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REVIEW

Short peptides as biosensor transducers

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Abstract This review deals with short peptides (up to 50 amino acids) as biomimetic active recognition elements in sensing systems. Peptide-based sensors have been developed in recent years according to different strategies. Synthetic peptides have been designed on the basis of known interactions between single or a few amino acids and targets, with attention being paid to the presence of peptide motifs known to allow intermolecular self-organization of the sensing peptides over the sensor surface. Sensitive and sophisticated sensors have been obtained in this way, but the use of designed peptides is limited by severe difficulties in their in silico design. Short peptides from random phage display have been selected in a random way from large, unfocussed, and often preexisting and commercially available phage display libraries, with no design elements. Such peptides often perform better than antibodies, but they are difficult to select when the target is a small molecule because of the need to immobilize it with considerable modifications of its structure. Artificial, miniaturized receptors have been obtained from the reduction of the known sequence of a natural receptor down to a synthesizable and yet stable one. Alternatively, binding sites have been created over a designed, stable peptide scaffold. Short peptides have also been used as active elements for the detection of their own natural receptors: pathogenic bacteria have been detected with antimicrobial and cell-penetrating peptides, but key challenges such as detection of bacteria in real samples, improved sensitivity, and improved selectivity have to be faced. Peptide substrates have been conjugated to fluorescent quantum dots to obtain disposable sensors for protease activity with high sensitivity. Ferrocene–peptide conjugates have been used for electrochemical sensing of protease activity.

Keywords Amino acids · Peptides, Biosensors, Electrochemical sensors · Mass sensitive sensors, Fluorescence · Luminescence, Optical sensors

Abbreviations

ACPP	activatable cell-penetrating peptide
AMP	antimicrobial peptide
CPP	cell-penetrating peptide
ELISA	enzyme-linked immunosorbant assay
Fc	ferrocene
FRET	Förster resonance energy transfer
LOD	limit of detection
MPA	mercaptoproprionic acid
PCR	polymerase chain reaction
QCM	quartz crystal microbalance
QD	quantum dot
SAM	self-assembled monolayer
SNAP-25	synaptosomal-associated protein 25
SPRI	surface plasmon resonance imaging
TAT	transactivator of transcription
TNT	2,4,6-trinitrotoluene

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Introduction

Peptides represent a clear option if one wishes to mimic the molecular recognition mechanism occurring in biomolecules

such as enzymes, antibodies, drug receptors, and transmembrane proteins [1–3]. The high specificity or high catalytic activity of these macromolecules or both are generated by binding sites in which multiple interactions can be formed with the target molecule, and there has been a considerable effort to understand the mechanism that controls recognition with the view of generating artificial macrostructures for the purpose of binding and/or catalysis. An example that has attracted significant research is represented by the olfactory receptor proteins [4]. These very complex molecules are difficult to purify from natural sources and impossible to synthesize, yet their availability for specific applications would be invaluable. For these reasons the design of short, peptide-based, artificial receptors capable of highly specific recognition is one of the goals of biotechnology to obtain competitors, inhibitors, mimotypes, drugs, reagents for affinity purification systems, and active elements in biosensors [5]. Short peptides represent an excellent opportunity for the design of artificial receptors because of (1) the number of different molecules that can be obtained by combining the 21 natural amino acids, (2) the availability of both molecular biology and chemical techniques for the fast screening of peptide libraries, (3) the possibility of automated synthesis, and the low cost (in comparison, e.g., with monoclonal antibody technologies) for the preparation of relatively large amounts of highly purified peptides, (4) the ease of modification to further enhance binding, and (5) relatively easy modeling. A growing number of biomimetic, peptide-based sensing systems have been reported in the recent literature, and the wide range of target analytes includes whole cells, proteins, small organic molecules such as drugs, hormones, and pollutants, and ions. This review deals with short peptides, meaning peptides that could be available via automated chemical synthesis (two to 50 amino acids), and relevant examples will be reported according to the source of biomimetic inspiration that allowed their design and according to the method adopted for their selection and synthesis as well:

- Designed synthetic peptides: we include here binder elements designed on the basis of known interactions between single or a few amino acids and targets, with no attempt to build highly organized binding sites, but rather with attention being paid to the presence of peptide motifs known to allow intermolecular self-organization of the sensing peptides over the sensor surface.
- Short peptides from random phage display: here the peptides are selected in a random way from large, unfocussed, and often preexisting and commercially available phage display libraries, with no design elements.
- Peptide receptors for ligand sensing: artificial, miniaturized receptors can be obtained from the reduction of the known sequence of a natural receptor down to a synthesizable and yet stable one. Alternatively, a binding site can be created over a designed, stable peptide scaffold.
- Peptide ligands for receptor sensing: short peptides can also be used as active elements for the detection of their own receptors. Thus, antimicrobial peptides (AMPs) and cell-penetrating peptides (CPPs) are used for sensing bacterial cells, antigenic peptide sequences for antibody monitoring, and peptide substrates for enzyme detection.

Designed synthetic peptides

The simplest, but nevertheless very effective, level of design for peptides involved as active elements in sensors is found in ion-selective electrodes for metals such as copper, nickel, and zinc. The topic was reviewed by Chow and Gooding [6] in 2006, and we report here some interesting recent examples (see also Table 1). Even in the very simple tripeptide Gly-Gly-His, the design embodies ion selectivity, as the peptide interacts selectively with copper, whereas its isomer Gly-His-Gly cross-reacts with copper and zinc. On this basis, a field-effect transistor selectively sensitive to copper and a polypyrrole nanowire electrode were built [7, 8]. The mechanism of copper complexation by the peptide has been studied directly on the surface of a gold microcantilever [9], which gave different responses depending on the copper concentration and time. The results are consistent with the presence of two different copper–peptide complexes: a monodentate, low-affinity complex is formed in a fast step by histidine (Fig. 1) and then the system evolves in a slow step towards a high-affinity tetradentate complex, which gives rise to a different response from the cantilever, owing to the change in volume of the peptide monolayer.

This result also explains the ion selectivity of the peptide, since the Gly-His-Gly isomer is likely unable to form the tetradentate complex for steric reasons, and monodentate complexation by histidine is not selective. A higher level of design is embodied in an octapeptide that forms aggregate nanofibrils covering the surface of the electrode, and changes its conformation upon binding of copper, thus modifying the electrode activity [10]. Ion-sensitive peptides have been designed also taking inspiration from more complex naturally occurring motifs: the zinc-finger peptide sequence has been used to build up a zinc Förster resonance energy transfer (FRET) fluorimetric sensor [11]. The zinc-finger peptide changes its conformation upon zinc binding, and the addition of a fluorophore/quencher couple at the peptide ends allows zinc detection by measuring the fluorescence quenching. Peptide sequences known to interact with nickel, from tetanus toxin (P12), hepatitis C virus, fragment (NS4/7), and a poly-H sequence, have been

Table 1 Sensors based upon designed synthetic peptides

Reference	Target	Sensing peptide	Sensor	Sensitivity	Selectivity
[7]	Cu^{2+}	3-mer GGH	Silicon nanowire FET	1 nM to 1 mM	Cu^{2+} in the presence of Zn^{2+}
[8]	Cu^{2+}	3-mer GGH	Polypyrrole nanowire electrode	20–300 nM	
[9]	Cu^{2+}	3-mer GGH	Microcantilever	1 μM to 1 mM	
[10]	Cu^{2+}	8-mer NCGAITIG	Ion-selective electrode	15–50 μM	
[11]	Zn^{2+}	Zinc-finger peptide	FRET fluorimetric		
[12]	Ni^{2+}	Tetanus toxin (P12); HCV sequence (NS4/7); poly-H sequence	SPRI microarray	LOD 10 μM, similar to what it is usually obtained with label-free biosensors for ions	
[14]	VOCs	Short-chain polypeptides: PAC1, PAC2, PAM1, PAM2	QCM	Milligram per milliliter	PAC1, PAC2, PAM2 to acetic acid; PAC1, PAC2 to butyric acid; PAM1, PAM2 to dimethyl amine
[15]	Dioxins and PCBs	[N]NFQG-aa-[C]	QCM		
[16]	Dioxins	FLDQPhenylglycine and other designed pentamers	On-bead fluorescence microscopy	LOD 0.05 ng/ml	Very detailed data on cross-reactivity with chlorinated dioxins (+) PCBs (−), chlorinated dibenzofurans (+)
[17]	Atrazine, diuron	EYYY and other 4-mers from combinatorial libraries	SPR	200 μM to 1 mM	
[27]	Carbonic anhydrase	42-mer helix–loop–helix peptide	Assembly of gold nanoparticles	LOD 10 nM, dynamic range 10–100 nM	Partial cross-reactivity with human albumin

aa amino acid, *FET* field-effect transistor, *FRET* Förster resonance energy transfer, *HCV* hepatitis C virus, *LOD* limit of detection, *PCBs* polychlorinated biphenyls, *QCM* quartz crystal microbalance, *SPR* surface plasmon resonance, *SPRI* surface plasmon resonance imaging, *VOCs* volatile organic compounds

exploited on surface plasmon resonance imaging (SPRI)-based microarrays for multiparametric and simultaneous analyses of peptide–metal interactions or of metal levels to monitor a large panel of metal-mediated biological activities [12]. A Pb^{2+}-binding peptide has been linked with peptide nanotubes, and an electrode surface was coated with such material. After complexation of Pb^{2+} by the sensing peptide, lead was reduced to obtain lead nucleation on the peptide nanotubes. The presence of the metal junctions on the surface was detected as a resistor in the equivalent circuit, allowing unprecedented sensitivity for Pb^{2+} in the 0.01–10 nM range [13].

Small organic molecules have also been detected with designed oligopeptides. Simultaneous detection and identification of various classes of volatile organic compounds has been performed by a quartz crystal microbalance (QCM) with a sensor module coated with synthetic short peptides derived from in silico design and selection, together with conducting polymers [14]. Picogram per milliliter dioxin pentapeptide binders have been obtained by a combinatorial peptide library screening [15] and designed modification of the binders with nonnatural amino acids [16]. The sensitivity obtained with such short sequences including nonnatural amino acids is a clear advance with respect to the previous results obtained by the same authors in detection of atrazine and other chlorinated herbicides with fully natural oligopeptides [17].

Peptides with an amino-terminal cysteine [18], lipoyl helical peptides [19, 20], and amphiphilic β-peptides with an acid-terminal thiol group [21] are capable of forming self-assembled monolayers (SAMs) on gold surfaces, providing an advantageous means of manipulating the surface properties of metal nanoparticles. Recently, to reproduce the chemical and structural complexity of proteins, a nanoengineering approach was used to design peptides able to cap such surfaces, conferring stability [22], minimizing nonspecific interactions [23], and providing an appropriate template to incorporate several molecular receptors or recognition groups [23, 24]. Most of these sequences, such as CALNN and 3-MPA-$(Ser)_5$-OH (where MPA is mercaptoproprionic acid), are provided by rational or combinatorial designs. We report two biosensors that exploit peptides to establish SAMs as the molecular recognition elements for *Botulinum* toxin A and vancomycin. In the first one, SAMs displaying an immobilized, fluorescent peptide that mimics the toxin's natural partner (synaptosomal-associated protein 25, or SNAP-25) were clustered on gold surfaces and interfaced with arrayed microfluidic channels (Fig. 2).

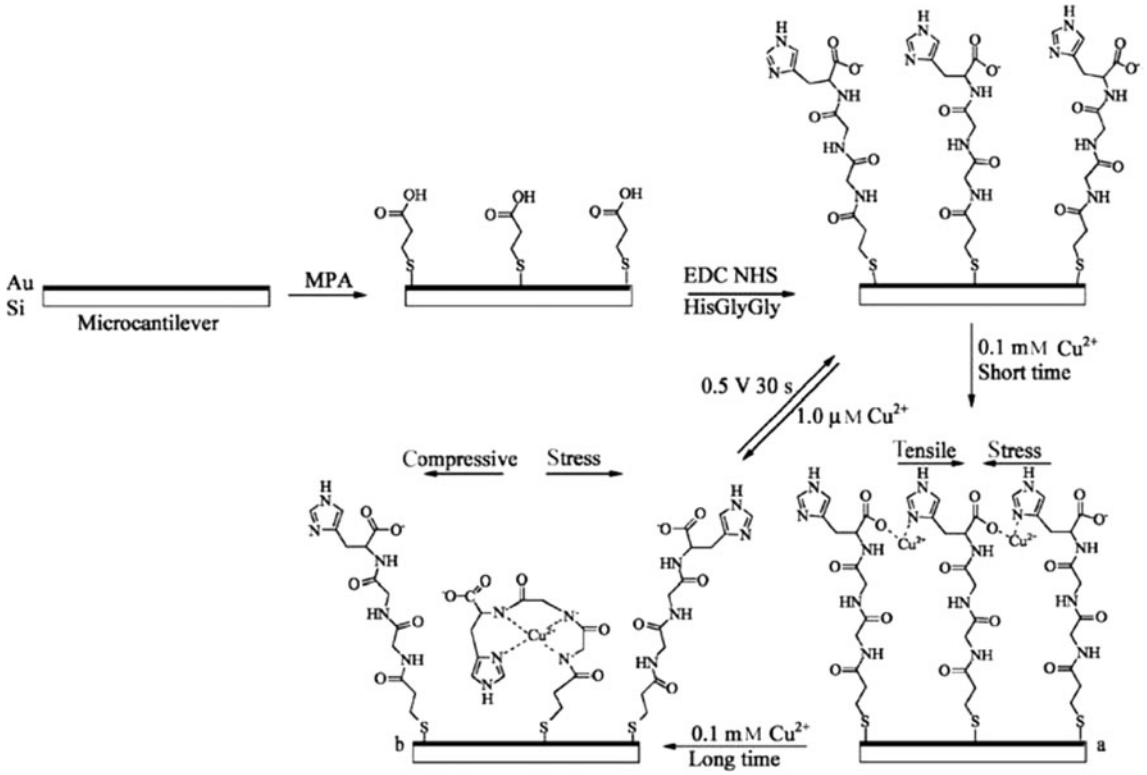

Fig. 1 Selective binding of copper to a microcantilever coated with the Gly-Gly-His tripeptide. *EDC N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide, *MPA* mercaptoproprionic acid, *NHS N*-hydroxysuccinimide. (From Xu et al. [9], with permission from Elsevier)

Measuring fluorescence intensities resulting from toxin cleavage of the surface substrate, the sensor could detect the holotoxin down to 3 pg/mL—ten times below the desired limit of detection (LOD) [25].

In the second example, a gold-coated QCM detects binding of vancomycin, a glycopeptide antibiotic, with a D-Ala-D-Ala-containing peptide immobilized as a stable and highly packed SAM, which is formed thanks to the presence of a designed spacer, containing also several poly(ethylene glycol) units in order to modulate its polarity (Fig. 3). The advantages of this method are high sensitivity and real-time analysis and no labeling of the analyte [26].

A very high and sophisticated level of peptide design has been used in the development of JR2EC and KE2C, a couple of 42 amino acid synthetic peptides [27]. The peptides are unfolded at neutral pH, owing to a very high number of negative charges, whereas in the presence of Zn^{2+}, complexation of the carboxylate groups occurs, and the peptides fold in a helix–loop–helix conformation. The folded peptides aggregate in a dimeric coiled coil, even when they are immobilized on gold nanoparticles, which for this reason aggregate. If the loop region of the peptide is designed in a way to bind the target, then its recognition interferes with aggregation, and the gold nanoparticles are shifted towards a free form, which differs greatly in spectral properties from the aggregate (Fig. 4).

The proof of concept was given using carbonic anhydrase as a target, by using a chemically modified peptide including a carbonic anhydrase inhibitor moiety at the loop level [28].

Designed peptides are thus powerful tools for the development of sensors for ions, small molecules, and even proteins, and for introducing high levels of organization in the sensing surface of the device. However, natural peptide motifs represent the almost unique source of inspiration for the design: peptide binding motifs and peptide self-organizing motifs originate from the knowledge of similar interactions in proteins. This is the main shortcoming because of the enormous difficulties still to be overcome in theoretical approaches towards fully artificial designed peptides. The in silico design of a peptide capable of binding a chosen target requires optimizing iteratively an objective function (e.g., the binding affinity) upon a library of candidate peptides differing in their primary sequence. Even though important successes have been obtained with these approaches, computational and experimental protein designers struggle with severe technical problems. First, embedding a recognition site in a fixed scaffold leads to a suitable candidate only if the protein scaffold retains its stability in spite of the mutations. However, stable scaffolds are normally large and difficult to synthesize or express in large quantities. Moreover, in computational approaches one has to cope with the combinatorial explosion associated with the

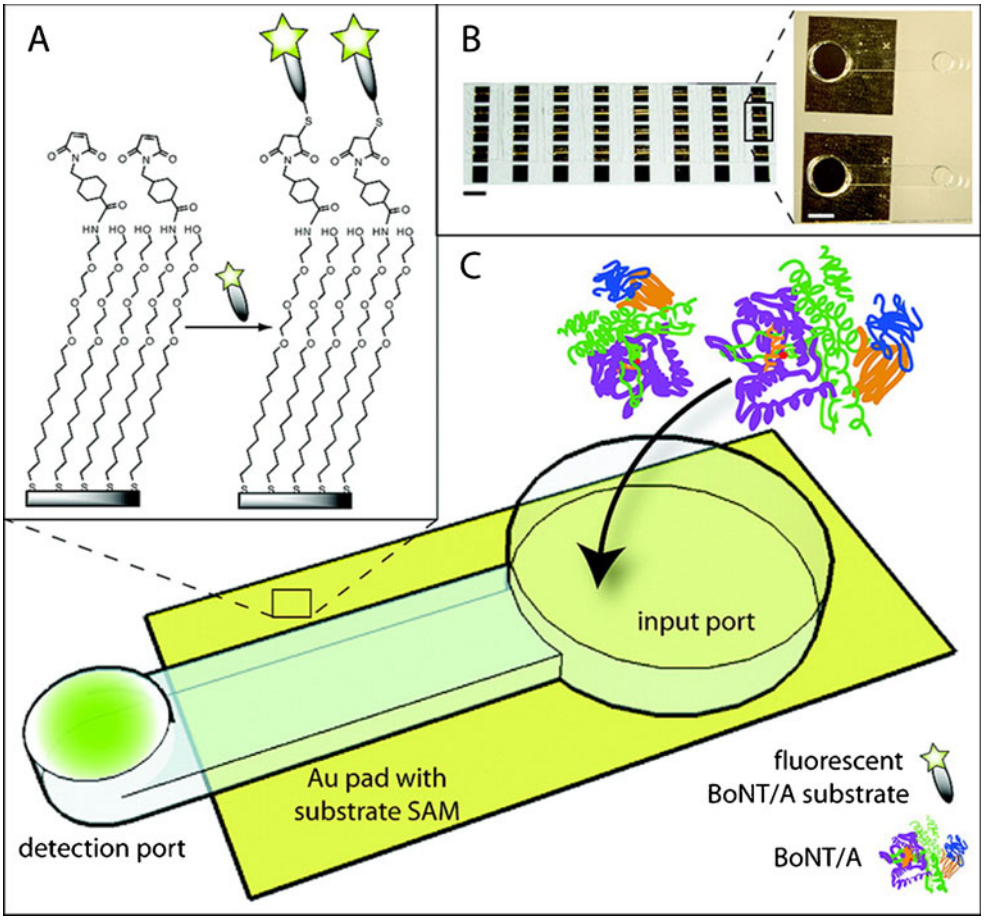

Fig. 2 Sensor for *Botulinum* neurotoxin A (*BoNT/A*). *A* self-assembled monolayer (*SAM*) formation on gold yields mixed monolayers of amine- and hydroxyl-terminated alkanethiols presenting the BoNT/A sensing peptide. *B* polydimethylsiloxane microchannels on 40 arrayed gold pads (10.5 mm^2) (*scale bar* 5 mm) with the image in the *inset* representing two neighboring channels (*scale bar* 1 mm). *C* BoNT/A is added at the input port and incubated on SAMs. Cleavage of the immobilized peptide substrate releases fluorescent fragments into solution. The fragments are concentrated at the detection port via evaporation. (From Frisk et al. [25], with permission from the American Chemical Society)

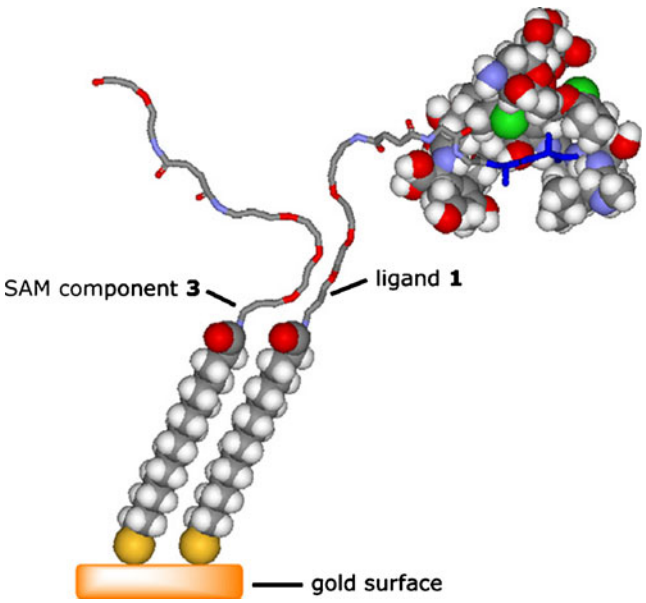

Fig. 3 Model of binding of vancomycin to stable, well-packed, and mixed SAMs presenting D-Ala-D-Ala (*blue sticks*). The mixed SAMs are linked by ligand 1 (a short ethylene glycol oligomer) and SAM component 3 (thiolipoic acid). (From Tseng et al. [26], with permission from Elsevier)

mutational space: if one chooses ten putative mutation sites along the peptide sequence, the number of possible combinations one should consider is 10^{20}.

Short peptides from random phage display

"The key characteristics of evolving organisms are replicability and mutability. How can a chemical replicate, or mutate? Take a protein as an example: it cannot replicate or mutate directly, of course; but it is associated with a cell that can. Linkage of the protein with the generic machinery that encodes it thus confers on it the key properties of replicability and mutability" [29, 30].

In peptide phage display, a property such as recognition and binding, related to the primary sequence, is linked to the gene encoding for it. Phages are viruses that infect bacteria (typically *Escherichia coli*), and they are used as expression vectors for a foreign DNA strand, which will be expressed by the infected host as a peptide. In phage display the foreign gene is spliced into the gene for one of the phage coat proteins, and the required amino acid sequence is fused to the endogenous one, to give a hybrid fusion protein. The hybrid coating protein is then incorporated into phage

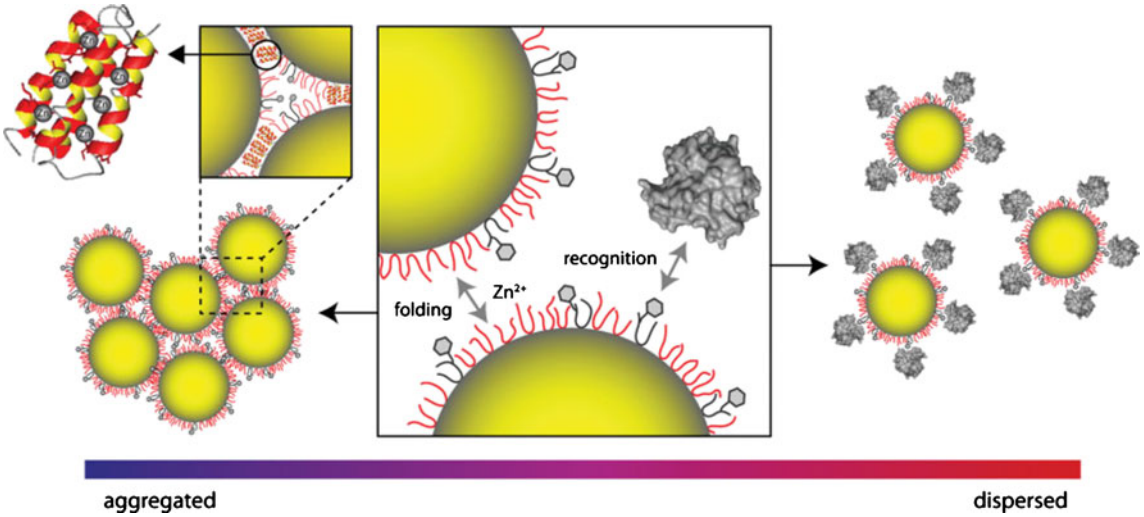

Fig. 4 Zn^{2+}-promoted folding of helix–loop–helix peptides allows nanoparticle aggregation. Recognition of the target shifts the equilibrium towards dispersed nanoparticles. (From Aili et al. [27], with permission from Wiley)

particles when they are released from the cell, and the peptide is displayed on the surface of the virion (Fig. 5). Phage display libraries are mixtures containing as many as 10^9 members of different phage clones, each displaying a different peptide generated by randomizing the gene sequence. The selection of binder peptides through the library involves necessarily a binding event between the displayed peptide and an immobilized version of the target to allow the physical separation of the best performing binders from the library mixture. Both the fused expression protein on the phage and the immobilization system of the target (which is modified by a reactive linker) can interfere in the binding event.

This may represent the main shortcoming of the approach if the target is a small molecule. Very few functional groups are usually found in a small organic target molecule, such as a drug, and each of them may offer a fundamental interaction point with potential receptors. The linker has to be introduced by modification of such functional groups, and it may be larger than the target itself. Biotin is often found at the linker end, to allow immobilization on avidin-coated surfaces. Cross reactivity with the linker, and even with

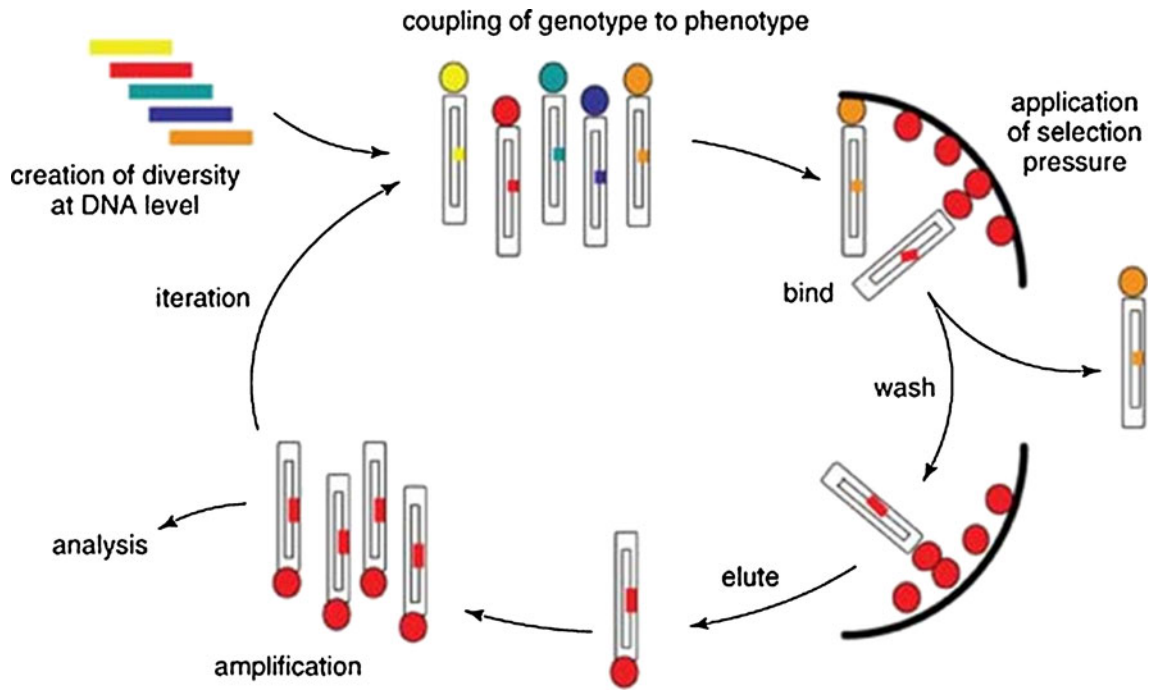

Fig. 5 The cycle of peptide phage display for the selection of binder peptides. (From Bradbury [30], with permission from Wiley)

avidin, if the target is displayed on avidin–biotin surfaces, is often a problem. It is rather difficult to select peptide binders that are able to discriminate between the immobilized and the free target, as has been reported in the selection of antitestosterone and antimethotrexate peptides [31, 32]. A typical example of the difficulties connected with phage display for small molecules is the development of a 2,4,6-trinitrotoluene (TNT) biosensor [33]. To allow immobilization, the methyl group of TNT was replaced by a benzenesulfonic acid moiety, suitable for covalent linking to carrier proteins by sulfonamide formation. The phage libraries were selected for albumin conjugates of such a molecule. All the stronger binders selected shared an Arg-His consensus dyad (a bidentate cation and a hydrogen-bond acceptor for synergistic recognition of the nitro group): this can be regarded as a correct binding response from the library, as also the immune system and the bidentate anion moieties react in a very similar way [34]. However, use of the selected peptides in fluorimetric flow sensors resulted in LODs as high as 12 mg/ml, most likely due to the inability of the peptides to recognize fully the unmodified, free TNT.

The proximity between the many peptide molecules displayed on the phage capsid may lead to multivalency in target binding: a tetrapeptide binder was selected for a porphyrin target by phage display, but two molecules of the peptide were involved in π-stacking interactions with a single porphyrin molecule [35]. The peptide was exploited best by reproducing the proximity between the peptide molecules on polyvalent latex (polystyrene) beads which were linked to a QCM.

However, the main advantage in the use of phage display lies in the generation of many binder peptides from fully random libraries, both without the inclusion of any design in the peptide structure and without containing elements of designed structure [36–38]. Nondesigned peptide libraries may offer peptides able to discriminate between fine structural differences in the target, and even between different conformations. A label-free and time-resolved biosensor based on reflectometric interference spectroscopy exploits phage-display-selected peptides to probe the conformational changes occurring inside the estrogen receptor α on binding various ligands [39]. A library of phage-displayed peptides against the two forms of the same receptor was selected, yielding two different sets of binder peptides capable of discriminating between the different conformations. Biotinylated peptides α/β were immobilized on the sensor surface to interact with high or low affinity with estrogen receptor α depending on the receptor conformations adopted in the presence of agonists or antagonists [40].

A selection of biosensor systems developed with random phage display peptides is reported in Table 2.

Despite the high diversity of the targets, which include whole cells, proteins, and small organic molecules, all the systems reported in Table 2 have been obtained from commercially available phage display random peptide libraries without any kind of design, and without any need for complex stages of biological work, as would be required for the development of monoclonal antibodies. This is clearly an advantage of the random phage display technique, resulting in low cost and ease of standardization. In principle, one could try to fish out peptide receptors for any kind of target from the same commercial library. The results are, however, rather unpredictable, and the peptides may perform much better than antibodies [41, 44], or may be far worse [33, 46].

Peptide receptors for ligand sensing

Phage display represents a key technique also in the development of libraries of designed receptor peptides. Two approaches have been mostly followed to obtain artificial, designed receptors rather than random short binder peptides. In the first approach, a stable peptide scaffold is identified, inspired by stable natural peptide domains, or derived from a fully artificial design, and then certain positions in the amino acid chains are randomized to obtain a library of receptors sharing the same constant region (in analogy to the monoclonal antibody and antibody fragment technologies). The second approach is target-focused: a natural receptor of the target is identified and reduced to the length of a synthesizable peptide, or miniprotein. Libraries of mutated receptors can then be generated to improve binding and selectivity.

Many different nonantibody scaffolds have been obtained in recent years by the first approach (Table 3) [47, 48]. Affibodies are 58 amino acid three-helix bundles randomized at 13 positions by phage display [49, 50]. They are particularly suitable for the recognition of proteins, and several examples of fluorimetric microarray biosensors have been reported to perform with extremely high sensitivities, down to femtomolar sensitivity [51, 52]. The highest sensitivities have been obtained by submitting the phage-display-selected affibodies to chemical oligomerization before immobilization over the sensor surface, thus generating avidity phenomena. Affibodies are also largely used as biosensor probes in imaging techniques [53].

α-Helix coiled coils have been widely considered: the coiled-coil motif (Fig. 6) occurs when a seven amino acid sequence including hydrophobic residues such as valine, alanine, and leucine at positions 3, 4, and 5 is repeated along a peptide.

The amphiphilic helix tends to associate via its hydrophobic side with another helix, giving rise to parallel or more often antiparallel coils. If the heptad repeat is interrupted by loop sequences, a typical and stable helix–loop–helix motif is obtained, and binding sites can be generated at

Table 2 Sensors based upon random phage display peptides

Reference	Target	Sensing peptide	Sensor	Sensitivity
[41]	Murine myofibers	7-mer ASSLNIA	Acoustic wave	Murine skeletal proteins 10 μg/ml, apparent K_d 280 μg/ml Fast response (100 s), whereas a similar, antibody-based sensor shows 100-min equilibration time
[42]	*Ps. Aeruginosa* whole cells	9-mer QRKLAAKLT resembling the sequence of natural CPP	QCM	
[43]	SW620 metastatic cells	12-mer	Potentiometric	100 cells/ml blood
[44]	*Anthrax* protective antigen	16-mer HKHAHNYRLPASGGGKK	Square wave voltammetric	K_d 4.06 pM, dynamic range 4–120 pM, LOD 5.2 pM
[45]	Enterotoxin B	12-mer	Fiber optical. The RAPTOR optical probe was used [41]	Dynamic range 10^{-3}-1 μg/well, LOD 1.4×10^{-3} μg/well
[46]	Cardiac troponin I	12-mer FYSHSFHENWPS	QCM and EIS	QCM: dynamic range 0.1-6 μg/ml, LOD 0.11 μg/ml EIS: LOD 0.34 μg/ml
[47]	Streptavidin	12-mer from filamentous R5C2 phages	Optofluidic ring resonator	LOD 100 pM, apparent K_d 25 pM
[32]	Methotrexate	11-mer SIFPLCNSGAL selected directly on a QCM device	QCM and SPR	Range 2–50 μM, K_d 22.3 μM
[33]	TNT	12-mers sharing a HR consensus dyad	Fluorimetric flow sensor	Dynamic range 6–100 mg/ml, LOD 12.5 mg/ml
[35]	Porphyrin	5-mer HASYS peptide immobilized on latex beads to allow 2:1 peptide-to-target complex formation	QCM	Dynamic range 0.01-100 μg/ml, LOD 0.01 μg/ml
[40]	EDCs	Peptide α/β I	RIFS	ERα-LBD 100-μg/mL incubated with 10 μg/mL β-estradiol and with 10 μg/mL tamoxifen Discrimination between agonists and antagonists of ERα

CPP cell-penetrating peptide, *EDCs* endocrine-disrupting chemicals, *EIS* electrochemical impedance spectroscopy, *ERα* estrogen receptor α, *LBD* ligand binding domain, *RIFS* reflectometric interference spectroscopy, *TNT* 2,4,6-trinitrotoluene

Table 3 Sensors based upon designed receptor peptides

Reference	Target	Sensing peptide	Sensor	Sensitivity	Selectivity
[51]	*Taq* DNA polymerase and IgA	58-mer affibodies from phage display	SPR and microarrays with fluorimetric detection	*Taq*: LOD 30 pM IgA: LOD 3 pM	
[52]	IgA, IgE, IgG, TNF-α, insulin, *Taq* DNA polymerase	$(58\text{-mer})_n$ multimeric affibodies from phage display, by oligomerization of the peptides over the microarray slides	Microarrays with fluorimetric detection	LODs: IgA 600 fM, IgE 20 pM, IgG 70 fM, TNF-α 20 pM, insulin 60 pM, *Taq* 10 pM	No cross-reactivity between the targets
[53]	HER-2 expressing tumor cells	58-mer affibodies from phage display	NIR quantum dots and iron oxide based nanoprobes		
[54]	HIV-1 pseudopeptide adhesion inhibitors	25-mer coiled coil from gp41 HIV protein. The peptide acts as an ion channel	Voltammetric	Down to 100 nM for high-affinity inhibitors	
[55]	Antibodies	15-mer gramicidin A. 6.3 helical ion channel displaying an antigen	Voltammetric		
[56]	C-reactive protein	35-mer helix–loop–helix chemically modified coiled coils	Reflectometric interference sensor	K_d ranging from 7 to 11 nM, dynamic range 870 pM to 87 nM	
[57]	Caffeine	35-mer K coiled-coil randomized peptide from phage display	SPR	Caffeine K_d 70 μM	Full cross-reactivity with theophylline
[59]	*Botulinum* neurotoxin	22-mer synaptotagmin-derived peptide	Microfluidic channel/ fluorescently labeled antibody	A spiked 2 pg /ml toxin solution in milk allowed the detection of 16 pg toxin	*Botulinum* neurotoxin type B heavy chain
[60]	Phosphoryl peptides	33-mer WW phosphoprotein binding domain	Hybrid fluorescence chemosensor	Micromolar K_d values	
[61]	Nitric oxide released by captured cells	12-mers derived from the integin-binding fibronectin domain and laminins	Electrochemical	Estimated 400 nM nitric oxide released by a cell monolayer captured over the electrode surface by the receptor peptide	
[62]	Alcohols associated with *Salmonella typhimurium* in beef	LUSH protein sequence (81–93)	QCM	1-3 ppm	3-Methyl-1-butanol, 1-hexanol
[63]	VOCs xylene, ammonia, trimethylamine, acetic acid	Human olfactory receptor protein (P30953) fragments: horp61(61–72), horp103(103–8), horp109(109–14), horp193(193–99)	QCM	The sensitivity is given only in a relative form	horp61 for trimethylamine, horp103 for *o*-xylene, horp109 for ammonia, horp193 for acetic acid
[64]	VOCs 1-hexanol, 1-pentanol	Mouse olfactory receptor (OR744) sequence 210-216	QCM	2-5 ppm	1-Hexanol, 1-pentanol

HER-2 human epidermal growth factor receptor type 2, *NIR* near infrared, *TNF-α,* tumor necrosis factor α

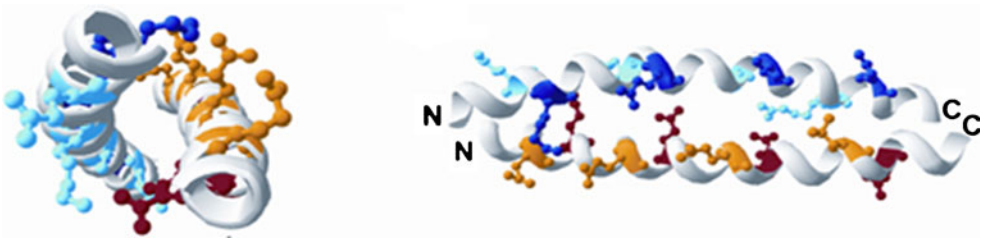

Fig. 6 A coiled-coil superhelical structure

the loop level. Very interesting applications can be developed when the coil also acts as an ion channel: in this way the binding activity is directly obtained within the signal-generating molecule, and target detection can be performed by electrochemical approaches [54, 55]. The coiled-coil peptide can also be chemically modified in order to add interacting groups, and a reflectometric interference sensor for C-reactive protein has been generated in this way [56]. Our research group has patented a library of coiled-coil receptors for the detection of small organic targets [57]. Peptides containing 35 amino acids and based upon the sequence of the EK leucine zipper [58] have been randomized at the central heptad, and xanthine-binding peptides have been selected.

Reduction of natural receptors has been used in the development of a microfluidic accumulation channel/fluorimetric sensor for *Botulinum* neurotoxin B [59]. The toxin receptor synaptogamin II was reduced to a 22 amino acid peptide allowing the detection of 16 pg of target.

A series of phosphopeptide sensors was obtained by reducing the WW phosphoprotein binding protein to 33 amino acid peptides [60]. Hybrid sensors were then obtained by modification of the 33 amino acid receptor domain with a fluorescent stilbazole having a zinc(II) dipicolylamine phosphate binding group. Binding of phosphorylated peptides to the receptor resulted in fluorescence quenching.

A sensor for the release of nitric oxide from living cells was developed by reducing the fibronectin and laminin binding domains to cell-binding dodecapeptides [61]. An electrode for nitric oxide sensing was coated with such peptides to allow adhesion of a monolayer of living cells, and their nitric oxide release activity was monitored by the electrode.

One of the most effective sensing systems is the mammalian olfactory system, capable of discriminating over 10,000 distinct odors and of detecting some odorants at parts per billion levels. A fascinating challenge is represented by the development of an "electronic nose" integrating biomaterials as olfactory receptors, odorant-binding protein [62], and synthetic olfactory-receptor-based polypeptides [63, 64] with a QCM for gas sensing. In early studies, odorant binding domain fragments were employed to "sniff" gases such as trimethylamine and ammonia [63]. Recently, a biomimetic sensor has detected alcohols at low concentrations as food contaminants [62, 64].

Peptide ligands for receptor sensing

Identification of natural peptide ligands for cell-surface elements, transmembrane proteins, antibodies, and enzymes provides a miscellaneous set of peptide sequences, complementing the receptor binding sites and allowing design, synthesis, and coating onto various surfaces for biosensor development (Table 4).

Antimicrobial and cell-penetrating peptides

AMPs and CPPs are able to interact with cell-surface elements. The availability of robust and portable biosensors to detect pathogenic bacteria is of growing importance in the environmental, food, and human diagnostics areas. Current methods to identify the presence of bacterial cells include enzyme-linked immunosorbent assays (ELISAs) and real-time polymerase chain reactions (PCRs). The ELISA platforms exploit the high specificity of antibodies but suffer from instability under extreme environments and from high costs. On the other hand, PCR may allow single-cell detection, but nucleic acid extraction protocols restrict its use as a portable device. To overcome many of these limitations, AMPs were evaluated as alternative recognition molecules.

AMPs are part of the host's innate immune system in many eukaryotic organisms as a means of defense against microbial infections. Many AMPs are cationic and bind to the negatively charged bacterial membrane, leading to cell lysis. This natural efficiency of binding the lipopolysaccharide component of bacterial cell walls has been exploited to develop biosensor assays to detect, to classify, and to quantify Gram-negative bacteria.

In most of these studies, AMPs were immobilized onto glass slides to detect directly fluorescently labeled cells or in a sandwich assay using a fluorescently labeled antibody, developing an optical biosensor capable of performing multitarget analyses in parallel. The goal of the early studies was to apply AMPs as a recognition element for *Escherichia coli* O157:H7 and *Salmonella typhimurium*, which are considered the most dangerous food-borne pathogens in both developing and developed countries. LODs of 5×10^4 cells per milliliter were obtained [65–67].

The ability of AMPs to bind multiple microbial species with different affinities was also exploited in the screening

Table 4 Sensors based upon peptide ligands for detection of receptors

Reference	Target	Sensing peptide	Sensor	Sensitivity
[79]	mAb against the FLAG peptide	CDYKDDDDK (FLAG) peptide	Liquid crystal	LOD 27 ng/mL, response time <1 h
[12]	mAb (12 F10) against HCV protein ARFP (A97)	Polypeptide from HCV ARFP	SPRI microarray	LOD 10^{-9} M
[80]	HIV-1 PR	Protease substrate DABCYL-SQNYPIVQ-EDANS	FRET	
[81]	Trypsin	Trypsin substrate DABCYL-GPAX$_{AA}$LAIG-EDANS	FRET	
[82]	*Botulinum* neurotoxins A, B, F	SNAP-25 (187–203 and derivates) VAMP-2 (35–70 and derivates) VAMP (60–94 and derivates)	FRET	SNAP-25 fragment is specifically cleaved by serotype A, VAMP-2 fragment by toxin B, and VAMP fragment by type C
[83]	Caspase 3	Peptide substrate SGDEVDSG	QD-FRET-based composite sensors	
[84]	*Botulinum* neurotoxin LcA	SNAP-25 (187–202) modified	QD-FRET	LOD 0.35 nM
[86]	Subtilisin	Decapeptide substrate	X-ray photoelectron spectroscopy.	LOD 0.037 μM
[87]	MMP-7	Helix peptide with sequence RPLALWRSC	Cyclic voltammetry	LOD 3.4 pM
[88]	Proteases	Fc–peptide monolayers	Cyclic voltammetry	1-1,000 nM
[89]	Plasmin	Fc–peptide conjugate (KTFK)	Cyclic voltammetry	LOD 50 ng/mL
[90]	Papain	Fc–peptide conjugates	Cyclic voltammetry	LOD 4 nM
[91]	Caspase 3 (detection of apoptotic cells)	Fc–peptide conjugate (GDGDEVDGC)	Cyclic voltammetry	
[92]	HIV-1 PR	Fc–pepstatin	Cyclic voltammetry	LOD 4 nM
[93]	HIV-1 PR	Fc–pepstatin conjugates	Cyclic voltammetry	LOD 0.8 pM
[94]	Protein kinases	Peptide substrates for tyrosine kinases Abl and Src	QD-FRET	Subnanomolar LOD
[95]	Protein kinases	ARCs. ARCs are potent inhibitors of protein kinases	FRET	Nanomolar LOD
[97]	MMP-1	Peptide substrate of catalytic domain (AMFLEA)	FT-SPR and ESI-MS	Micromolar LOD
[98]	β-galactosidase	20-mer YHNN and QYHH selected from a microarray based on β-galactosidase binding		Nanomolar LOD

ARCs adenosine analogue–oligoarginine conjugates, *ARFP* alternate reading frame protein, *DABCYL* 4-(dimethylaminoazo)benzene-4-carboxylic acid, *EDANS* 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid, *ESI-MS* electrospray ionization mass spectrometry, *Fc* ferrocene, *FT* Fourier transform, *LcA* light chain protease serotype A, *mAb* monoclonal antibody, *MMP* matrix metalloproteinase, *SNAP-25* synaptosomal-associated protein 25, *VAMP* vesicle-associated membrane protein

of challenging species such as intracellular pathogens and enveloped viruses by an array-based system using multiple AMPs [68].

A first step towards the development of AMP-based, label-free biosensors was made by immobilizing an AMP within nanostructured electroactive thin films for detection of *Leishmania* cells [69]. The most recent study has reported a portable, label-free, real-time sensing platform that implements impedance spectroscopy to distinguish pathogenic bacteria with a clinically relevant limit of one bacterium per microliter (Fig. 7) [70].

Two AMP classes are mainly employed for all these purposes: linear, cationic peptides forming amphipathic α-helices upon interaction with membranes (magainins, dermaseptins, cecropins, sheep myeloid antimicrobial peptide 29, PGQ, parasin, mellitin) and short loop peptides (polymyxins and bactenecin) (Fig. 8). Their small molecular size, the feasibility to synthesize variants of these peptides and to anchor them on different surfaces, and the intrinsic stability under harsh environmental conditions, such as the presence chemical and thermal denaturants, epitomize the advantages of their use in multianalyte detection assays for targets of biodefense interest. However, exploiting AMPs in pathogenic sensor devices faces a number of key challenges that include the detection of bacteria in real water samples, the increase of sensitivity and selectivity, and the reusability of the devices.

CPPs are typically short, cationic sequences and may be derived from natural sources or designed. They have been used to overcome the lipophilic barrier of the cellular

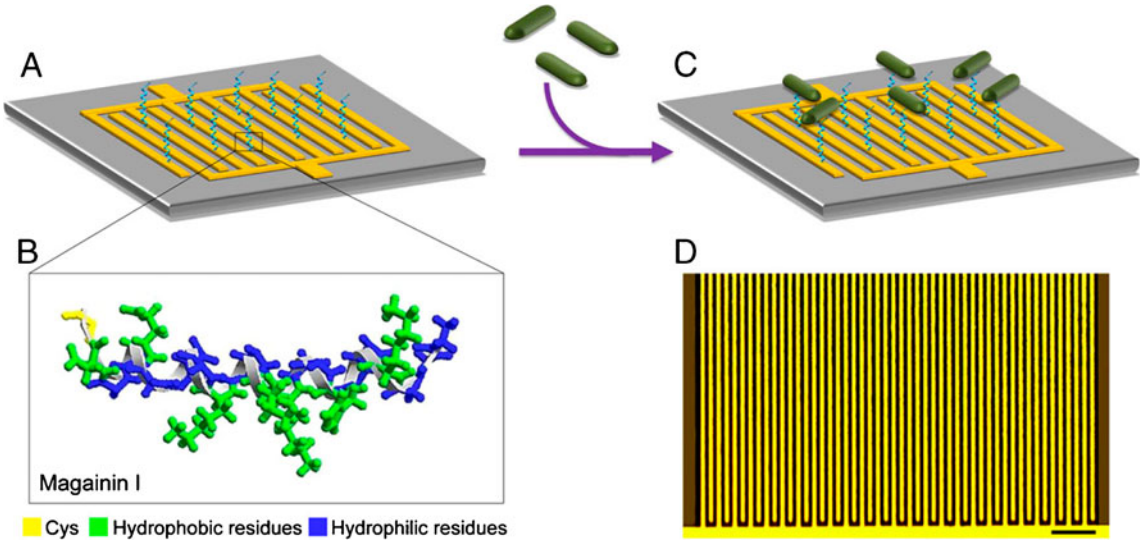

Fig. 7 Antimicrobial peptide (AMP)-based electrical detection of bacteria. *A* AMPs immobilized on an interdigitated microelectrode array. *B* magnified image of the AMP magainin I in helical form, modified with a terminal cysteine residue, and with clearly defined hydrophobic and hydrophilic faces. *C* detection of bacteria is achieved via binding of target cells to the immobilized AMPs. *D* optical image of the interdigitated microelectrode array (*scale bar* 50 μm). (From Mannoor et al. [70], with permission from the National Academy of Sciences USA)

membranes and deliver large molecules and even small particles inside the cell for new cellular imaging tools, biosensors, or biomolecular delivery systems [71–73]. A first example of a CPP is a peptide derived from the transactivator of transcription (TAT) protein of HIV-1. TAT was immobilized on a gold surface in a peptide SAM that retains the ability of the full-length TAT protein to bind specifically the transactivation response element RNA [74, 75]. Better understanding of the behavior of TAT peptide is of enormous interest for the design new sensing and therapeutic strategies and will expand our knowledge of CPPs.

Activatable CPPs (ACPPs) were developed to ameliorate the in vivo pharmacokinetic and toxicity problems posed by CPPs [76]. An ACPP consists of a polycationic CPP, a cleavable linker, and a polyanionic inhibitory domain (Fig. 9).

Cleavage of the linker by specific proteases (mostly tumor-associated matrix metalloproteinases) dissociates the polyanion and enables the cleaved ACPP to enter cells. Yet,

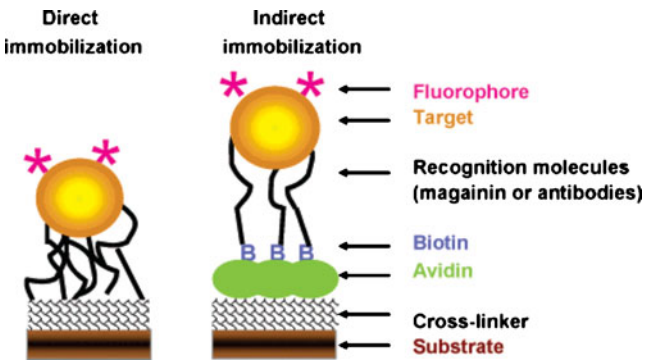

Fig. 8 Immobilization of AMPs on glass slides for detection of bacterial binding. In the direct method, unmodified recognition molecules are attached to a thiol-modified substrate through a heterobifunctional cross-linker. The maleimide moiety of the linker targets thiol functionalities on the substrate, whereas its *N*-hydroxysuccinimidyl moiety targets primary amines on the magainin. In the indirect immobilization, NeutrAvidin is covalently attached to the slide surface. Biotin-labeled peptides are subsequently incubated with the NeutrAvidin-coated surface, yielding peptide immobilized by an avidin–biotin bridge. (From Kulagina et al. [65], with permission from the American Chemical Society)

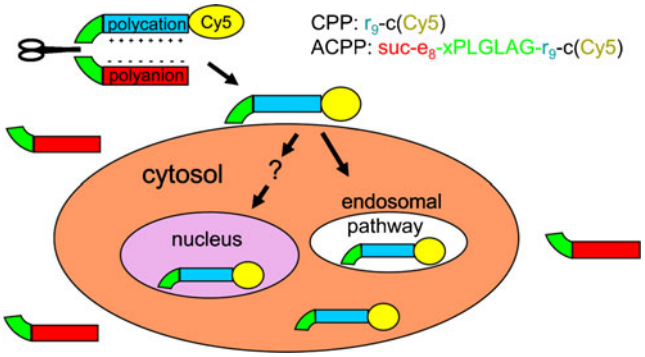

Fig. 9 Activatable cell-penetrating peptides selectively unmask cell-penetrating peptides upon protease cleavage of a linker. While the linker (*green*) between the polyanion (*red*) and polycation (*blue*) sequences remains intact, cell uptake is blocked and the entire molecule can enter the extracellular space of tissues and wash out. Once a protease (*scissors*) cuts the linker, the polyglutamate dissociates, allowing the polyarginine and its payload (*yellow*, in this example Cy5) to immediately adhere to cells and eventually become endocytosed. *ACCP* activatable cell-penetrating peptide, *CCP* cell-penetrating peptides (From Aguilera et al. [76], with permission from the Royal Society of Chemistry)

enzyme activation may represent a source of trouble, because peptide substrates that are very specific for a single protease remain a challenge and both cleavage and uptake result from a plurality of proteases.

CPPs and ACPPs for the preparation of nanoparticles designed for cancer research have been described in a recent review [77]. Dendrimer nanoparticles coated with ACPPs have been labeled with both Cy5 and gadolinium, and used as a dual probe for magnetic resonance imaging and in vivo fluorescence in clinical detection and staging of tumors, providing a correlation between tomographic imaging, which can detect a wide variety of tumors from different anatomical sites, and high but superficial resolution imaging, which is effective for intraoperative surgical guidance [78].

Antigens

Antibody titration for serological monitoring of exposure to exogenous antigens has been widely performed with classic immunological techniques such as immunoprecipitation, ELISA, and immunoelectrophoresis. As antibody-mediated immune response occurs via the recognition of specific epitopes, reduction of the antigen to minimal sequences has often been addressed. Such short peptides are also used in biosensor systems. The optical properties of liquid crystals and the specific epitope–antibody interactions have been exploited to develop an optical immunobiosensor which can be used to detect monoclonal anti-FLAG M2, a model antibody [79]. The oligopeptide antigen is immobilized as a monolayer at a liquid crystal/aqueous interface. Anti-FLAG antibody binds to the immobilized FLAG epitope and triggers an orientational transition of the liquid crystal, generating changes in the optical images of liquid crystals from dark to bright under crossed polarizers. Antibodies against the hepatitis C alternate reading frame protein (A97) have been detected with a polypeptide from the protein [12]. Pyrrole-modified peptides were immobilized on the gold surface of the chip by electrochemical copolymerization of pyrrole, and an SPRI microarray was developed.

Enzyme substrates and inhibitors

Proteases are important physiological enzymes, and are involved in the control of a large number of vital processes, such as cell growth, cell death, hemostasis, tissue remodeling, and immune defense. The current critical need for high-throughput and sensitive monitoring of protease activity has highlighted some biosensing devices that exploit a surface-immobilized peptide substrate to detect recognition/cleavage by protease targets. Although proteolytic assays have been performed by a plethora of different methods, fluorescent and electrochemical techniques are still predominant.

FRET-labeled peptide substrates have been applied for the detection of a number of proteases, including HIV protease [80], trypsin [81], and *Botulinum* neurotoxins [82].

Specific peptide substrates have been conjugated to photoluminescent semiconductor nanocrystals or quantum dots (QDs), an appealing nanomaterial in FRET-based biosensing applications. QDs manifest unique physicochemical properties as FRET donors and acceptors and as scaffolds for a variety of biological molecules. Several groups exploited QD-conjugated peptide substrates to successfully monitor the proteolytic activity of caspases [83]. As a recent example, a QD-FRET sensor monitored the protease activity of *Botulinum* neurotoxin A, the so-called light chain protease serotype A, by exploiting a specific region of the toxin substrate SNAP-25. SNAP-25 (187–202) was mutated (E194Q) to increase the sensitivity to protease activity, and lengthened with a helical spacer and a polyhistidine fragment to further enhance affinity and self-assembly (Fig. 10) [84]. QD-FRET methods are achieving impressive results, reducing the scale of electroluminescent charged-coupled devices down to microchip detection platforms [85], but are not readily applicable for assays in complex, turbid, and colored fluids such plasma and blood.

Ferrocene (Fc) displays a simple and environment-insensitive electrochemical behaviour, and for this reason Fc cyclic voltammetry is an emerging biosensor technique, taking advantage of the Fc end-labeling strategy. Basically, a peptide fragment containing the Fc moiety is cleaved by proteases, causing a decreased electrochemical response. The preparation of Fc–peptide-modified gold electrodes is effortless and, at the same time, the sequence of the peptide is specifically designed to act as a substrate for the target protease. Monolayer-modified electrodes have been developed for monitoring the action and activity of wide-ranging and relevant enzymes: matrix metalloproteinases, which are often overexpressed in diseases such as cancer (Fig. 11) [87]; serine proteases, involved in the tightly coordinated coagulation cascade [88, 89]; cysteine proteases [90] such as caspase 3, related to cellular apoptosis [91]; HIV-1 protease [92]; integrating metal nanoparticles with carbon nanotubes on a gold electrode [93].

Further advancements of these detection strategies involve the improvement of efficient chemistries for attaching peptides to surfaces with intimate control over their stoichiometry, orientation, and affinity. Also, the tricky development of reusable activated surfaces is still unresolved.

Protein kinases mediate protein phosphorylation and play a pivotal role in the regulation of cellular processes. Their dysfunction causes many disease states, notably cancer. Thus, an increasing need has risen for improvement of sensor devices for detecting protein kinase activity and for high-throughput screening of inhibitors of protein kinases.

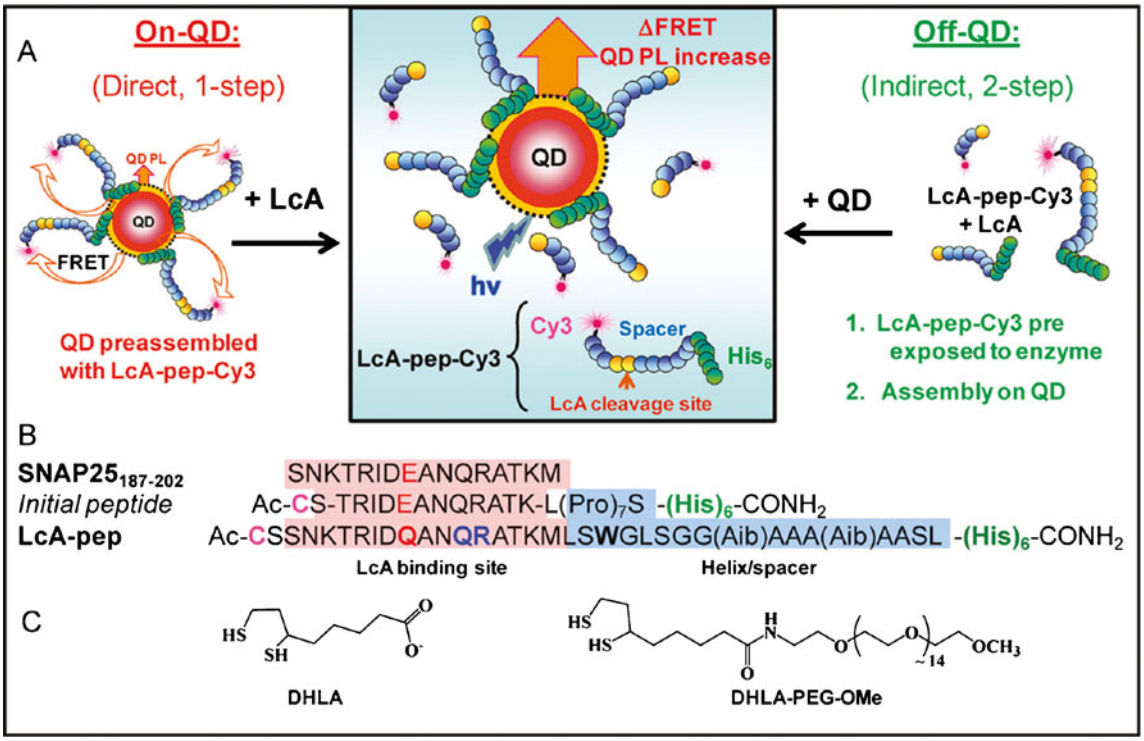

Fig. 10 Quantum dot (*QD*)–Förster resonance energy transfer (*FRET*) assay design components. *A* The two QD-FRET-based assay formats, on-QD (one step) and off-QD (two step), used to investigate light chain protease serotype A (*LcA*) enzymatic activity. *B* peptide sequences of the LcA substrate used in this study. *Aib* is α-aminoisobutyric acid, *Ac* is an N-terminal acetyl blocking group, and $CONH_2$ is a C-terminal amide blocking group. Colors are used to highlight the different functional modules. *C* chemical structures of the two capping agents, dihydrolipoic acid (*DHLA*) and DHLA-PEG, used to render the in-house-synthesized QDs hydrophilic. *PL* photoluminescence, *SNAP25* synaptosomal-associated protein 25. (From Sapsford et al. [84], with permission from the American Chemical Society)

Peptide substrates conjugated with QDs by metal-affinity-driven self-assembly [94] and adenosine analogue–oligoarginine conjugate bisubstrate inhibitors, which consist of adenosine mimics and D-arginine-rich peptides [95], were successfully employed for these purposes.

However, such methods are based on labeled peptide substrates. An approach to evaluate the activity of matrix metalloproteinases conveniently anchored on a gold surface that couples the Fourier transform surface plasmon resonance technique with electrospray ionization mass spectroscopy to

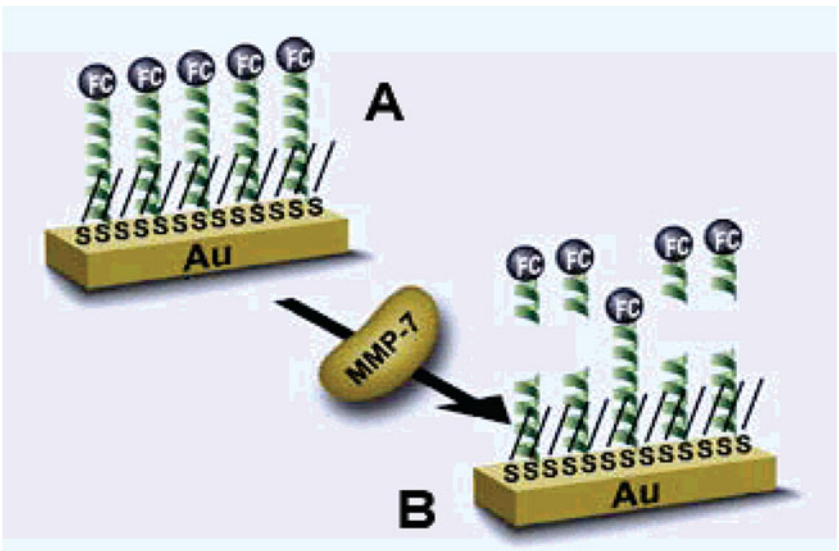

Fig. 11 Electrochemical polyelectrolytic beacons (EPB) for detecting matrix metalloproteinase (MMP) activity. Using the EPB offers the capability of "on–off" voltammetric signaling for the specific MMPs: *A* self-assembly of electrochemical ferrocene (*FC*)–peptide conjugates on a gold substrate; *B* cleavage of EPB in the presence of MMP-7. (From Liu et al. [87], with permission from the American Chemical Society)

detect the cleavage of unlabeled peptide substrates has been reported [98].

Peptides with high affinity and selectivity for enzyme epitopes outside the catalytic site represent a promising class of protein-anchoring molecules for the creation of peptide-modified surfaces that immobilize specific enzymes and optimize their orientation, improving their thermal and pH stability.[99]

Conclusion

This review shows that many different ideas on biomolecule–target interactions may lead to a sensing system based upon short peptides. Macromolecular targets, such as proteins and enzymes, can, in principle, be addressed by any of the available approaches. The availability of known natural peptide ligands and substrates allows binding, recognition, indirect detection by release of labeled peptide fragments, and direct detection with label-free systems. On the other hand, peptides from random library selection may bind the same macromolecular targets at different epitopes, thus leaving the target active site free for labeling with secondary reagents, or for detecting its own biological activity.

Whole microbial cells are optimal interactants in the world of AMPs, and their peculiar acidity and shape allow selective detection of cell-surface elements.

Sensing of low molecular weight organic molecules is an open challenge, when one attempts to fish out peptide transducers by selection techniques involving immobilization stages. A micromolar affinity barrier seems to affect in similar ways the worlds of antihapten antibodies and of peptide receptors. Reduction of natural receptors down to short peptide sizes may represent an opportunity, provided that such natural receptors are known. On the other hand, parts per billion LODs have been reported in a few articles on detection of volatile organic compounds with designed peptides: in our opinion, designed peptides may represent a key point in the advance of the field, as new computational and bioinformatics methods will became available.

Future developments in the field will also involve implementation of improved techniques for nanoscale-confined peptide synthesis, and micropatterning of phage-displayed peptides [95, 96]. On-chip combinatorial peptide libraries will allow us to fully exploit peptide diversity to prepare arrays of sensors for fingerprinting of complex mixtures.

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