluorescence analysis.

A ligand binding assay often involves a detection step, such as an ELISA, including fluorescent, colorimetric, bioluminescent and chemiluminescent ELISAs, a paper test strip or lateral flow assay, or a bead-based fluorescent assay.

In one example, a paper-based ELISA test may be used to detect the liberated reporter in urine. The paper-based ELISA may be created inexpensively, such as by reflowing wax deposited from a commercial solid ink printer to create an array of test spots on a single piece of paper. When the solid ink is heated to a liquid or semi-liquid state, the printed wax permeates the paper, creating hydrophobic barriers. The space between the hydrophobic barriers may then be used as individual reaction wells. The ELISA assay may be performed by drying the detection antibody on the individual reaction wells, constituting test spots on the paper, followed by blocking and washing steps. Urine from the urine sample taken from the subject may then be added to the test spots, then streptavidin alkaline phosphate (ALP) conjugate may be added to the test spots, as the detection antibody. Bound ALP may then be exposed to a color reacting agent, such as BCIP/NBT (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride), which causes a purple colored precipitate, indicating presence of the reporter.

In another example, volatile organic compounds may be detected by analysis platforms such as gas chromatography instrument, a breathalyzer, a mass spectrometer, or use of optical or acoustic sensors.

Gas chromatography may be used to detect compounds that can be vaporized without decomposition (e.g., volatile organic compounds). A gas chromatography instrument includes a mobile phase (or moving phase) that is a carrier gas, for example, an inert gas such as helium or an unreactive gas such as nitrogen, and a stationary phase that is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The column is coated with the stationary phase and the gaseous compounds analyzed interact with the walls of the column, causing them to elute at different times (i.e., have varying retention times in the column). Compounds may be distinguished by their retention times.

A modified breathalyzer instrument may also be used to detect volatile organic compounds. In a traditional breathalyzer that is used to detect an alcohol level in blood, a subject exhales into the instrument, and any ethanol present in the subject's breath is oxidized to acetic acid at the anode. At the cathode, atmospheric oxygen is reduced. The overall reaction is the oxidation of ethanol to acetic acid and water, which produces an electric current that may be detected and quantified by a microcontroller. A modified breathalyzer instrument exploiting other reactions may be used to detect various volatile organic compounds.

FIG. 7 is a mass spectrum that may be used to detect a target activity, as described in step 125. Mass spectrometry may be used to detect and distinguish reporters based on differences in mass. In mass spectrometry, a sample is ionized, for example by bombarding it with electrons. The sample may be solid, liquid, or gas. By ionizing the sample, some of the sample's molecules are broken into charged fragments. These ions may then be separated according to their mass-to-charge ratio. This is often performed by accelerating the ions and subjecting them to an electric or magnetic field, where ions having the same mass-to-charge ratio will undergo the same amount of deflection. When deflected, the ions may be detected by a mechanism capable of detecting charged particles, for example, an electron multiplier. The detected results may be displayed as a spectrum of the relative abundance of detected ions as a function of the mass-to-charge ratio. The molecules in the sample can then be identified by correlating known masses, such as the mass of an entire molecule to the identified masses or through a characteristic fragmentation pattern.

When the reporter includes a nucleic acid, the reporter may be detected by various sequencing methods known in the art, for example, traditional Sanger sequencing methods or by next-generation sequencing (NGS). NGS generally refers to non-Sanger-based high throughput nucleic acid sequencing technologies, in which many (i.e., thousands, millions, or billions) of nucleic acid strands can be sequenced in parallel. Examples of such NGS sequencing includes platforms produced by Illumina (e.g., HiSeq, MiSeq, NextSeq, MiniSeq, and iSeq 100), Pacific Biosciences (e.g., Sequel and RSII), and Ion Torrent by ThermoFisher (e.g., Ion S5, Ion Proton, Ion PGM, and Ion Chef systems). It is understood that any suitable NGS sequencing platform may be used for NGS to detect nucleic acid of the detectable analyte as described herein.

Analysis may be performed directly on the biological sample or the detectable analyte may be purified to some degree first. For example, a purification step may involve isolating the detectable analyte from other components in the biological sample. Purification may include methods such as affinity chromatography. The isolated or purified detectable analyte does not need to be 100% pure or even substantially pure prior to analysis.

Detecting the detectable analyte may provide a qualitative assessment (e.g., whether the detectable analyte is present or absent) or a quantitative assessment (e.g., the amount of the detectable analyte present) to indicate a comparative activity level of the enzymes. The quantitative value may be calculated by any means, such as, by determining the percent relative amount of each fraction present in the sample. Methods for making these types of calculations are known in the art.

The detectable analyte may be labeled. For example, a label may be added directly to a nucleic acid when the isolated detectable analyte is subjected to PCR. For example, a PCR reaction performed using labeled primers or labeled nucleotides will produce a labeled product. Labeled nucleotides, such as fluorescein-labeled CTP are commercially available. Methods for attaching labels to nucleic acids are well known to those of ordinary skill in the art and, in addition to the PCR method, include, for example, nick translation and end-labeling.

Labels suitable for use in the reporter include any type of label detectable by standard methods, including spectroscopic, photochemical, biochemical, electrical, optical, or chemical methods. The label may be a fluorescent label. A fluorescent label is a compound including at least one fluorophore. Commercially available fluorescent labels include, for example,

fluorescein phosphoramidites, rhodamine, polymethadine dye derivative, phosphores, Texas red, green fluorescent protein, CY3, and CY5.

Other known techniques, such as chemiluminescence or colormetrics (enzymatic color reaction), can also be used to detect the reporter. Quencher compositions in which a “donor” fluorophore is joined to an “acceptor” chromophore by a short bridge that is the binding site for the enzyme may also be used. The signal of the donor fluorophore is quenched by the acceptor chromophore through a process believed to involve resonance energy transfer (RET), such as fluorescence resonance energy transfer (FRET). Cleavage of the peptide results in separation of the chromophore and fluorophore, removal of the quench, and generation of a subsequent signal measured from the donor fluorophore. Examples of FRET pairs include 5-Carboxyfluorescein (5-FAM) and CPQ2, FAM and DABCYL, Cy5 and QSY21, Cy3 and QSY7.

In various embodiments, the cyclic peptide may include ligands to aid it targeting particular tissues or organs. When administered to a subject, the cyclic peptide is trafficked in the body through various pathways depending on how it enters the body. For example, if cyclic peptide is administered intravenously, it will enter systemic circulation from the point of injection and may be passively trafficked through the body.

For the cyclic peptide to respond to enzymatic activity within a specific cell, at some point during its residence time in the body, the cyclic peptide must come into the presence of the enzyme and have an opportunity to be cleaved and linearized by the enzyme to release the linearized reporter or therapeutic molecule. From a targeting perspective, it is advantageous to provide the cyclic peptide with a means to target specific cells or a specific tissue type where such enzymes of interest may be present. To achieve this, ligands for receptors of the specific cell or specific tissue type may be provided as the tuning domains and linked to polypeptide.

Cell surface receptors are membrane-anchored proteins that bind ligands on the outside surface of the cell. In one example, the ligand may bind ligand-gated ion channels, which are ion channels that open in response to the binding of a ligand. The ligand-gated ion channel spans the cell’s membrane and has a hydrophilic channel in the middle. In response to a ligand binding to the extracellular region of the channel, the protein’s structure changes in such a way that certain particles or ions may pass through. By providing the cyclic peptide with tuning domains that include ligands for proteins present on the cell surface, the cyclic peptide has a greater opportunity to reach and enter specific cells to detect enzymatic activity within those cells.

By providing the cyclic peptide with tuning domains, distribution of the cyclic peptide may be modified because ligands may target the cyclic peptide to specific cells or specific tissues in a subject via binding of the ligand to cell surface proteins on the targeted cells. The ligands of tuning domains may be selected from a group including a small molecule; a peptide; an antibody; a fragment of an antibody; a nucleic acid; and an aptamer.

Once cyclic peptide reaches the specific tissue, ligands may also promote accumulation of the cyclic peptide in the specific tissue type. Accumulating the cyclic peptide in the specific tissue increases the residence time of the cyclic peptide and provides a greater opportunity for the cyclic peptide to be enzymatically cleaved by proteases in the tissue, if such proteases are present.

When the cyclic peptide is administered to a subject, it may be recognized as a foreign substance by the immune system and subjected to immune clearance, thereby never reaching the specific cells or specific tissue where the specific enzymatic activity can release the therapeutic compound or reporter molecule. To inhibit immune detection, it is preferable to use a biocompatible carrier so that it does not elicit an immune response, for example, a biocompatible carrier may include one or more subunits of poly(ethylene glycol) maleimide. Further, the molecular weight of the poly(ethylene glycol) maleimide carrier may be modified to facilitate trafficking within the body and to prevent clearance of the cyclic peptide by the reticuloendothelial system. Through such modifications, the distribution and residence time of the cyclic peptide in the body or in specific tissues may be improved.

In various embodiments, the cyclic peptide may be engineered to promote diffusion across a cell membrane. As discussed above, cellular uptake of cyclic peptides has been well documented. See Gang. Hydrophobic chains may also be provided as tuning domains to facilitate diffusion of the cyclic peptide across a cell membrane may be linked to the cyclic peptide .

The tuning domains may include any suitable hydrophobic chains that facilitate diffusion, for example, fatty acid chains including neutral, saturated, (poly/mono) unsaturated fats and oils (monoglycerides, diglycerides, triglycerides), phospholipids, sterols (steroid alcohols), zoosterols (cholesterol), waxes, and fat-soluble vitamins (vitamins A, D, E, and K).

In some embodiments, the tuning domains include cell-penetrating peptides. Cell-penetrating peptides (CPPs) are short peptides that facilitate cellular intake/uptake of cyclic

peptides of the disclosure. CPPs preferably have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or has sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. See Milletti, 2012, Cell-penetrating peptides: classes, origin, and current landscape, Drug Discov Today 17:850–860, incorporated by reference. Suitable CPPs include those known in the literature as Tat, R6, R8, R9, Penetratin, pVEc, RRL helix, Shuffle, and Penetramax. See Kristensen, 2016, Cell-penetrating peptides as tools to enhance non-injectable delivery of biopharmaceuticals, Tissue Barriers 4(2):e1178369, incorporated by reference.

In certain embodiments, a cyclic peptide may include a biocompatible polymer as a tuning domain to shield the cyclic peptide from immune detection or inhibit cellular uptake of the cyclic peptide by macrophages.

When a foreign substance is recognized as an antigen, an antibody response may be triggered by the immune system. Generally, antibodies will then attach to the foreign substance, forming antigen-antibody complexes, which are then ingested by macrophages and other phagocytic cells to clear those foreign substances from the body. As such, when cyclic peptide enters the body, it may be recognized as an antigen and subjected to immune clearance, preventing the cyclic peptide from reaching a specific tissue to detect enzymatic activity. To inhibit immune detection of the cyclic peptide, for example, PEG tuning domains may be linked to the cyclic peptide. PEG acts as a shield, inhibiting recognition of the cyclic peptide as a foreign substance by the immune system. By inhibiting immune detection, the tuning domains improve the residence time of the cyclic peptide in the body or in a specific tissue.

Enzymes have a high specificity for specific substrates by binding pockets with complementary shape, charge and hydrophilic/hydrophobic characteristic of the substrates. As such, enzymes can distinguish between very similar substrate molecules to be chemoselective (i.e., preferring an outcome of a chemical reaction over an alternative reaction), regioselective (i.e., preferring one direction of chemical bond making or breaking over all other possible directions), and stereospecific (i.e., only reacting on one or a subset of stereoisomers).

Steric effects are nonbonding interactions that influence the shape (i.e., conformation) and reactivity of ions and molecules, which results in steric hindrance. Steric hindrance is the slowing of chemical reactions due to steric bulk, affecting intermolecular reactions. Various

groups of a molecule may be modified to control the steric hindrance among the groups, for example to control selectivity, such as for inhibiting undesired side-reactions. By providing the cyclic peptide with tuning domains such as spacer residues between the cyclization linker and residues and the cleavage site and/or any bioconjugation residue, steric hindrance among components of cyclic peptide may be minimized to increase accessibility of the cleavage site to specific proteases. Alternatively, steric hindrance can be used as described above to prevent access to the cleavage site until an unstable cyclization linker (e.g., an ester bond of a cyclic depsipeptide) has degraded. Such unstable cyclization linkers can be other known chemical moieties that hydrolyze in defined conditions (e.g., pH or presence of a certain analyte) which may be selected to respond to specific characteristics of a target environment.

In various embodiments, cyclic peptides may include D-amino acids aside from the target cleavage site to further prevent non-specific protease activity. Other non-natural amino acids may be incorporated into the peptides, including synthetic non-native amino acids, substituted amino acids, or one or more D-amino acids.

In some embodiments, tuning domains may include synthetic polymers such as polymers of lactic acid and glycolic acid, polyanhydrides, polyurethanes, and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof.

One of skill in the art would know what peptide segments to include as protease cleavage sites in a cyclic peptide of the disclosure. One can use an online tool or publication to identify cleavage sites. For example, cleavage sites are predicted in the online database PROSPER, described in Song, 2012, PROSPER: An integrated feature-based tool for predicting protease substrate cleavage sites, PLoSOne 7(11):e50300, incorporated by reference. Any of the compositions, structures, methods or cyclic peptides discussed herein may include, for example, any suitable cleavage site, as well as any further arbitrary polypeptide segment to obtain any desired molecular weight. To prevent off-target cleavage, one or any number of amino acids outside of the cleavage site may be in a mixture of the D and/or the L form in any quantity.

## Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

## Equivalents

Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains important information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

What is claimed is:

1. An engineered cyclic peptide comprising:

one or more cleavage sites cleavable within a target environment and generally resistant to cleavage outside a target environment, wherein cleavage of the one or more cleavage sites releases a linearized peptide reactive with the target environment.

2. The engineered cyclic peptide of claim 1 wherein the target environment is a tumor.

3. The engineered cyclic peptide of claim 1 wherein the target environment is a biological fluid.

4. The engineered cyclic peptide of claim 3 wherein the biological fluid is blood.

5. The engineered cyclic peptide of claim 1 wherein the cleavage site is cleaved by an enzyme present in the target environment.

6. The engineered cyclic peptide of claim 5 wherein the enzyme is known to be expressed with a certain disease or medical condition.

7. The engineered cyclic peptide of claim 6 wherein the linearized peptide is a therapeutic peptide operable to treat the disease or medical condition.

8. The engineered cyclic peptide of claim 1 wherein the linearized peptide is bioactive within the target environment.

9. The engineered cyclic peptide of claim 1 wherein the linearized peptide is cleavable in response to pH of the target environment.

10. The engineered cyclic peptide of claim 1 wherein the cyclic peptide is a cyclic depsipeptide and the one or more cleavage sites comprise an ester bond.

11. The engineered cyclic peptide of claim 1 wherein the cyclic peptide is a macrocyclic peptide.

12. The engineered cyclic peptide of claim 1 further comprising a carrier.

13. The engineered cyclic peptide of claim 12 wherein the carrier comprises a poly ethylene glycol (PEG) scaffold of covalently linked PEG subunits.

14. The engineered cyclic peptide of claim 1 wherein the linearized peptide is a detectable reporter

15. The engineered cyclic peptide of claim 14 wherein the detectable reporter comprises one selected from the group consisting of: a volatile organic compound; an elemental mass tag; a peptide comprising one or more D-amino acids; a nucleic acid; and a neoantigen.

16. The engineered cyclic peptide of claim 14 wherein the detectable reporter comprises an elemental mass tag comprising an element of atomic number greater than 20.

17. The engineered cyclic peptide of claim 14 wherein the reporter comprises an antigen detectable by a hybridization assay.

18. The engineered cyclic peptide of claim 1 wherein the one or more cleavage sites comprise a plurality of different cleavage sites.

19. The engineered cyclic peptide of claim 18 wherein the plurality of different cleavage sites are cleaved by different enzymes.

20. The engineered cyclic peptide of claim 18 wherein cleavage of two or more of the plurality of different cleavage sites is required to release the linearized peptide.

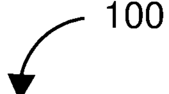
21. The engineered cyclic peptide of claim 20 wherein the two or more of the plurality of different cleavage sites must be cleaved in a specific order to release the linearized peptide.

22. The engineered cyclic peptide of claim 21 further comprising a tuning domain that modifies a distribution or residence time of the engineered cyclic peptide within a subject when administered to the subject.

23. The engineered cyclic peptide of claim 22 further comprising a plurality of tuning domains wherein the tuning domains comprise ligands for receptors of a specific cell or a specific tissue type.

24. The engineered cyclic peptide of claim 23 wherein the ligands promote accumulation of the engineered cyclic peptide in the specific tissue type or body compartment, wherein the ligands each comprise one selected from the group consisting of a small molecule; a peptide; an antibody; a fragment of an antibody; a nucleic acid; and an aptamer.

25. The engineered cyclic peptide of claim 22 further comprising a plurality of tuning domains wherein the tuning domains comprise hydrophobic chains that facilitate diffusion of the engineered cyclic peptide across a cell membrane.



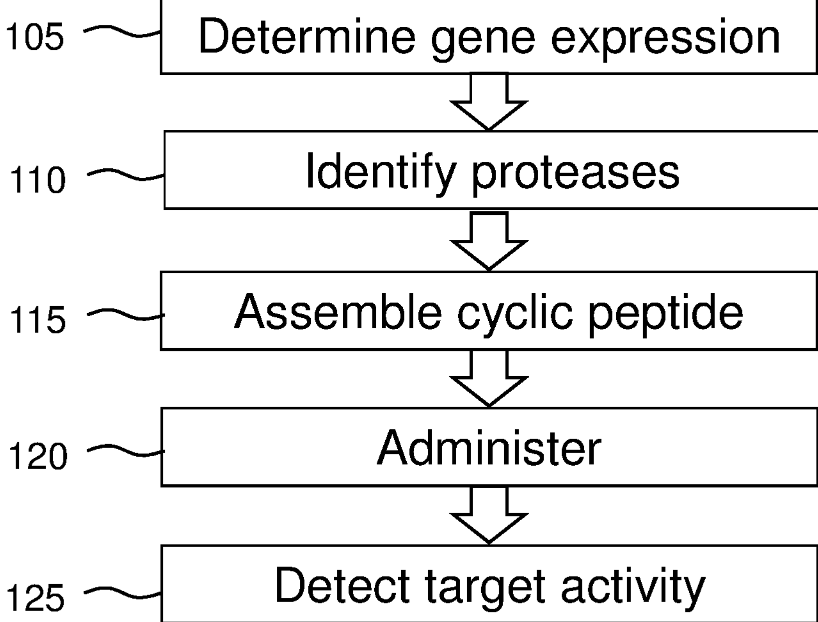




FIG. 1

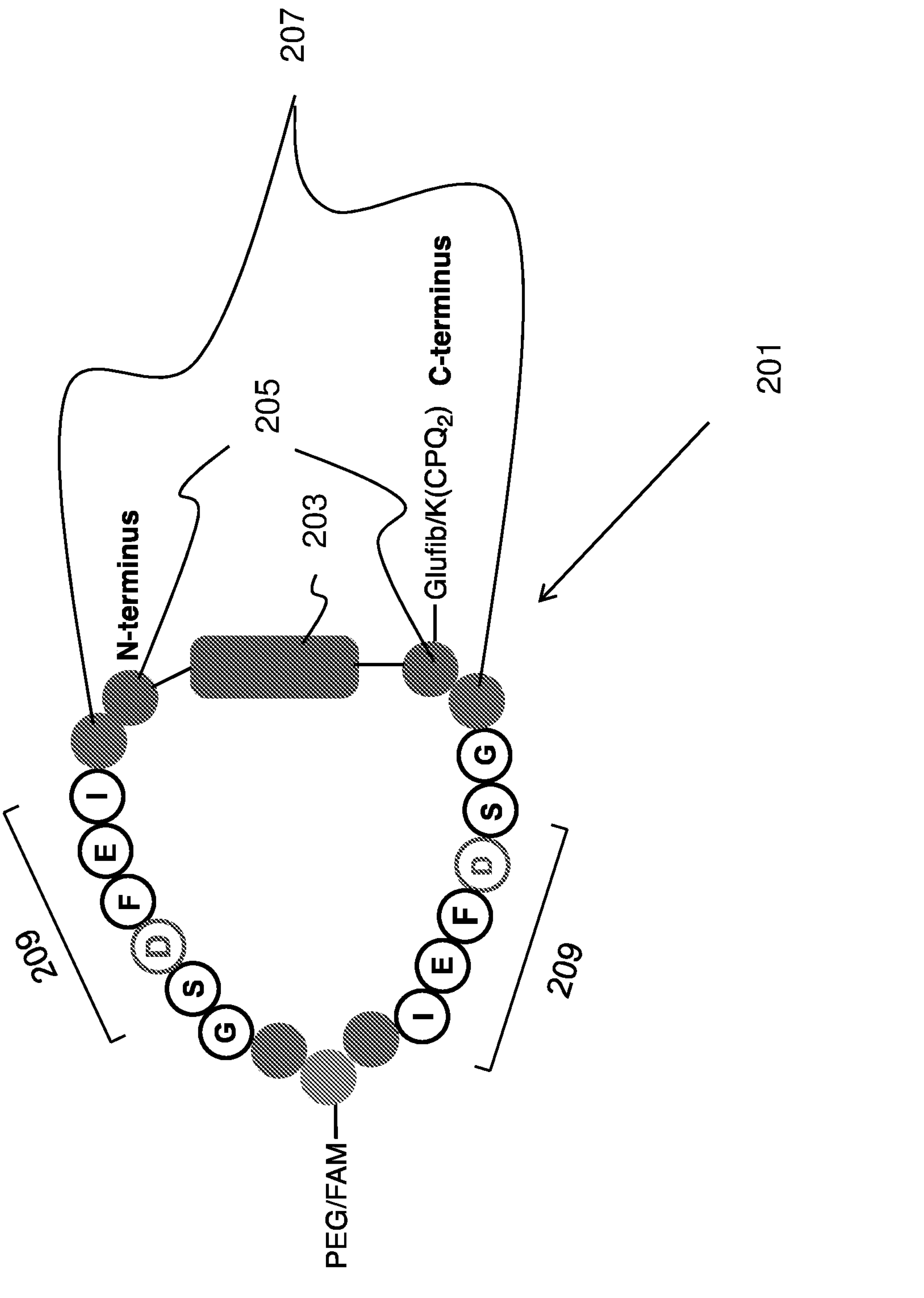



FIG. 2

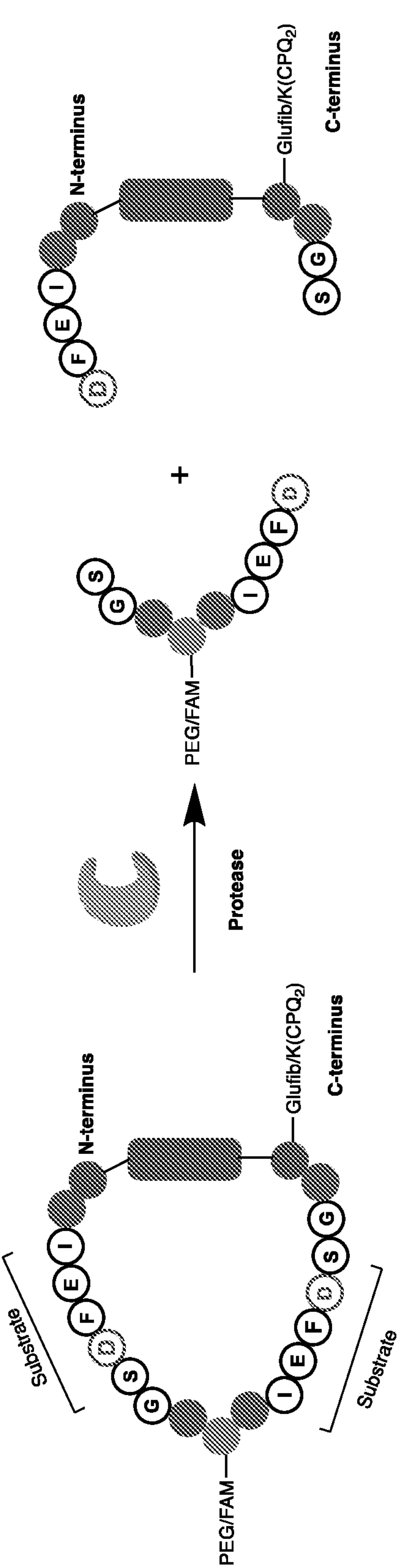



FIG. 3

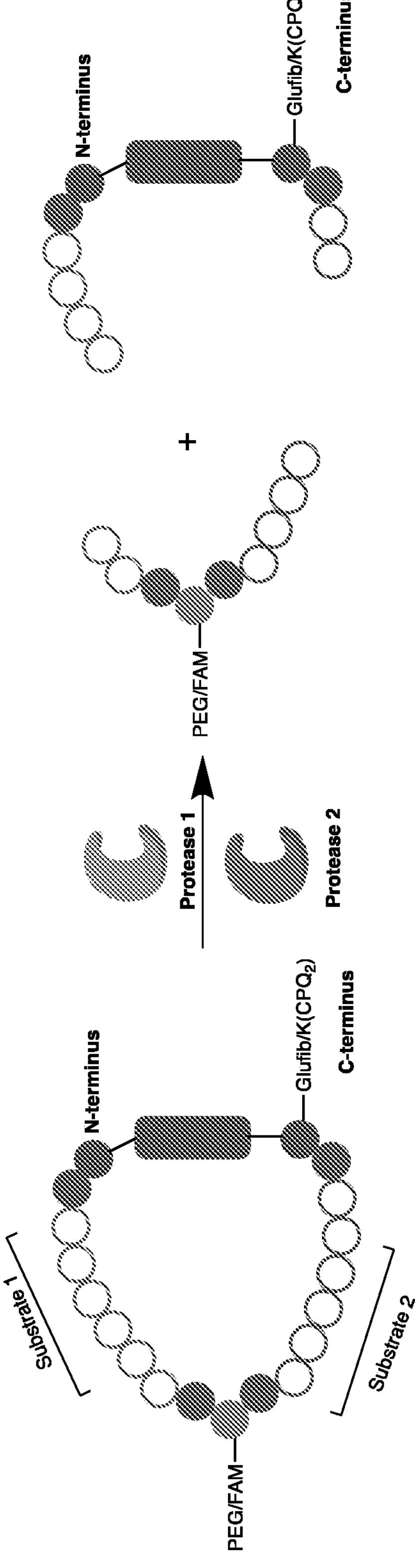



FIG. 4

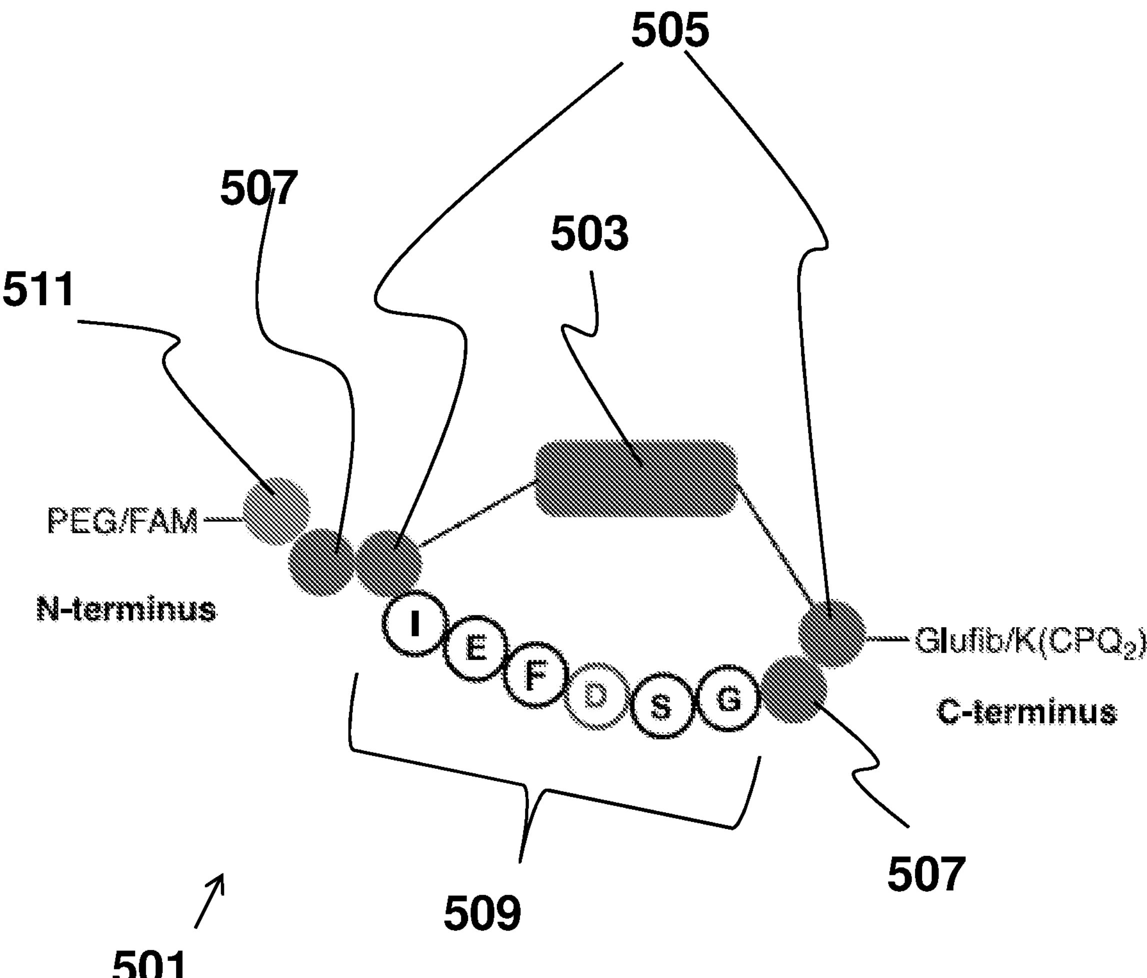



FIG. 5

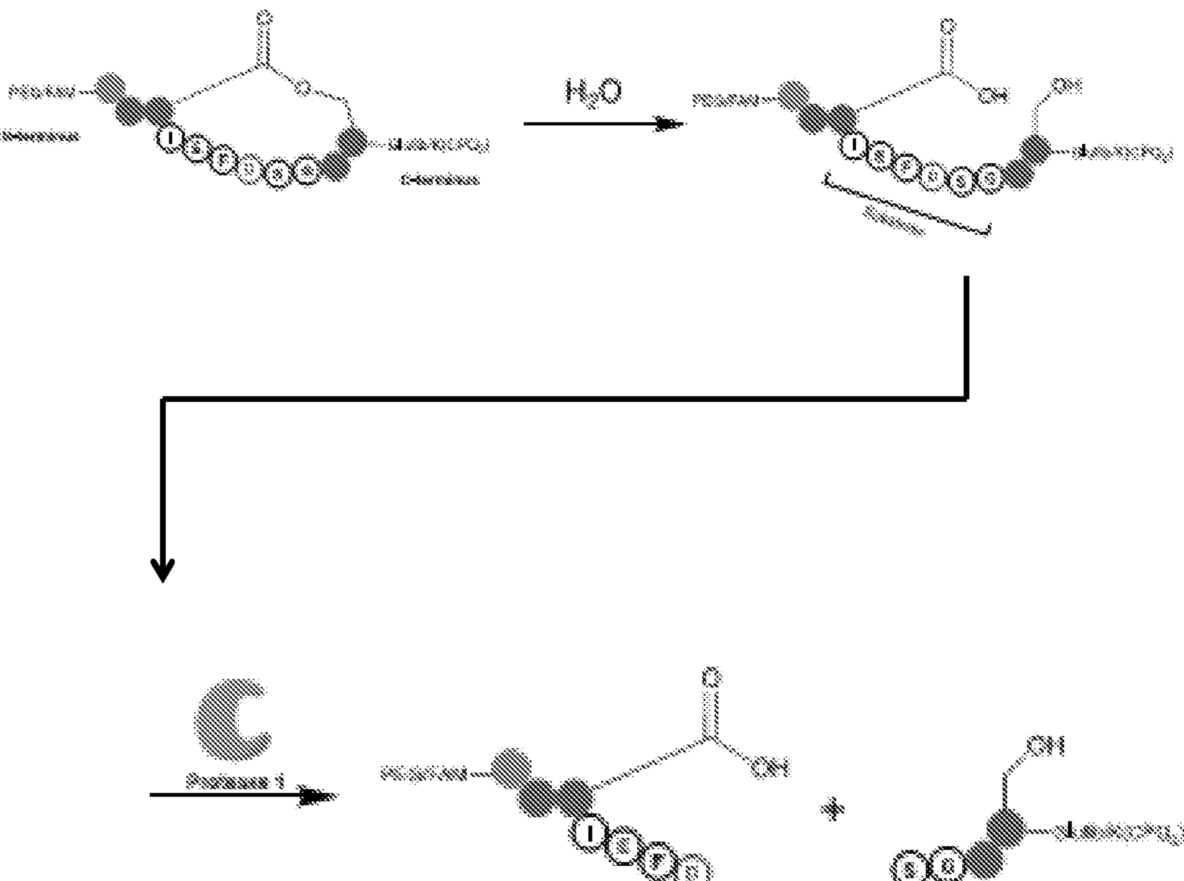



FIG. 6

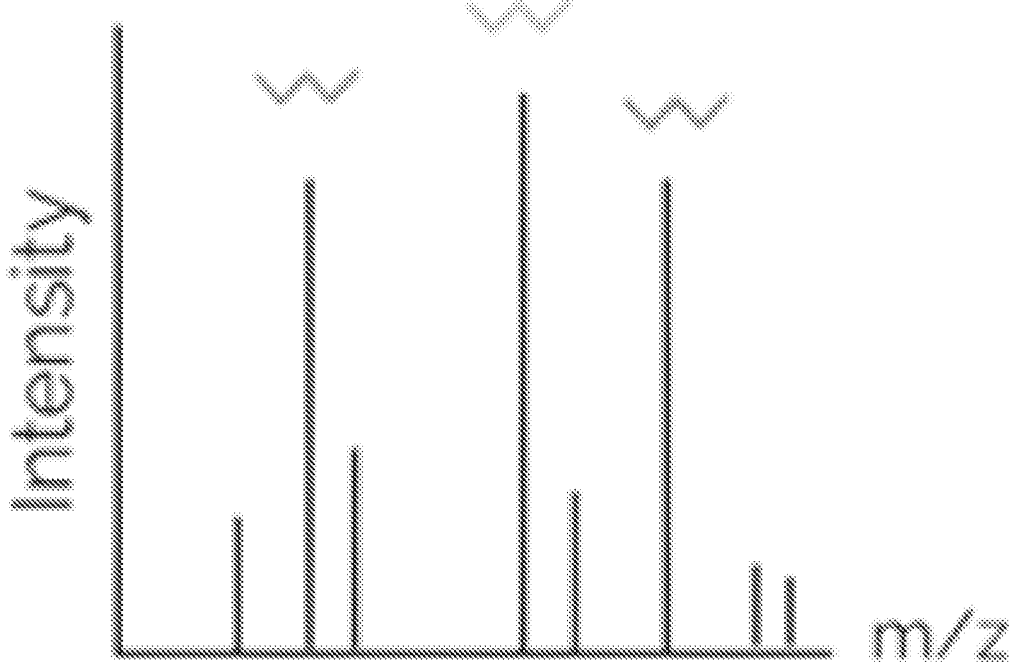
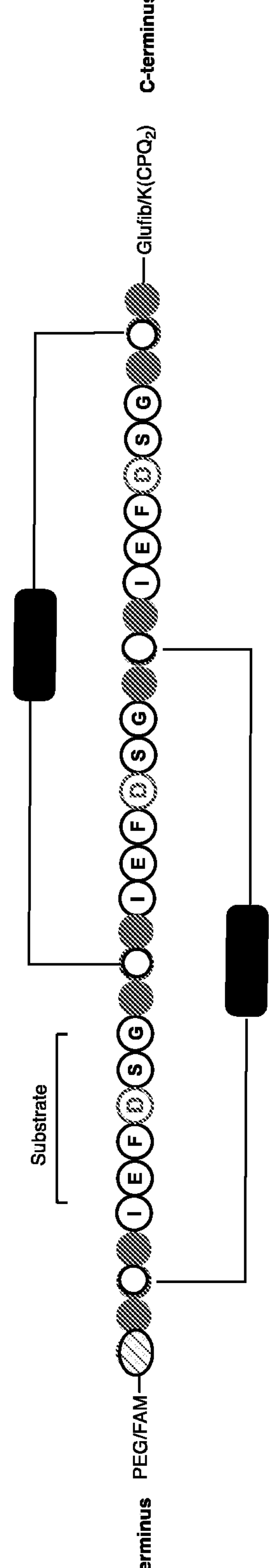



FIG. 7



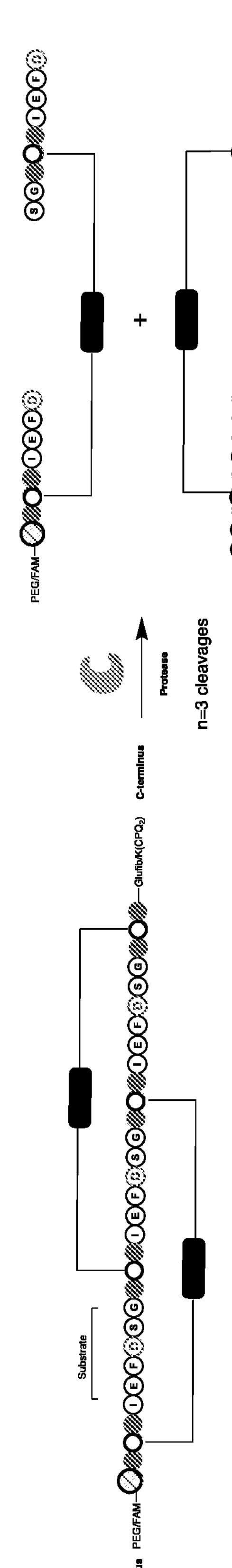

= Cyclization Linkers

= Cyclization residue

= Spacer residue

= Bioconjugation residue

FIG. 8



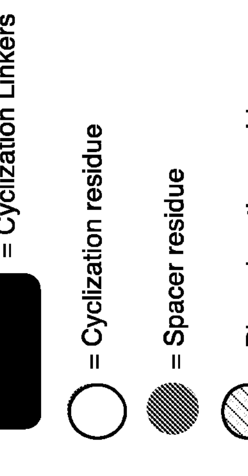
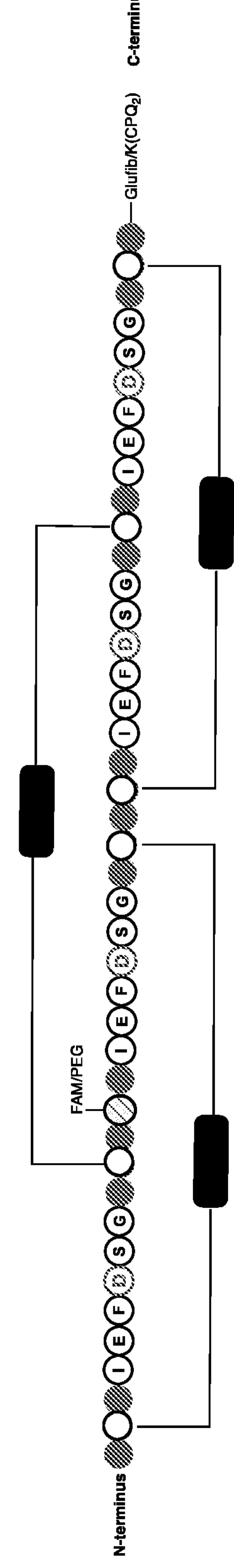




FIG. 9




= Cyclization Linkers

= Cyclization residue

= Spacer residue

= Bioconjugation residue

FIG. 10

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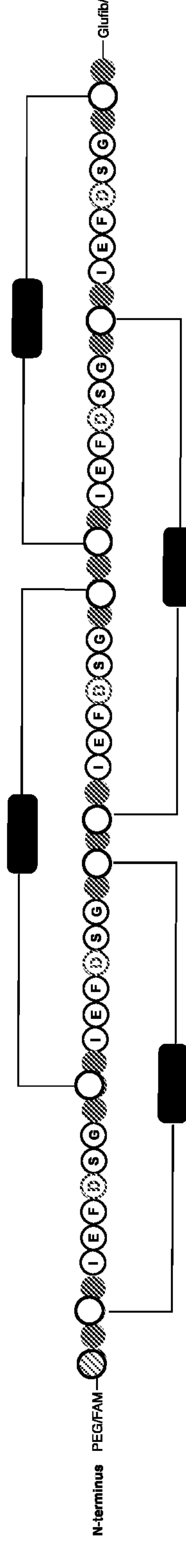


= Cyclization Linkers

= Cyclization residue

= Spacer residue

= Bioconjugation residue

FIG. 11

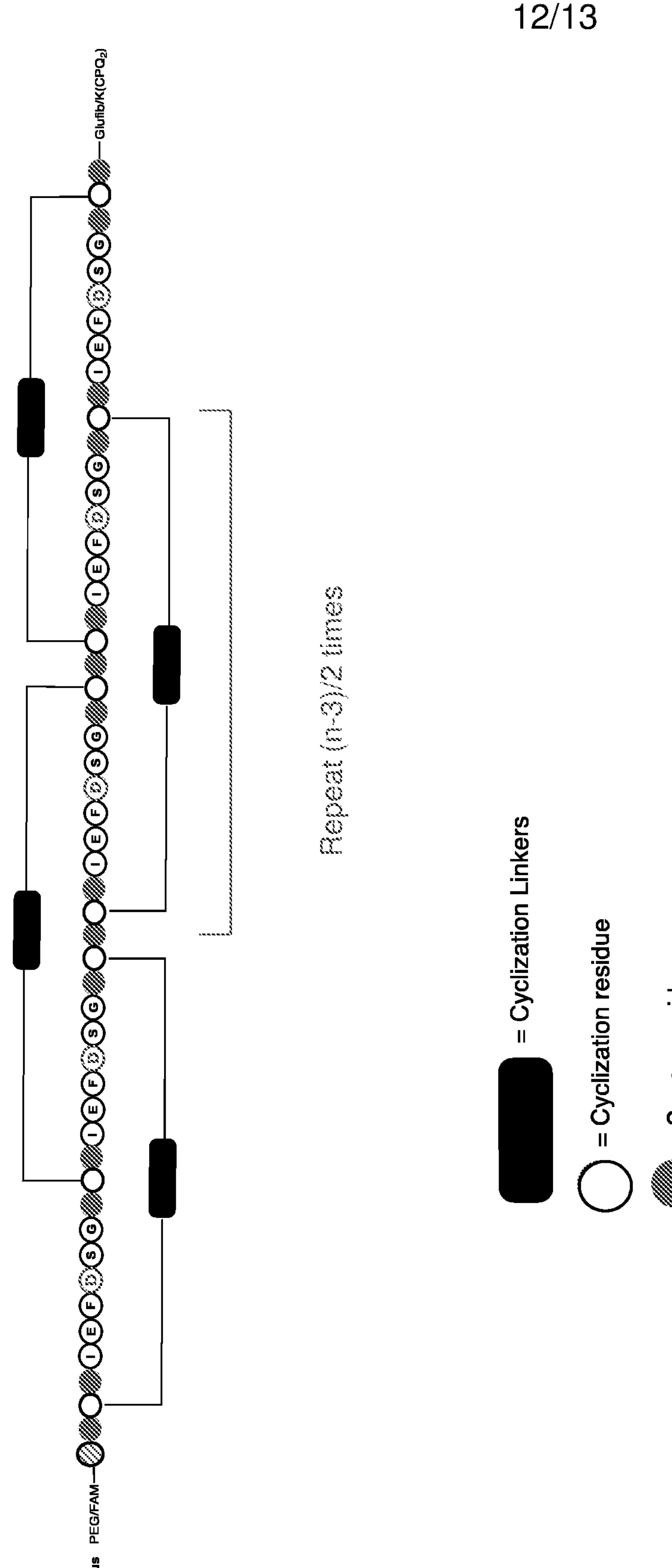


FIG. 12

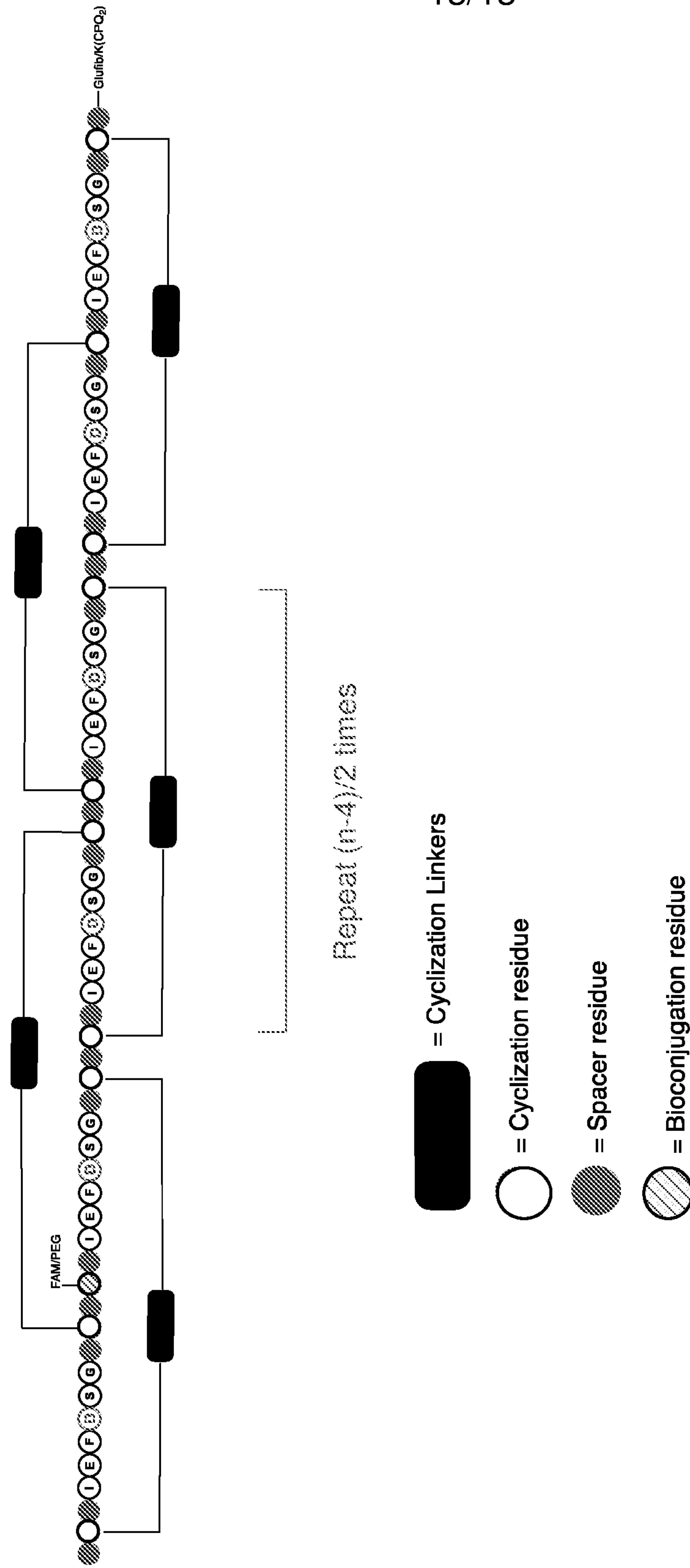


FIG. 13