Peptide-based biosensors

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Abstract

Peptides have been used as components in biological analysis and fabrication of novel biosensors for a number of reasons, including mature synthesis protocols, diverse structures and as highly selective substrates for enzymes. Bio-conjugation strategies can provide an efficient way to convert interaction information between peptides and analytes into a measurable signal, which can be used for fabrication of novel peptide-based biosensors. Many sensitive fluorophores can respond rapidly to environmental changes and stimuli manifest as a change in spectral characteristics, hence environmentally-sensitive fluorophores have been widely used as signal markers to conjugate to peptides to construct peptide-based molecular sensors. Additionally, nanoparticles, fluorescent polymers, graphene and near infrared dyes are also used as peptide-conjugated signal markers. On the other hand, peptides may play a generalist role in peptide-based biosensors. Peptides have been utilized as bio-recognition elements to bind various analytes including proteins, nucleic acid, bacteria, metal ions, enzymes and antibodies in biosensors. The selectivity of peptides as an enzymatic substrate has thus been utilized to construct enzyme sensors or enzyme-activity sensors. In addition, progress on immobilization and microarray techniques of peptides has facilitated the progress and commercial application of chip-based peptide biosensors in clinical diagnosis.

Key Words: peptides, biosensor, protein, protease, kinase, fluorophore

1. Introduction

Sensors are integrated systems that receive a certain signal or stimulus (such as bio/chemical substances, actions, processes and changes in environment.) and respond in a distinct manner. In the biochemical field, sensors are usually defined as a device which includes both a receptor (bio-recognition element) and a transducer, providing specific quantitative or semiquantitative analytical information. The general function of biosensors involves a

receptor in the most general sense recognizing an analyte, then a transducer either triggers a measurable signal or catalyzes a reaction related to the analyte concentration to generate a signal [1]. In clinical diagnosis, a sensitive, quick, convenient and versatile molecular biosensor has been desired to simplify the testing process, reduce the cost and shorten testing time [2]. Artificial peptides provide an opportunity to develop the desired molecular biosensor due to their desirable properties such as diversified structure, high affinity to proteins, matured synthesis protocol and modified approach [3-4].

Peptides are formed by natural or synthetic short polymers of amino acids which are linked by peptide bonds with shorter lengths than those of proteins [5]. Peptides have the same building block as proteins, therefore, it is possible for the peptides with a specific sequence to substitute for proteins in biological analysis [6]. Peptides with specific sequences can provide high affinity to particular analytes, and be obtained by screening and optimization of artificial peptide libraries. In addition, peptides have shown further advantages including high stability, standard synthetic protocol, easy modification and large chemical versatility. For example, peptides with short chains of amino acids generally have better chemical and conformational stability than proteins.

Peptides can be prepared with arbitrary sequences according to standard Fmoc and t-Boc solid-phase peptide synthesis (SPPS) protocols [7-9]. The SPPS protocol, typically involves repeated cycles of coupling-wash-deprotection-wash, carried out to couple an Fmoc or *t*-BOC N-protected amino acid unit to the free N-terminal amine of a peptide attached on a solid-phase Wang-resin. The SPPS protocol also provides an opportunity to attach arbitrarily a wide range of functional molecules at two terminal positions of a peptide sequence or particular amino acid residue with an additional functional group, such as in lysine. Furthermore, peptides thereby modified in a specific manner [10] can also retain their high affinity to the target analyte.

Peptide sequences that are specific enzyme substrates, play a critical role in assays of enzymatic activity and screening of enzymatic inhibitors. Due to these unique properties, peptides are excellent candidates for developing sensitive, fast, and convenient biosensors.

Peptides generally do not generate a measurable signal directly in response to a binding event, and therefore conjugation with a signal marker is an efficient strategy to convert the information of analyte/binding into a measurable signal. To date, several methods have been utilized to construct peptide-based biosensors via conjugating a peptide with signal markers. These biosensors can be used for detecting various analytes including metallic ions, proteins, proteases, kinases, bacillus species, nucleic acids and antibodies. Environmentally sensitive fluorophores are common signal markers which are widely used when conjugated with peptides. Their fluorescence emission can be affected by changes in the local environment caused by the affinity or interaction between conjugated peptide and analytes. The method of conjugating peptides to environmentally-sensitive fluorophores has been utilized to develop various peptide-conjugated molecular probes/sensors, [11] including ion sensors, DNA sensors, redox sensors and protein sensors. This work was reviewed by Choulier [3] and Vazquez [4]. Apart from fluorophores, other materials have also been used as signal markers, such as near-infrared dyes [12], nanoparticles [13], quantum dots [14] graphene [15-16], polydiacetylene (PDA)-liposomes [17], lanthanide chelators [18] and electrochemical markers [19]. Although limited, peptide-based biosensors without signal markers have also been developed. For example, an optical sensor of proteases based on photonic crystals was realized through immobilization of a peptide substrate in the silicon-based pores of the photonic crystal filter [20].

Peptides play various roles in peptide-based biosensors, including acting as the receptor (bio-recognition element), an enzymatic substrate (linker) and framework (scaffold) [21]. The

remainder of this review expands on peptide-based biosensors according to these roles that peptides may play in the sensing process.

2. Peptides as recognition elements in biosensors

Since peptides share the same chemical structure with proteins, peptides are an ideal candidate to substitute for protein as the receptor (biorecognition element) in biosensors. Artificial peptides can be obtained through standard solid-phase synthesis protocols to provide a specific sequence or screening a library of peptides. These peptide-based molecular biosensors have been developed for convenient, fast detection of various analytes including proteins, antibodies, DNA, and metallic ions [22].

2.1. Peptide-based protein sensors

Spectral properties of fluorophores are highly dependent on the surrounding environment. Therefore, fluorophores conjugated to peptides have been utilized to develop novel protein molecular sensors to target specific proteins [23-24]. Design strategies including the excimer [25], fluorescent resonance energy transfer (FRET) or probe-quencher pair strategies [26], have been used in protein molecular biosensors, which are summarized in Table 1. An excimer is a short-lived dimeric or heterodimeric molecule formed from two species. The wavelength of an excimer's emission is longer than that of the excited monomer's emission. Therefore, the wavelength shift in emission from transformation between the excimer state and the monomers can be utilized in providing a signal output for biosensors. FRET refers to the phenomenon of energy transfer between two chromophores separated by only a short distance (typically in the range of 1-10 nm) through non-radiative dipole–dipole coupling. The spectral overlap of the donor emission spectrum and the acceptor absorption spectrum, is required for the FRET effect. Therefore, the fluorescence quenching or

enhancement caused by the FRET process, can be utilized in the design of signal transducers in biosensors. In comparison to FRET, probe-quencher pair strategies usually depend on a static quenching which occurs when the molecules form a non-fluorescent complex in the ground state.

Fig. 1A illustrates the mechanism of a general protein biosensor based on environment-sensitive fluorophores. The initial process for protein detection is the affinity/recognition between the peptide and analyte. Upon recognition, the binding event induces a change in the spectral properties of the fluorophores. The emission of fluorophores is usually enhanced and blue shifts after affinity/recognition in a polar solvent. Computational studies have showed that the spectral change is caused by the insertion of the peptide-conjugated environment-sensitive fluorophore in the hydrophobic binding groove of the target protein [27]. The environment-sensitive fluorophore strategy has been utilized for detection of cyclin A [27] and HIV-1 specific monoclonal antibodies [28]. Recently, a novel environment-sensitive fluorophore, tetraphenylethylene (TPE), was conjugated with a small peptide sequence referred to as AP2H to form a fluorescent probe for tracking tumor marker in live cancer cells. The binding of the TPE-AP2H probe with the target cancer-related protein LAPTM4B can switch on the fluorescence of TPE due the inhibition of internal rotations within the TPE framework [29]. Environment-sensitive europium chelate was also used to conjugate with a peptide as a molecular bisosensor for detection of cyclin A [30]. The quantum yields (Φ) and emission lifetimes (τ) of the $^5D_0 \rightarrow {}^7F_j$ emission band of europium ion is significantly sensitive to the local environment [31]. Therefore europium chelate-peptide bioconjugates can respond to the binding affinity between the peptide and target protein. The strong binding affinity between the peptide and cyclin A leads to a significant enhancement of emission of europium ion under excitation with UV or two-photon (near infrared) light, and was applied in imaging in live HeLa cells.

Excimer-type protein molecular biosensors are composed of a peptide and two identical fluorophores, which are attached to the opposite ends of the peptide (Fig. 1B). In the presence of the target analyte, the interaction between the analyte and peptide forces the fluorophores to seperate, resulting in a shift of the emission peak of the fluorophores to that of the monomer. Plaxco *et al.* reported an excimer-type peptide beacon for detection of anti-HIV antibody. In their work, two pyrene units were attached at the opposite ends of a short peptide sequence, inducing the peptide to fold and form an excimer of pyrene units with the emission band at 475 nm through hydrophobic interaction of the pyrene units. In the presence of anti-HIV antibodies, the binding interaction of the anti-HIV antibody with the peptide forces the peptide into an extended formation disrupting the excimer of pyrene units, resulting in a shift of the emission band to 375 nm and decreasing the emission intensity of the excimer band [25].

The peptide beacon concept was further extended to develop an HIV sensor based on the probe-quencher pair model. In probe-quencher pair protein-sensors, fluorophores and quencher units are attached at various residues of the peptide sequence. Affinity between a peptide sequence and target protein influences the distance between the fluorophore and quencher, resulting in a change in the fluorescence emission. Plaxco *et al.* developed a probe-quencher pair model molecular biosensor based on a peptide beacon for detection of HIV. This system consisted of a ruthenium (II) bisbipyridine-phenanthroline chelate as a long-lived fluorophore 'probe' and methyl viologen as electron-accepting quencher. The probe and quencher were attached at the opposite ends of the peptide beacon. The binding interaction between HIV antibody and the peptide inhibited the electron transfer between the probe and the quencher, resulting in enhancement of fluorescent emission of the probe (Fig. 1C). The HIV molecular biosensor could achieve a 6-fold increase in fluorescent emission intensity

after binding to HIV antibodies. The detection limit for anti-HIV antibody using such a system could be as low as 250 pM [26].

In the probe-quencher pair peptide molecular biosensors, fluorescence emission can be significantly affected by the position, type and property of fluorophores. All of these factors have been investigated in detail. Baltzer *et al.* developed a series of human carbonic anhydrase II (HCA II) molecular biosensors based on a 42-residue helix-loop-helix polypeptide LA-42b. In this series of peptide-based sensors, lysine residues with active amine groups were inserted into various positions of a 42-residue peptide to conjugate with the probe and quenchers, respectively. Various types of probe-quencher pairs and spacers with differing length of quencher were also investigated in the HCA II biosensors. The HCA II biosensors with a variety of structures were compared and investigated to determine the major factors to influence fluorescent emission, including fluorophore location [32], probe-quencher pairs [33] and spacer length of the quencher [34].

Probe-quencher peptide molecular biosensors have also been applied in a competitive binding format. Qing *et al.* developed a graphene oxide-based fluorescent biosensor for analysis of peptide–receptor interactions, and applied this biosensor to imaging of the somatostatin receptor subtype 2, overexpressed in tumor cells [35]. In this biosensor, the fluorescence of FITC-labelled octreotide was quenched by graphene oxide due to the high adsorption affinity of octreotide to graphene oxide. In the presence of the antibody anti-octreotide, the binding of target antibody with FITC-labelled octreotide competitively released FITC-labelled octreotide from the graphene oxide surface, resulting in recovery of the fluorescence.

Intermolecular antenna energy transfer is also a common strategy in peptide-based fluorescent molecular sensors. In this strategy, a matching donor-acceptor pair was used in the sensor. Upon binding to the analyte, the distance between the donor and acceptor was reduced, resulting in an energy transfer from donor to acceptor, giving rise to significant

enhancement of the fluorescent emission of the acceptor. Mascarenas and Vazquez demonstrated that energy transfer from a tryptophan, acting as the antenna in a target protein, to a lanthanide ion chelated to a moiety on the peptide-based molecular sensor, can excite the fluorescent emission of the chelate for detection of the target protein. The cyclin A molecular sensor was fabricated by conjugating a peptide sequence with a high-affinity for cyclin A with DOTA[Tb^{3+}]. The cyclin A recognition induced energy transfer from the tryptophan to DOTA[Tb^{3+}] complex and excited the Tb^{3+} ion, resulting in an increase of fluorescence emission [36]. A detection limit as low as 30 μg/mL was achieved for cyclin A by the molecular biosensor.

In an intermolecular energy transfer strategy, a novel poly-diacetylene (PDA)-liposome-peptide sensor for detection of bacterial lipopolysaccharide was reported by Schmuck *et al.* based on tuning the absorbance of the acceptor. The molecular sensor was formed with a PDA-liposome tagged with a pentalysine oligopeptide conjugated to naphthalic acid fluorophore [37]. In the "off state", the emission band of the naphthalic acid overlaps with the absorbance band of the PDA-liposomes, and the fluorescence of naphthalic acid was quenched. In the presence of lipopolysaccharide, the fluorescence of naphthalic acid was turned "on" because the affinity/recognition between pentalysine oligopeptide and lipopolysaccharide lead to the blue shift of the absorption of PDA-liposomes [17] and restoration of the fluorescence of naphthalic acid.

Peptide-based protein sensors have also been constructed based on an electrochemical platform to harness advantages including fast measurement, portable device and low cost in clinical diagnosis. An electrochemical peptide-based protein biosensor can be realized based on bio-conjugation strategy. As shown in Figure 2, by conjugating an electrochemical marker with a peptide sequence that has a high affinity for the target protein, and immobilizing the peptide bioconjugate on the electrode of the sensor, the binding of the analyte with the

peptide sequence can considerably change the electron transfer from the electrochemical marker to the electrode, which would be detected by the sensor. For example, Lin *et al.* reported an epidermal growth factor receptor (EGFR) sensor, in which the ferrocene moiety was attached to the peptide sequence with high specific affinity to EGFR, and immobilized on a gold electrode through Au-S covalent attachment. Upon binding of the peptide with EGFR, a change in conformation of the peptide on the electrode surface occurred, which resulted in an increase in current [38]. The same strategy was also applied in other electrochemical peptide-based sensors. Electrochemical peptide-based sensor has been constructed successfully recently for the detection of HIV [39] and amyloid β 1-42 [40].

Compared with protein-based biosensors, peptide-based biosensors show a great advantage in imaging in *vivo*, and convenient fast detection in *vitro*, due to the low molecular weight of peptide. On the other hand, the peptide only represents a limited fragment of the protein and therefore may simulate only partial function of the protein, possibly limiting the application of peptide-based biosensors in protein detection.

2.2. Peptide-based metallic ion sensors

Protein structure and biological function depend on the interactions between proteins and metallic ions. Hence, as potential substitutes for proteins, peptides with special sequences can also have the capability to interact with metallic ions. The capability of peptides to capture metallic ions can be utilized to construct peptide-based metallic ion sensors by conjugating the special peptide sequence with signal markers. The peptide sequences used in metallic ion sensors can be obtained by optimizing peptide libraries or using special protein fragments. A wide variety of metallic ions, including Zn(II) [41], Cu(II), Hg(II), Cd(II), Ca(II), Mg(II), Na(I), K(I), Mn(II), Co(II), Al(III), Ni(II), Pb(II) [42-43] and Ag(I), can be detected by such metallic ion sensors, as summarized in Table 2.

In metallic ion sensors based on environment-sensitive fluorophores, it is essential for a specific peptide sequence to bind the target metallic ion (Fig. 3) [44-45]. The coordination of the peptide and metallic ion can give rise to a highly ordered, stable conformation of the peptide [46], resulting in a change to the micro-environment where the fluorophore is located. The binding status of the metallic ion and the peptide-based molecular sensor can be reflected in the spectral change of the environment-sensitive fluorophores [47]. The strategy has been employed to develop a metallic ion sensor via immobilization of peptide molecular sensor on latex beads [48].

Peptide-based fluorescence resonance energy transfer (FRET) metallic ion sensors are usually composed of a donor-acceptor pair and a peptide sequence [49]. The peptide sequence binds with the target metallic ions, and acts as a scaffold to induce a close distance between the donor and receptor, resulting in an increase in the energy transfer from donor to acceptor. Berg *et al.* developed a zinc ion sensor based on zinc finger consensus peptide and lissamine (as donor) - fluorescein (as acceptor) pair. The peptide folded upon binding with zinc ions [49] bringing the lissamine and fluorescein into closer proximity, giving rise to an increase of fluorescent emission intensity of the acceptor fluorescein. Dansyl and tryptophan are also a common donor-acceptor pair used in FRET metallic ion sensors [50]. Several reports described Hg (II) sensors using tryptophan as the donor and dansyl as the acceptor. Holcombe and co-workers used a peptide sequence from mercury binding protein to construct a high-specificity Hg (II) sensor [51]. Alternatively, in the design of Lee and co-workers, coordination of Hg (II) and two cysteine residues in a peptide sequence. In the presence of Hg (II), coordination of Hg (II) and two cysteine residues in the peptide sequence folded the peptide bringing the donor-acceptor pair closer, giving rise to increased fluorescent emission intensity of the acceptor fluorophore [52].

In an alternative sensing mode to FRET, chelation enhanced fluorescence (CHEF) metallic ion sensors have also been developed, in which both fluorophores and peptide sequences were used to coordinate with metallic ions. In the presence of the target metallic ions, the fluorophore coordinated with metallic ions and induced a change in the electronic structure of the fluorophore, which changed the fluorescence emission [53]. Additionally, in CHEF metallic ion sensors, both the structure of the peptide sequence and position of the fluorophores in the peptide can play an important role in enabling the fluorophores to coordinate with metallic ions.

In the metallic ion sensors, signal markers used in the sensors were not limited to fluorophores, with gold nanoparticles widely used in peptide-based metallic ion sensors [54-55]. A colorimetric Zn^{2+} sensor was developed by Wang *et al.* based on the conjugation of gold nanoparticles and β-amyloid peptide (Aβ 1-16) [13]. In this colorimetric sensor, the peptides bind with metallic ions, inducing aggregation of the gold nanoparticles, giving rise to change in the UV-vis absorption spectra of the gold nanoparticles. In addition, a Zn^{2+} sensitive sensor with this approach is reusable because the binding/aggregation process can be easily reversed by the addition of a chelating ligand such as EDTA, with greater chelating effect for Zn^{2+} than the peptide.

The peptide-based metal ion sensors showed a much higher specificity to metal ions compared with chemical analytical methods using chemical ligands such as EDTA. On the other hand, the specificity of peptide-based sensors still depends on differences in binding capability of peptide with various metal ions, meaning that some interference from other transition metal ions is inevitable. In the field of detection of metal ions, the specificity and sensitivity of peptide-based sensors has not been sufficient to satisfy commercialization requirements meaning that there is still a reliance on conventional physical analytical methods such as atomic absorption spectroscopy.

2.3. Peptide-based nucleic acid and other sensor types

Recently, special peptide sequences with high affinity to DNA have attracted great attention due to their potential application in the development of nucleic acid molecular sensors (Table 3) [56-57]. These peptide sequences with high affinity to nucleic acids can be synthesized according to DNA-binding protein or transcription factors. Conjugation of fluorophores with peptides has also been used to design such nucleic acids molecular sensors.

Schmuck *et al.* reported a nucleic acid sensor for imaging nucleic acids in cells based on bioconjugates of molecular peptide beacon and pyrenes [58]. The molecular peptide beacon consisted of two Trp-Thr-Lys tripeptide units, with pyrenes conjugated on the opposite terminals of the peptide beacon. The excimer of pyrenes was formed when the molecular peptide beacon folded. In the presence of DNA, the molecular peptide beacon was forcibly extended and intercalated into DNA as shown in Fig. 4. This interaction between the peptide and DNA disrupted the excimer of pyrene and changed the fluorescence of the pyrene units.

A fluorescent peptide-based nucleic acid sensor was also reported by Kim *et al.* for monitoring interactions between RNA and RNA-binding proteins in a competitive format. The fluorescent peptide-based sensor was constructed with pyrene units as fluorophores conjugated with a peptide sequence from the first 22 amino acids of λN protein which has high affinity for target boxB RNA. In the absence of λN protein, the interaction of the RNA and the peptide can turn on the fluorescence of the pyrene unit in the molecular sensor through photo-induced electron transfer (PET). In the presence of λN protein, the peptide of the molecular sensor was displaced by λN protein, turning off the fluorescence of the pyrene unit in the molecular sensor [59].

Thompson also reported a DNA sensor (YOHIN) based on a conjugation strategy. This DNA sensor was composed of cyanine dye oxazole yellow (intercalating dye) and an amino acid sequence derived from 139-190 residues of the native Hin recombinase. Compared to native

Hin recombinase, the derivative peptide sequence retained high affinity and specificity for the target DNA. On the other hand, fluorescent emission of the intercalating dye molecules was only excited while bound to DNA. The DNA sensor demonstrated that the combination of DNA-recognition polypeptides and cyanine dyes can efficiently generate an array of both sequences for a wavelength specific sensor [60]. Similar peptide bioconjugates for DNA were also reported by Mascarenas *et al.* In this DNA sensor, a peptide derived from the basic region of a bZIP transcription factor, was conjugated with intercalating tripyrrole through an amine linker. Affinity of the sensor to DNA was enhanced by optimization of the molecular structure design, such as the spacer structure between the peptide and fluorophores [61]. In peptide-based nucleic acid biosensors, the manner of the interaction between the peptide sequence and nucleic acid is typically limited to intercalation with DNA, or affinity to specific nucleic acid. Therefore, reported peptide biosensors for DNA is limited to qualitative analysis of the nucleic acid, and cannot provide for detection of specific nucleic acid sequences.

An additional class of peptide-based biosensor using peptides as the recognition element were also reported. A replacement strategy of counter ion of the peptide was utilized to develop a fast and reliable sensor for monitoring heparin contaminants [62]. 6-methoxyquinolinium fluorophores, which are selectively quenched by chloride ions, were attached to a known heparin-binding peptide sequence. The introduction of the polyanionic glycosaminoglycans could replace chloride ions and then associate with the peptide sensor, giving rise to a significant increase in fluoresence of the 6-methoxyquinolinium fluorophore. In addition, penetrating peptides are a special class of peptides also used to form peptide-based biosensors [63] or to improve the performance of peptide-based molecular biosensors for imaging *in vivo* [64].

In summary, peptide-based molecular biosensors using the peptide as a recognition element

are still in the development stage, and activity is focusing on two main issues, specificity and signaling. Seeking new high affinity and highly specific peptide sequences for improvement of specificity and extension of the application range is ongoing. Improving the signal output system for these sensors including signal measurement platforms (such as electrochemical, chemiluminescent platforms) and signal markers (such as gold nanoparticles and carbon-based nanomaterials) are also ongoing issues. A major challenge in the development of most molecular biosensors using a peptide as the recognition element is that signaling output of these biosensors is weak, resulting in low sensitivity compared with other analytical methods. In peptide-based biosensors, one unit of analyte usually can only react with one peptide sequence to activate the signal marker or a pair of signal markers attached on the peptide sequence for signal output. Therefore, an efficient versatile signal amplification/enhancement strategy is still required to improve the sensitivity of these peptide-based molecular biosensors in the future.

3. Peptides as enzymatic substrate in biosensors

Peptides are important substrates for proteases and kinases, and have been widely used in monitoring enzyme activity, screening for enzyme inhibitors and controlling release of drugs. Enzyme-activity sensors of proteases and kinases, were usually constructed based on the enzymatic catalysis of peptide substrates [65]. Conjugation of signal markers to the peptide substrate is one of the most efficient ways to convert the enzymatic information into a measurable signal in enzyme sensors. There are a wide range of candidates that can be used as the signal marker for the protease sensors including IR fluorescence dyes, graphene, nanoparticles and electronic markers.

3.1. Protease/ protease activity sensor

Proteases are a type of enzyme that can hydrolysis peptide bonds to conduct proteolysis, and naturally in all organisms. In the human body, proteases play an important role in protein catabolism. A large number of diseases and their relevant therapy involve proteases. For example, matrix metallopeptidase-2 (MMP-2) and MMP-9 are thought to be important in tumor metastasis, various inflammatory and pathological processes [66-68]; MMP-1 is thought to be implicated in rheumatoid [69] and osteo-arthritis [70-71]; HIV-1 protease is an important target for drug therapy of HIV infection and AIDS [72-73]. Therefore, development of protease/protease activity sensors is significant for human health.

Protease-sensitive peptide sequences play an irreplaceable role in the development of protease sensors. Protease-sensitive peptide sequences can be cleaved accurately and efficiently by the protease at specific positions on the peptide, and the cleavage information can be efficiently conveyed through the change in the behavior of signal markers conjugated to the protease-sensitive peptide sequence. Hence, the conjugation strategy of fluorophores with the protease-sensitive peptide sequence, has been utilized to develop fluorescent protease molecular sensors for detection of proteases and measurement of protease activity [74] and screening for protease inhibitors [75-76]. A number of such proteases sensors are summarized in Table 4. Quenching and FRET models were also widely used in the design of fluorescent protease molecular sensors. In the two models, donor-acceptor pairs (or probe-quencher pairs) are normally attached at the opposite ends of protease-sensitive peptide sequence. In the presence of protease, cleavage of the peptide bio-conjugates can free the fluorescent molecules, giving rise to a considerable increase of fluorescent emission intensity. There are two quenching models generally employed, the self-quenching model and the heterogeneous quenching model. Both of these models have been utilized in the design of quenching model protease molecular sensors. For the self-quenching model of homogenous fluorophores, concentration-dependent self-quenching fluorophores were used to conjugate

with protease-sensitive peptide sequence in a close proximity or at a high local concentration. Cleavage by the protease can free the constrained fluorophores, resulting in an increase of fluorescent emission intensity.

Bradley *et al.* have developed an approach to construct endoproteinase AspN molecular sensors based on conjugating a self-quenching dye (Cy-5 or fluorescein) with a dendrimer-shaped peptide. The dendrimer-shaped peptide was synthesized using a standard solid-phase protocol based on dendrimerized-resin with various generations. The dendrimer-shaped structure of the molecular sensor gave a high local concentration of the self-quenching dyes. Enzymatic cleavage can free the fluorophores, which eliminates the self-quenching effect of the fluorophores, resulting in a nine-fold enhancement in fluorescence emission [77].

A protease sensor based on the close arrangement of a self-quenching dye via peptide self-assembly was reported by Edwards *et al.* In this protease sensor, two self-quenching fluorophores (LS276) were conjugated on both sides of a special peptide sequence cleavable by MMP-2/MMP-9, which could self-assemble into a triple-helical structure. Enzymatic cleavage disrupts the close triple-helical structure of the peptide probe and frees the self-quenching fluorophores (LS276) on the peptide, resulting in a five-fold fluorescence enhancement in tumor compared to muscle [78].

A schematic illustration of the heterogeneous quenching model is shown in Fig. 5A. A probe-quencher pair was attached at the opposite ends of a flexible peptide substrate in a protease molecular sensor. In the absence of protease, the fluorescence of the probe was quenched through static or collision-inducing interaction. Hydrolysis by the protease frees the fluorescent probes from the quenching state, resulting in an increase of fluorescence intensity of the probe molecules. The protease molecular sensors have been utilized to assay a wide range of proteases including HIV protease [79], rennin [80], hepatitis A 3C protease, interleukin 1β-converting enzyme [81], human cytomegalovirus protease [82], porcine pepsin

[79], neprilysin [83] and insulin-degrading enzyme [83]. In addition to common dye-quencher pairs, several novel probe-quencher pairs including poly(phenyleneethynylene)(PPE)-QSY7/Azo [77], FITC-gold nanoparticles [78-79], fluorescein amidite (FAM)-graphene oxide (GO) [80], FAM-single walled carbon nanohorns (SWCNHs) [81] and FITC-graphene [82], were also reported recently to be utilized to develop probe-quencher pair protease sensors with heterogeneous fluorophores.

The peptide-based biosensors based on nanomaterials have received much attention recently due to their unique and versatile properties. For example, gold nanoparticles have excellent biocompatibility and enable a surface plasmon resonance effect for usage in *vivo* and signaling; whereas iron oxide particles have superparamagnetic properties allowing for magnetic separation and recycling as well as imaging using MRI. Semiconductor quantum dots have tunable fluorescence with high quantum yield for sensitive signaling; and carbon-based nanomaterials such as graphene are excellent conductive materials for signaling and usage in electrochemical platforms. Consequently, these nanoparticles are also good candidate carriers of peptides for peptide-based molecular sensors [84], and provide opportunities in signal enhancement and code analysis of various analytes.

In one specific example, Chu *et al.* developed a GO-peptide conjugate biosensor for imaging caspase-3 activation in live cells. In the molecular protease sensor, a caspase-3-cleavable peptide sequence conjugated with FAM dye was immobilized covalently on GO. The cleavage by caspase-3 of the peptide sequence freed the FAM dye and restored fluorescent emission of the FAM dye. Recently, Lee and Kim *et. al.* reported a GO-peptide conjugate biosensor for optical detection of cell-secreted proteases based on the same strategy. The difference is that metalloprotoporphyrins and QXL_{570} as quenchers were used to covalent link with the peptide to quench the photoluminescence of GO in the visible and near-infrared

region. In the presence of the target protease, the cleavage of the peptide sequence by the protease freed the quencher and restored the photoluminescence of the GO [85].

The FRET quenching approach has also been utilized in the development of peptide-based protease sensors because peptides are sufficiently small to be tied to donor-acceptor pairs within the range of the 'Förster radius' which is one important condition for the FRET quenching approach. In such protease biosensors, donor-acceptor pairs are attached at the opposite ends of the protease-sensitive peptide sequence [86] (Fig. 5B). Protease biosensors in this format have been used to assay bacillus anthracis protease [87], MMP-9 [88] and HIV protease [89].

The other requirement for the FRET quenching model is that there should be spectral overlap between emission of the donor and absorbance of the acceptor, which gives vast options for matching donor-acceptor pairs in protease sensors. Apart from fluorescent dye molecules, semiconductor quantum dots (QD) [90] and near-infrared (NIFR) fluorescent probes have also been utilized to develop novel peptide-based FRET protease sensors. The absorbance and emission wavelengths of QD can be adjusted by particle size and surface modification. Therefore, QD with emission at a specific wavelength can be matched with fluorescent dyes to develop FRET protease sensors. Medintz *et al.* have been focusing on developing peptide-based FRET protease biosensors using the pairing of QD with fluorescent dyes (Fig. 6) [74, 91]. The QD-based protease sensors are formed by attaching a large number of fluorescent dye-conjugated peptide substrates on the surface of QD. Enzymatic cleavage induces termination of the FRET effect between QD and the dyes, resulting in the recovery of fluorescent emission of the QD. The reaction velocity and kinetic parameters of protease action for a range of proteases including caspase-1, thrombin, collagenase and chymotrypsin, has been quantitatively measured by the QD-based protease sensors.

The optical signal of the protease sensors based on fluoreophore conjugation to cleavable peptide sequences is not limited to the visible wavelength range; near-infrared fluorophores have also been used in the protease biosensors [92-93]. Common models used in visible fluorescent protease sensors including locally concentrated self-quenching, probe-quencher pairs and FRET quenching, were also applied to the design of NIFR protease sensors for diagnosis of tumors and HIV. Additionally, the application of NIFR protease molecular sensors was further extended into vivo imaging of tumors by using NIFR protease sensors as an NIR probe. Weissleder *et al.* synthesized a NIFR probe that consisted of a polylysine chain grafted to methoxypolyethyleneglycol (MPEG) and auto-quenching Cy5.5 dyes (Fig. 7) [94-95]. Tumor-associated proteases activate the NIRF probe through cleaving the backbone of the polylysines, giving rise to increased near-infrared emission intensity at the tumor location. A tumor with size of 1 mm could be detected by the NIRF probe.

In addition to fluorescent protease sensors, other modes of signal output were also used to develop peptide-based protease sensors including electrochemical [19, 96], electrogenerated chemiluminescence (ECL) [97], colorimetric assay [98] and element mass spectrometry [18]. Liu *et al.* developed an electrochemical assay for detection of active prostate-specific antigen (PSA) [19]. In their work, a ferrocene-functionalized PSA sensitive peptide was used to covalently modify a gold electrode of an electrochemical sensor (Fig. 8). PSA can cleave the peptide sequence, and then free electronically-active ferrocene from the gold electrode, resulting in the decrease of current intensity. A wide linear range of PSA at 0.5-40 ng/mL could be detected by the peptide-based electrochemical assay. The same strategy was also applied in the development of an ECL biosensor for detection of PSA by Qi *et al.* A PSA-sensitive peptide sequence conjugated with a Ru^{2+} chelate (as ECL marker), was immobilized on the surface of the gold electrode via S-Au bond. With the presence of PSA, the cleavage of the peptide by PSA releases Ru^{2+} chelate from the gold electrode, resulting in a decrease in

the chemiluminescence intensity. A wide linear range of PSA of 0.1-8 ng/mL and low detection limit of 0.038 ng/mL were achieved by the peptide-based ECL assay [97]. Recently, Qi and Zhang *et al.* reported a new ECL biosensor for the detection of PSA using immobilized gold nanoparticle-peptide-Fc (ferrocene carboxylic acid) bioconjugates as quencher, and $Ru(bpy)_3^{2+}$ as the ECL marker together on Nafion film. In the presence of PSA, the cleavage of the peptide by PSA removes the Fc quencher, and resulting in the recovery in the chemiluminescence intensity of $Ru(bpy)_3^{2+}$. A detection limit of 0.8 fg/mL for PSA was achieved, lower than with previously reported assays [99].

Ju *et al.* developed a colorimetric assay for the detection of MMP-7. In the assay, an MMP7-cleavable peptide sequence was conjugated with Au nanoparticles (Au Nps). This conjugation was then immobilized on the substrate of a chip through chelation between Ni^{2+} and His residue of the peptide sequence. In the assay, a fraction of the AuNps were released by cleavage of MMP-7 and removed through washing. The concentration of MMP-7 was inversely proportional to the color density from the remaining Au Nps on the chip [98]. In addition, a multiplexed protease assay can be realized based on lanthanide-coded strategy. Four protease-specific peptides were coded by Ln chelates (Ho, Tb, Pr and Eu) through conjugate of Ln chelates and matched peptide, and were immobilized on silica nanoparticles doped with $Ru^{2+}(bpy)_3$. Multiplexed proteases could then be assayed together. In the process of the assay, coded Ln chelates were released into solution through cleavage of proteases to the matched peptides, and were detected and quantified concurrently by element mass spectrometry [18].

3.2. Peptide-based kinase / kinase activity sensors

Kinases are a family of enzymes that transfer a phosphate group to a substrate to control the flow of information and regulate cellular metabolism, growth, differentiation, proliferation

and death. Several serious inflammatory condition and diseases, especially cancer, were reported to be caused by aberrations in kinase activity. Therefore, the development of efficient kinase sensors based on kinase recognition peptide sequences has been desired.

Fluorescent kinase sensors have been attracting great attention due to serial advantages including fast response and convenience. Hence, fluorescent kinase sensors analogous to protease sensors were used in detecting kinases, monitoring kinase activity [100] and screening for kinase inhibitors [101-102]. A few approaches including "self-reporting" [103], "deep-quench" [104] and "energy transfer", [105] have been utilized to design kinase sensors based on conjugation of environment-sensitive fluorophores with kinase recognition peptide sequences. A number of reported kinase molecular biosensors are summarized in Table 5.

Self-reporting kinase sensors were constructed based on the fact that tyrosine residues in the kinase recognition peptide sequence can quench fluorophores. In self-reporting kinase sensors, fluorophores were attached adjacent to the tyrosine residue in the kinase recognition peptide sequence. Phosphorylation of the tyrosine can alter the hydrophobic interaction between the fluorophores and the tyrosine, resulting in significant enhancement of fluorescence emission (Fig. 9). A light-regulated self-reporting tyrosine kinase sensor was further developed by Lawrence *et al.* by covalently modifying the tyrosine residue with a photolabile nitrobenzyl substituent, which can be dissociated under light irradiation [103]. Irudayaraj *et al.* reported a kinase biosensor for fluorescence lifetime imaging of single live cells. This kinase biosensor consisted of a kinase recognition peptide sequence and a cell-penetrating peptide sequence. A Cy5 dye was attached adjacent to the tyrosine residue in the kinase recognition peptide sequence. In this kinase sensor, phosphorylation of tyrosine by kinase can significantly increase the fluorescence lifetime of the adjacent Cy5 dye. The kinase sensor was successfully used to assess phosphorylation- and Abl kinase-dependence of the lifetime shifts in mouse embryonic fibroblast cells using fluorescence lifetime imaging

microscopy [64].

The deep-quench concept was recently proposed, and was utilized to develop a sensor of kinase activity. Lawrence *et al.* developed a kinase activity assay by dispersing a deeply quenched kinase-recognition peptide sequence labeled with pyrene in the solution of quenchers. With the presence of kinase, phosphorylation of serine disrupts the interaction between pyrene and the quencher through the formation of a new complex based on phospho-Ser binding domain, resulting in activation of the fluorescence emission of pyrene. In the deep-quench kinase assay, fluorophore-quencher pairs need be identified; a screened quencher of pyrene can provide significant fluorescent enhancement such as 21-fold for Ponceau S, 55-fold for Aniline Blue WS, and 64-fold for Rose Bengal.

Whitten *et al.* reported a fluorescent kinase sensor based on chelation of metallic ions and phosphate groups. In this kinase sensor, a kinase-recognition peptide sequence labeled with rhodamine was used as quencher to switch the fluorescence of a poly(p-phenylene-ethynylene)(PPE)-coated Ga(III)-modified polymer sphere. Kinase phosphorylation introduced the rhodamine-labeled kinase-recognition peptide sequence with phosphate groups, which was able to bind with Ga(III). The rhodamine-labeled kinase-recognition peptide sequence binding with Ga(III) gave rise to a close distance between the rhodamine and PPE, resulting in the quenching of PPE fluorescence on the polymeric sphere [106]. Katayama *et al.* also reported a fluorescent kinase sensor based on FRET from QD to fluorophore in a kinase-recognition peptide. In the performance of the kinase activity assay, a positively-charged protein kinase Cα (PKCα) recognition peptide was conjugated with tetramethylrhodamine (TAMRA) via a PEG-linker. Positively-charged TAMRA was then mixed with negatively-charged QD, and the electrostatic interaction between TAMRA and QD lead to FRET from QD to TAMRA. With the presence of PKCα, phosphorylation of kinase reduces not only the positive charge on the peptide, but also weakened the attracted

electrostatic interaction between QD and phosphorylated peptide, resulting in reduced FRET efficiency and restoration of the QD fluorescence [107].

The metallic ion chelation strategy has been utilized to develop a kinase sensor, whereby the mechanism relies on a spectral change of the fluorophores caused by phosphorylation. In the metallic ion chelation kinase sensor, the phosphorylation by the kinase can generate a negatively-charged phosphate group in the peptide sequence, which is involved in the chelation interaction between fluorophores and metallic ions, giving rise to a change in electronic structure in the fluorophores, resulting in the spectral change. Imperiali *et al.* have been developing chelation-enhanced fluorescence kinase sensors based on the chelation interaction of both phosphate groups and Sox amino acid to Mg(II). The kinase sensor was consisted of three main parts, the kinase-recognition peptide sequence with S/T/Y residue, a β-turn peptide sequence as the scaffold and a Sox group as fluorophore [108-109]. In the presence of a kinase, phosphorylation of the kinase can increase the binding affinity to Mg(II) due to the coordination of both Sox amino acid and phosphate groups to the metal ions. Strong binding between phosphorylated peptide molecular sensor and Mg(II) significantly enhanced the fluorescence intensity of Sox amino acid (Fig. 10). Nagano *et al.* developed a ratiometric kinase sensor based on chelation of both coumarin and phosphate groups to Cd(II). A Cd(II)-cyclen-appended aminocoumarin was used to conjugate with a kinase recognition peptide sequence to form the kinase molecular sensor. Phosphorylation of kinase generates negatively-charged phosphate groups which can replace aromatic 7-amino groups of the coumarin to coordinate with Cd(II), increasing the electron density of the 7-amino group, resulting in a shift of the excitation wavelength of the coumarin [110].

Lanthanide ions have also been utilized to develop fluorescent kinase sensors due to their unique optical properties. A lanthanide ion-chelation luminescence enhancement kinase sensor was developed by Sames *et al.* In this kinase molecular sensor, an iminodiacetate

moiety was attached to a kinase-recognition peptide sequence and close to the tyrosine residue. Kinase phosphorylation of the tyrosine residue increased the affinity of the peptide to lanthanide ions due to coordination between phosphorylated tyrosine and the iminodiacetate moiety to lanthanide ions. The chelation of the phosphorylated peptide sensor and the lanthanide ions (Tb(III) or Eu(III)) induced energy transfer from carbostyril aspartamide as an antenna to the lanthanide ion, giving rise to a significant luminescence enhancement of the lanthanide ion (Fig. 11) [111].

In addition, a peptide-based kinase sensor also was constructed based on electrochemical signaling. In the electrochemical kinase sensor, kinase-recognition peptide was immobilized on the gold electrodes of a silica chip. A special 5'-γ-ferrocenoyl-ATP (Fc-ATP) as a co-substrate was used to transfer kinase phosphorylation into a measurable electrochemical signal. The phosphate group and ferrocenoyl moiety of Fc-ATP were transferred into the peptide substrate immobilized on Au electrodes, and measured by the electrochemical sensor [112].

In summary, the peptide-based enzyme biosensor has been successful in the detection of proteases and kinases due to the key advantage of the peptide being the enzymatic substrate. Protease biosensors have matured as a technology to the point that several peptide-based protease molecular biosensors have been commercialized successfully as detection kits for disease indicating proteases including MMP and PSA. At the same time, the protease biochip based on peptide immobilization [113-114] for point-of-care detection has also been developed, and holds good prospects for commercialization in the near future. In the application of protease sensors in *vivo*, proteases are particularly relevant as tumor markers in cancer, motivating the development of protease probes for imaging in *vivo* [84, 115], which has become a new hot area of protease sensor research.

In comparison to protease sensors, the kinase biosensors are at a relatively immature development level with significant room for development. The mechanism of signal transduction in the kinase biosensor involves transferring the change in chemistry resulting from phosphorylation into a measurable signal, making it more complicated than the peptide cleavage mechanisms in protease biosensors [105]. Efforts towards improved design of the signal transduction mechanism can significantly improve the sensitivity of the kinase biosensor.

4. Peptides as the framework for biosensors

Short peptide sequences are sufficiently flexible to be folded as scaffolds, and have been used as a framework in molecular sensors.

4.1. Redox sensor

Peptide-based redox molecular sensors have been developed based on the redox property of cysteine residues. Thiol and disulfide structure of the cysteine has been used to control the static contact and separation of signal markers conjugated on a peptide. Chelation fluorescence enhancement and probe-quencher pair models were utilized to develop redox sensors by Winther's and Schneider's groups, respectively. Winther *et al.* developed a protein disulfide isomerase (PDI) sensor based on the probe-quencher pair model [116]. Fluorescent aminobenzoic acid residue and quencher nitrotyrosine were attached at the opposite ends of the sensor framework. The sensor framework was composed of two cysteines-containing peptides connected by a PEO spacer. Catalysis of PDI induces formation of a disulfide bond, giving rise to contact between the probe and quencher, resulting in an efficient quenching of fluorescence. Schneider *et al.* developed a Tb(III)-chelation fluorescent enhancement redox sensor based on the energy transfer strategy. In this case, carbostyril chromophore and Tb(III)-chelation were attached at the opposite ends of a flexible peptide sequence that was

flanked by a pair of cysteines. In the oxidized state, disulfide bond formation induced a close spatial distance between the carbostyril chromophore and Tb(III)-chelation moiety, resulting in an efficient energy transfer and fluorescent enhancement of the Tb(III)-chelation moiety (Fig. 12) [117].

4.2. Cyclodextrin sensor of peptide

Ueno *et al.* reported cyclodextrin-based sensors using a peptide framework. Cyclodextrins (CDs) can form an inclusion complex with a variety of organic compounds in the hydrophobic cavity. Therefore, the recognition of CD to guest molecules can be utilized to develop chemical sensors. In Ueno's peptide-based molecular sensor, CD was used as a receptor. Both CDs and fluorophores were conjugated with an α-helix peptide [118-123]. Various signalling strategies including FRET, probe-quencher pairs and excimers, were utilized to design the CD-based sensors. For example, in probe-quencher pair model sensors, CD was flanked by fluorophores, with quenchers on a peptide sequence. Guest molecules replaced the quencher by insertion into the cavity of CD, separating the probe-quencher pairs. This separation of probe-quencher pair enhanced the fluorescence intensity of the probe (Fig. 13A) [124]. In a FRET model sensor, the replacement of the donor by guest molecules breaks the energy transfer from donor to acceptor, resulting in a decrease in fluorescence intensity (Fig. 13B) [125]. In excimer model sensors, the guest can free one of fluorophores from the cavity of the CD and form an excimer with other fluorophores, resulting in spectral change of fluorophores (Fig. 13C) [126].

In both of these subclasses of peptide-mediated redox sensors, the peptide merely supplied the thiol group of the cysteine and is not directly involved in the signal transduction process of cyclodextrin sensor. Hence the peptide in this case does not play an irreplaceable role, and is in competition with other flexible building block option in redox and cyclodextrin sensors.

5. Conclusion and Outlook

Peptide-based sensors have been developing at a rapid pace particularly in the last two decades, and have shown great potential to be applied in various fields.Fluorescent molecular sensors in particular have demonstrated great promise in biological analysis due to the versatility in configuration. On the other hand, peptide-based biosensors based on electrochemical signalling could be utilized to develop commercial portable mini-biosensors or biochips in the future. In addition, with the immense amount of research into cellular function and enzymology, protease-cleavable peptide substrates as linkers could act as transducers for signal generation in biosensors [127], and give essentially limitless options for novel designs of peptide-based biosensors. Progress of the biochip technique also brings great challenges and opportunity to push the development of peptide-based biosensors [128-130]. Fast responsive, portable and convenient peptide-based chip-biosensors will almost certainly provide the next major step change in biological analysis and clinical diagnosis based on peptides [131].

Acknowledgement

The authors would like to gratefully acknowledge the Australian Research Council for providing financial support through the Linkage Project Grant Scheme (LP100100477).

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Tables

Table.1. Peptide-based protein molecular sensors.

Analyte	Interaction mode	Signal output	Signal strategy	Signal marker 1	Signal marker 2	Reference
Anti-HIV protein p17 antibody	Peptide beacon	Wavelength shift	Excimer	Pyrene	Pyrene	[25]
Anti-HIV protein p17 antibody	Peptide beacon	Fluorescence	Electron-accepting quenching	Ru (II) chelate	Phenanthroline	[26]
Cyclin A	Affinity	Normalized fluorescence	ESF	Dap(4-DMAP), and Dap(NBD)		[27]
Anti-HIV protein p17 antibody	Affinity	Color and fluorescence	ESF	Cy-3		[28]
LAPTM4B protein	Affinity	Fluorescence turn on	ESF	Tetraphenylethylene		[29]
Cyclin A	Affinity	Luminescence	ESF	Eu (III) chelate		[30]
HCAII	Affinity	Fluorescence	ESF	Dansyl group		[32-34]
Cyclin A	Affinity	Luminescence	ESF	Tb (III) chelate		[36]
Bacterial lipopolysaccharide	Affinity	Color change	PDA liposomes	PDA		[37]
EGFR	Affinity	Current	Electrochemical	Ferrocene		[38]
HIV anti-p24 antibodies	Affinity	Current	Electrochemical	MB		[39]
Amyloid 1–42	Affinity	Current	Electrochemical	Ferrocene		[40]

Abbreviation: HCAII = Human carbonic anhydrase II; EGFR = Epidermal growth factor receptor; ESF = Environmental sensing fluorophore; PDA = Polydiacetylene; MB = Methylene blue

Table. 2. Peptide-based metal ion sensors.

Analyte	Signal output	Signal strategy	Signal marker 1	Signal marker 2	Reference
Zn	Wavelength shift	Gold particle aggregation	Gold particle		[13]
Cu	Fluorescence	Quenching	Dansyl		[132]
Cu	Fluorescence	Quenching	DNS		[44]
Zn and Cd	Fluorescence	ESF	Dansyl		[45]
Zn	Fluorescence	ESF	8-Hydroxyquinoline		[53]
Ag	Fluorescence	ESF	Dansyl		[47]
Cu and Zn	Fluorescence	ESF	Dansyl		[48]
Zn	Fluorescence	ESF	DMB, DNS and CMN		[46]
Cd	Fluorescence	FRET	Dansyl	Trp	[50]
Hg	Fluorescence	FRET	Dansyl	Tryptophan	[51]
Hg	Fluorescence	FRET	Dansyl	Tryptophan	[52]
Cd, Co and Ni	Wavelength shift	Gold particle aggregation	Gold particle		[54]
Cu	Wavelength shift	Gold particle aggregation	Gold particle		[55]

Abbreviation: ESF = Environmental sensing fluorophore; DMB = 4-(dimethylamino)-benzamide; DNS = 5-(dimethylamino) naphthalenesulfonamide (dansyl); CMN = 3-carboxamido coumarin; FRET = Fluorescent resonance energy transfer.

Table. 3. Peptide-based nucleic acids molecular sensors.

Analyte	Interaction mode	Signal output	Signal strategy	Signal marker 1	Signal marker 2	Reference
Double-stranded DNA	Insertion	Wavelength shift	Excimer	Pyrene	Pyrene	[58]
Box B RNA	Affinity	Fluorescence	Competitive format	Pyrene		[59]
Heparin	Electrostatic interaction	Fluorescence	Quenching	Quinolinium		[62]

Table. 4. Peptide-based protease molecular sensors.

Analyte	Interaction mode	Signal output	Signal strategy	Signal marker 1	Signal marker 2	Reference
Trypsin and chymotrypsin	Cleavage	Element Mass Spectrometry	Lanthanide-Code	Lanthanide ions		[18]
Papain Cathepsin B	Cleavage	Fluorescence	Energy transfer	Benzoxazol-5-yl-alanine derivatives	Tyr NO2	[74]
AspN Endoproteinase and Chymotrypsin	Cleavage	Fluorescence	Self-quenching	Cy-5 or Fluorescein		[77]
MMP-2 and MMP-9	Cleavage	Fluorescence	Self-quenching	Dye Ls276		[78]
Aspartyl Proteinases	Cleavage	Fluorescence	Quenching	*o*-aminobenzoyl	DNPED	[79]
Renin	Cleavage	Fluorescence	Quenching	1,5AEDANS	DABCYL	[80]
ICE	Cleavage	Fluorescence	Quenching	1,5AEDANS	DABCYL	[81]
Cytomegalovirus protease	Cleavage	Fluorescence	Quenching	EDANS	DABCYL	[82]
Neprilysin and insulin-degrading enzyme	Cleavage	Fluorescence	Quenching	Alexa-350	DABCYL	[83]
Secretase and caspases	Cleavage	Fluorescence	Quenching	PPE	QSY-7	[133]
Trypsin, chymotrypsin, proteinase K and thermolys	Cleavage	Fluorescence	Quenching	FITC	Gold nanoparticles	[134]
PSA	Cleavage	Fluorescence	Quenching	FITC	Gold nanoparticles	[135]

Caspase-3	Cleavage	Fluorescence	Quenching	FAM	Graphene oxide	[136]
Chymotrypsin and MMP-2	Cleavage	Fluorescence	Quenching	Metalloprotoporphyrins, QXL $_{570}$	Graphene oxide	[85]
Thrombin	Cleavage	Fluorescence	Quenching	FAM	SWCNHs	[137]
Trypsin, chymotrypsin, V8 protease, plasmin, thrombin and pepsin	Cleavage	Fluorescence	FRET	Mca-fluorophore	Dinitrophenyl	[86]
Prokaryotic enzyme	Cleavage	Fluorescence	FRET	FITC	Dabcyl (Dbc)	[138]
MMP-1 and MMP-9	Cleavage	Fluorescence	FRET	5FAM	Cy5	[88]
HIV protease	Cleavage	Fluorescence	Quenching	EDANS	DABCYL	[89]
MMP-7	Cleavage	NIR fluorescence	FRET	Cy5.5	NIR8Q20	[92]
Thrombin	Cleavage	NIR fluorescence	Self-quenching	Cy 5.5		[93]
Tumor proteasee	Cleavage	NIR fluorescence	Self-quenching	Cy 5.5		[94-95]
PSA	Cleavage	Current	Electrochemical	Ferrocene		[19]
Trypsin and α-Thrombin	Cleavage	Current	Electrochemical	Ferrocene		[96]
MMP-7	Cleavage	Color density	Silver enhancement on Au particle	Au particle		[98]
PSA	Cleavage	Luminescence	Electrogenerated luminescence	Ru (II) chelate		[97]
PSA	Cleavage	Luminescence	Electrogenerated luminescence	Ru (II) chelate	Ferrocene	[99]

Abbreviation: ICE = Interleukin 1 beta converting enzyme; DNS = 5-(dimethylamino) naphthalenesulfonamide (dansyl); FRET = Fluorescent resonance energy transfer; DABCYL = 4-(4-N,N-dimethylaminophenyl)azobenzoic acid; FITC = Fluorescein isothiocyanate; 1,5AEDANS = 5-((2-

amino-ehyl)amino)-naphthalene-1-sulfonic acid; PPE = *poly*(phenyleneethynylene); DNPED = *N*-2,4-dinitrophenyl ethylenediamine; SWCNHs = Single-walled carbonnanohorns

Table. 5. Peptide-based kinase molecular sensors.

Analyte	Interaction mode	Signal output	Signal strategy	Signal marker 1	Signal marker 2	Reference
PTKs	Phosphorylation	fluorescence	Self-report	Cascade yellow Y-2 derivative		[103]
PKA	Phosphorylation	Fluorescence	Deep quench	Pyrene		[104]
c-Abl1	Phosphorylation	Fluorescent lifetime		Cy5		[64]
PKA and PTP-1B	Phosphorylation	Fluorescence	FRET	PPE	Rhodamine	[106]
PKCα	Phosphorylation	Fluorescence	FRET	Quantum dot	TAMRA	[107]
PKA, PKCα and Abl	Phosphorylation	Fluorescence	Chelation-enhanced fluorescence	Sox		[108-109]
PKA	Phosphorylation	Ratiometric	Chelation-enhanced fluorescence	Aminocoumarin		[110]
src and abl PTKs	Phosphorylation	Luminescence	Energy transfer	Lanthanide ion chelate		[111]
Src, Erk1, and CDK2/cyclin A	Phosphorylation	Current	Electrochemical	Ferrocene		[112]

Abbreviation: PTKs = Protein tyrosine kinases; PKA = Protein kinase; Abl1 = c-Abl kinase; PTP-1B = Protein tyrosine phosphatase 1B; PKCα = Protein kinase Cα; Src = Sarcomarelated kinase; Erk1 = Extracellular signal-regulated kinase 1; CDK2/cyclin A = cyclin A-dependent kinase 2; PPE = *poly*(p-phenylene-ethynylene); TAMRA = Tetramethylrhodamine.

Figure Legends

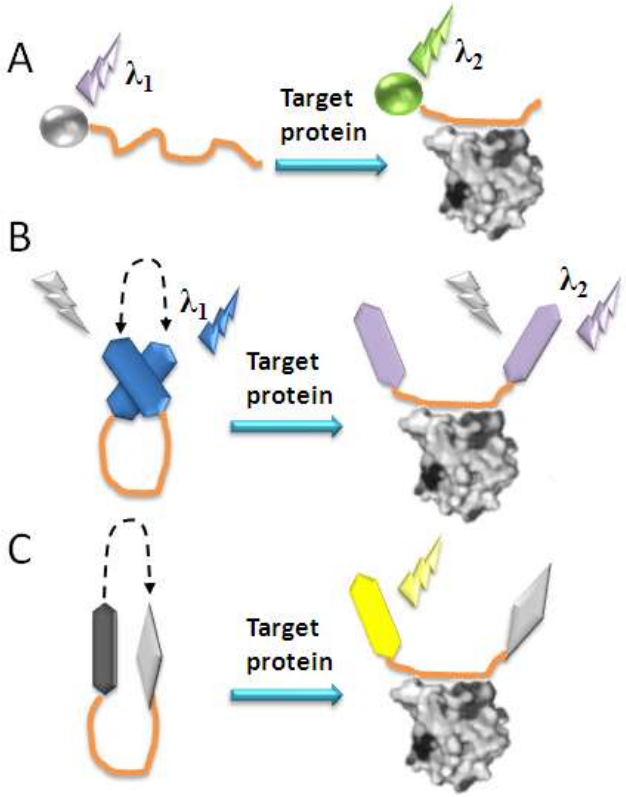

Fig. 1. Schematics of a range of peptide-based protein sensors with fluorescence readout: (A) Peptide probe with environment-sensitive fluorophores as signal markers to respond to the target protein; (B) Excimer-pair peptide sensor; the peptide binds to the target protein, changing the spectral property of the fluorophores; (C) FRET/probe-quencher pair peptide sensor, where the distance between the donor-acceptor/probe-quencher was changed by binding of the peptide to the protein.

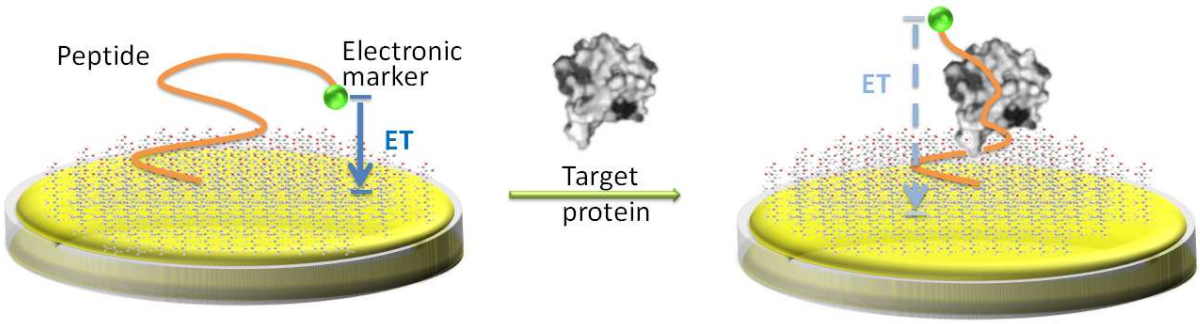

Fig. 2. Schematic diagram of an electrochemical peptide-based protein sensor. The electron transfer (ET) from electronic marker to the electrode is influenced by the binding/affinity between the peptide and the target protein.

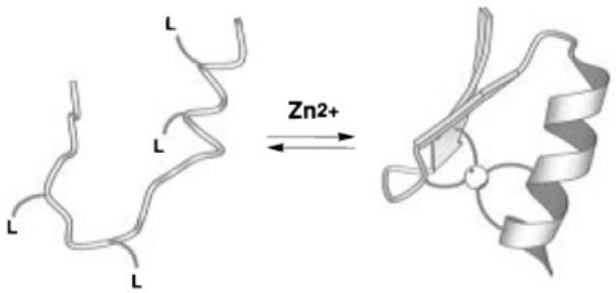

Fig. 3. The coordination of a metallic ion to the peptide sequence induces a change in the secondary/tertiary structure of the peptide, and affects the spectral properties of the environment-sensitive fluorophores. (From Walkup *et al.* [46], with permission from ACS)

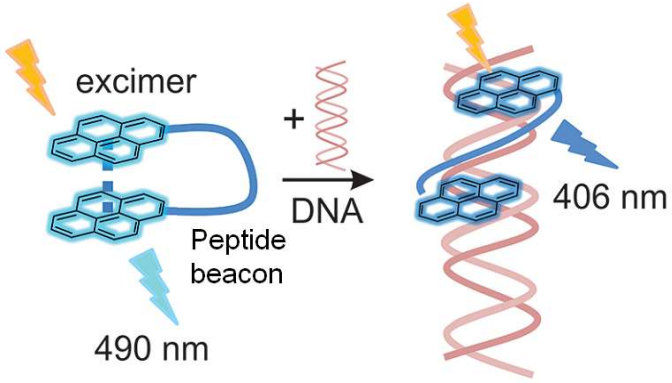

Fig. 4. Detection mechanism for DNA using a peptide-based nucleic acid sensor based on excimer of pyrene units. (From Walkup *et al.* [58], with permission from ACS)

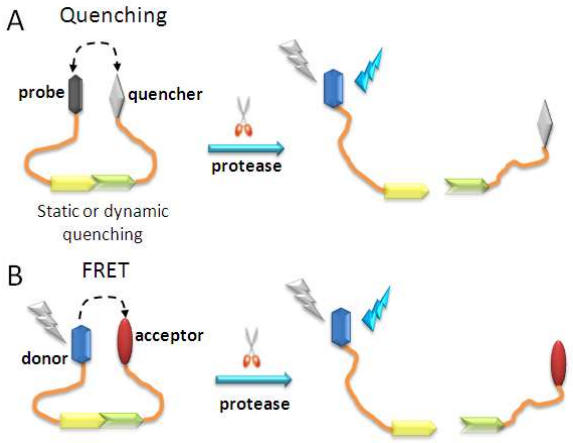

Fig. 5. Signaling mechanism using a protease-cleavable peptide strategy. Donor-acceptor/probe-quencher pairs are attached on a peptide in close proximity, and cleavage of the peptide by the protease releases the donor/probe, resulting in an increased fluorescence emission intensity.

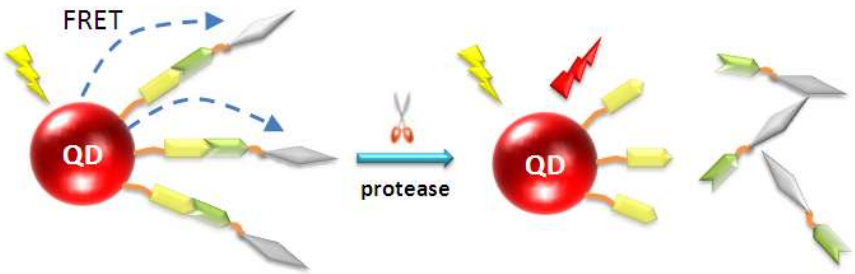

Fig. 6. Schematic of QD-based protease activity sensors. QD fluorescence is quenched by the attached quencher on the surface of QD connected by FRET. QD fluorescence is recovered after the protease cleaves the peptide linkers.

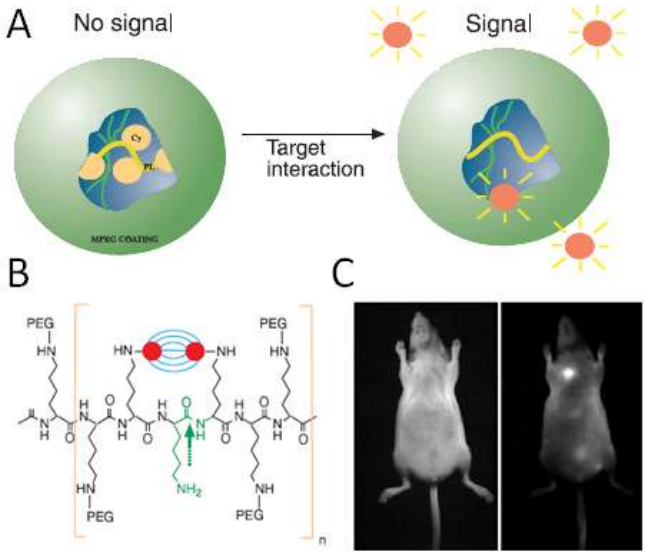

Fig. 7. (A) Schematic diagram of probe activation process; (B) Chemical structure of the NIR probe; (C) Brightfield image and NIRF image of the LX-1 tumor implanted into a nude mouse. (From Weissleder *et al.*[95], with permission from Nature)

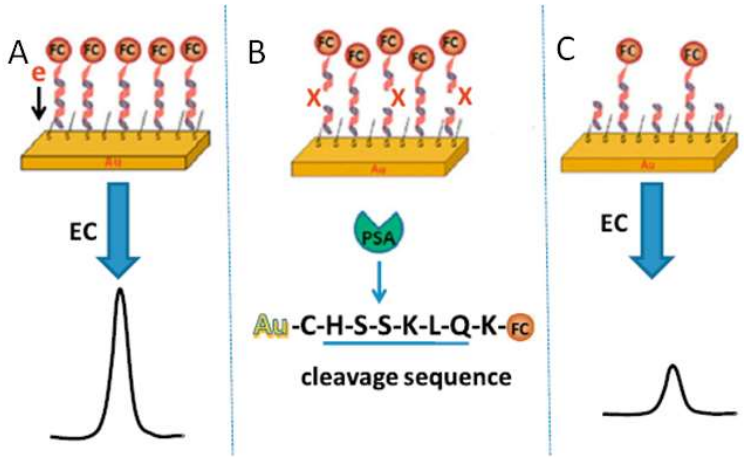

Fig. 8. Schematic illustration of the electrochemical assay for active PSA. (A) Self-assembly of FC-functionalized peptide probes on a gold electrode surface and electrochemical readout of redox activity of FC. The PSA cleaves the peptide sequence to remove the FC segments from the gold electrode surface (B), and reduces electrochemical readout of redox activity of FC (C). (From Zhao *et al.*[19], with permission from Elsevier)

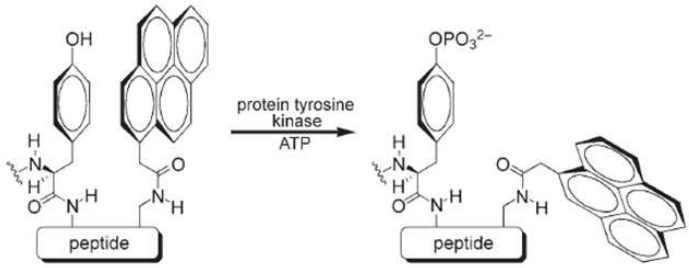

Fig. 9. Mechanism of self-reporting kinase sensors. Phosphorylation of tyrosine plays a quenching role to alter the stacking interactions between the tyrosine side chain and the pyrene fluorophores, resulting in a change in the emission properties of the pyrene fluorophores.

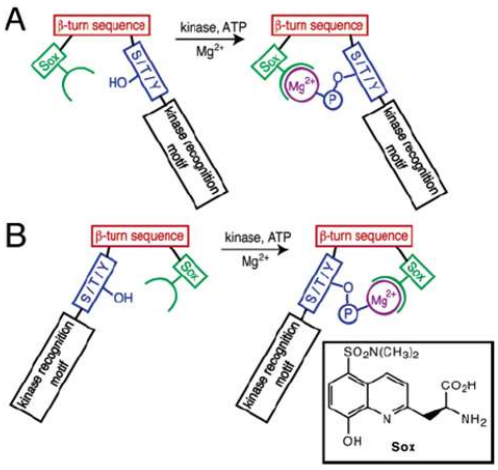

Fig. 10. Structure of metal-ion-chelation enhanced protein kinase sensors. This sensor consists of Sox, peptide scaffold and kinase recognition sequence connected with serine/threonine. Phosphorylation increases the binding affinity to Mg^{2+}. The coordination to the metal ions enhances the fluorescent intensity of the Sox amino acid. (From Shults *et al.* [108], with permission from ACS)

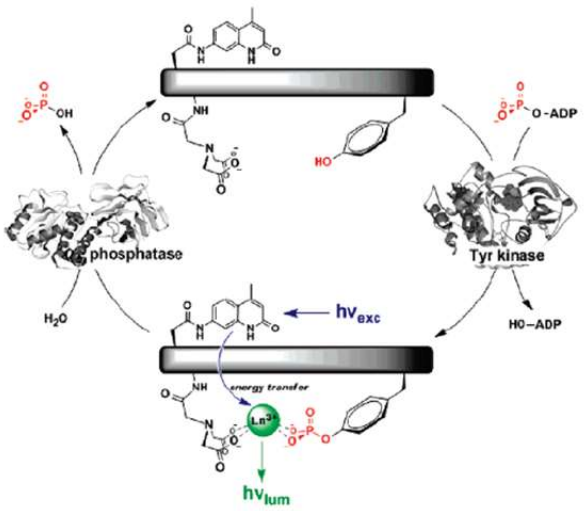

Fig. 11. Mechanism of lanthanide-chelation kinase sensors. Phosphorylation of tyrosine increases affinity to lanthanide ions through coordination, giving rise to energy transfer, resulting in fluorescence emission of lanthanide ions. (From Tremblay *et al.* [111], with permission from ACS)

Fig. 12. Mechanism of a peptide-based redox probe based on energy transfer. The formation of the disulfide bond in oxidised state reduces the distance between the donor and acceptor, leading to occurrence of FRET, resulting in a change in the spectral property of the fluorophores.

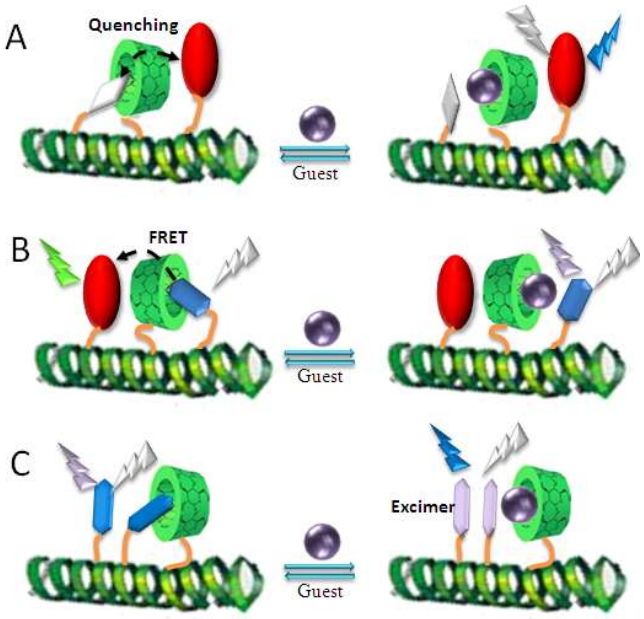

Fig. 13. Mechanism for chemical sensors based on peptide scaffolds with cyclodextrin and fluorophores. The sensors were designed based on (A) the probe-quencher pair, (B) FRET and (C) excimer strategies, respectively.

Graphical abstract