# Recognition-induced covalent capturing and labeling as a general strategy for protein detection

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## ABSTRACT

In this work we have developed a peptide-based method for protein detection, termed as "Recognition-induced Covalent Capturing and Labeling" (RCCL). In this method, upon binding of the peptide with the target protein, electrochemically controlled and metal catalyzed oxidative cross-linking can be induced between the peptide and the target protein. Specifically, the peptide and the target protein are cross-linked by the formation of dityrosine between tyrosine moieties of the two molecules. Meanwhile, the dityrosine formed in this manner also has fluorescent signal readout. Therefore, the proposed method needs only one probe for the target protein, and the initial non-covalent molecular recognition can be finalized by cross-linking between the peptide and the target, while the dityrosine formed between peptide and protein can also act as a signal reporter, thereby greatly simplifying the design. Moreover, the robust covalent capturing via RCCL also enables detection in complex biological and clinical samples. These results point to the prospect of using RCCL as a promising method in protein detection in the future.

## 1. Introduction

Protein bioanalysis is an indispensible tool for biomedical research, and its clinical application is also essential for disease screening and diagnosis (He et al., 2015; Wu and Qu, 2015). Over the last few decades, the technology of protein biosensing has evolved dramatically (Howes et al., 2014; Muenzer et al., 2013; Ren et al., 2014). Many aspects of protein biosensing have benefited from this progress (Peng et al., 2015), while the essential mechanism follows the classic design: two targeting ligands (usually antibodies) bind with the target protein, thereby coupling bio-recognition with subsequent signal generation and signal amplification. For the classic design we have the following considerations: First, antibodies need various other biosensing elements of signal amplification to achieve satisfactory analytical properties (Zhu et al., 2015), and high performance is often accompanied by relatively complicated design. Second, recent development of biosensing progresses introduce and promote the synthetic targeting agents (Feng et al., 2014; Toh et al., 2015; Wu et al., 2015),

on the basis of their readiness of chemical modification; but their application using the classic design may be hampered with several factors: (1) the classical design requires at least two different probes for the same target protein, but the proper synthetic candidates are not always available; (2) the classical design employs the non-covalent but strong binding between antibody and protein to work properly. Synthetic probes are somewhat inferior in terms of the strength of binding. This may greatly restrict the application in bio-sample analysis, which requires violent rinsing to reduce background interference. Such rinsing may dissolve the biosensing complex formed between the synthetic probe and the target protein. In light of these technological restrictions, we propose a design more suitable for the recently introduced synthetic protein-targeting probes. In this design, only one probe is required for each target protein, and probe/protein binding can induce cross-linking between the peptide probe and the target protein, so the performance of synthetic probes can be augmented by covalent bonds. Moreover, the product of cross-linking can have direct fluorescent signal readout, so the complexity of the design is greatly reduced.

This method, termed "Recognition-induced Covalent Capturing and Labeling" (RCCL), is first made possible by the fact that a lot of protein-targeting peptide probes have been screened out or

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designed during the search of protein-targeted therapeutic and biochemical reagents (Gray and Brown, 2014; Hosseinkhani et al., 2013; Linh and Tap, 2015; Milroy et al., 2014; Qin, 2015). Second, function groups of many proteins and peptides have chemo-selective reactivity under certain conditions (Chen et al., 2011; Schoneich, 2000). These interesting features of protein, peptide and their interaction have been noticed and taken advantage of in this work. Here tyrosine is selected to exemplify the proposed strategy. Tyrosine can be catalytically oxidized to form 3,4-Dihydroxyphenylalanine (DOPA) or dityrosine product, depending on the absence/presence of a second tyrosine in the vicinity (Dalsgaard et al., 2011). This enables the peptide probe to covalently capture target after the initial peptide/protein binding, via a dityrosine ligation driven by proximity. At the same while, surplus probes can mask themselves by the formation of DOPA. This points to another favorable fact that the original tyrosine, the dityrosine indicating the presence of target protein, and DOPA indicating its absence, all can give out fluorescent peak responses of distinct quantum yields at distinct wave lengths (Andreev et al., 2002; Kehr et al., 1972). To guarantee a quantitative conversion of protein abundance to signal readout, the RCCL mechanism is further secured by electrochemical control. In biosensing, electrochemistry is mostly employed as a form of signal readout. But electrochemistry is also apt for controlling the reductive/oxidative status, therefore the state of activity of bio/chemo-catalysts. So Cu (I)/(II) ion as a catalyst is incorporated into the sequence of the peptide probe. In this manner, through electrochemically controlling the availability of the catalyst, the ligation step can be controlled spatiotemporally to take place only at the correct stage of protein sensing. The above aspects constitute the proposed RCCL method, which has the following merits as a novel strategy of protein detection: (1) only one probe is required for each target protein; (2) the dityrosine formed between peptide and protein can also act as a signal reporter, so the design is greatly simplified; (3) noncovalent probe/target binding is finalized by covalent capturing immediately. Violent rinsing then becomes feasible, enabling biosample analysis. Using this method, several different disease-associated biomarker proteins have been quantitatively determined and their detection in complicated biological and clinical samples has been preliminarily realized.

## 2. Material and methods

### 2.1. Chemicals and biological samples

Peptide probe $NH_2$-GHK-$G_6$-TSFAEYWNLLSP-K(11-mercaptoundecanoic acid, MUA)-COOH, $NH_2$-GHK-$G_6$-DESDPEELMYWWEFLSED-PSS-K(MUA)-COOH, $NH_2$-GHK-$G_6$-RGTFEGKF-K(MUA)-COOH for murine double minute 2 (MDM2), G Protein alpha (G-pro) and amyloid beta 1–42 (A beta), respectively, were manufactured by Shanghai Science Peptide as lyophilized powder, purity > 95%. Powder of the peptide probes was dissolved with 10 mM phosphate buffer solution (PBS) (pH 7.4) to the desired concentrations. MDM2 was purchased from ProSpec-Tany TechnoGene Ltd.; G-pro was from Novus Biologicals; A beta was from Sigma Aldrich. Analytical-grade was attained for all of the other reagents. The serum-spiked sample of MDM2 was prepared by dissolving the protein powder with 10 mM PBS (pH 7.4) and then diluting it to desired concentrations with the fetal bovine serum. The original solution (50 mM Tris–HCl, pH 8.0) of G-pro was first diluted with 10 mM PBS (pH 7.4) and subsequently diluted with fetal bovine serum to desired concentrations. A beta lyophilized powder was firstly dissolved in 1,1,1,3,3,3-hexafluoro-2-propa-nol (HFIP) at a concentration of 1 mg/mL to eliminate pre-existing aggregates. HFIP was subsequently evaporated under a gentle stream of high purity nitrogen, leaving behind a film that was later reconstituted

with DMSO to 2.2 mM. This resuspension was further monomerized via sonication for 1 min, followed by being diluted to 440 μM with 10 mM PBS, pH 7.4, the above prepared solution of A beta was transparent without evident aggregation. This stock solution was kept at 4 °C for store and could be diluted to the desired concentrations with fetal bovine serum. Redistilled water for preparation of all the solutions was prepared from distilled water with a Milli-Q purification system, the resistance of 18 MΩ cm was achieved to guarantee the purity. Cancerous tissue samples of patients with non-small cell lung cancer were obtained from the Clinical Laboratory of the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, after elected consent by the local ethical committee. Immediately after resection, the tissue samples were sliced, lysed and fractioned using a Nuclear Extract Kit (Active Motif, CA), the nuclear fraction was retained and $100 \times$ diluted with 10 mM PBS (pH 7.4) for MDM2 quantification.

### 2.2. Electrode treatment

Transparent Au slides (Aldrich, layer thickness 100 Å) were cut to fit the size of the cuvette used for fluorescence measurement. These slides were cleaned by sonicating for 20 min in ethanolamine (20 wt%) at around 50 °C, followed by immersion for 10 s in piranha solution (*Caution: piranha solution is dangerous and should be handled with care*). After dried under mild stream of nitrogen, the slides were immersed in the assembly solution (2.5 μM peptide probes and 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 10mM PBS, pH 7.4) at 4 °C for 16 h, TCEP was adopted to prevent disulphide formation between peptide probes. The slides were then immersed in 9-mercaptononanol (MN) solution (1 mM MN in 10 mM PBS, pH 7.4) at room temperature for 3 h.

### 2.3. Protein detection

The prepared slides were immersed in serum spiked samples or fractioned tissue samples containing the target enzyme and kept at 37 °C for proper time for the target/probe recognition and binding to proceed. Then, using the slides as the working electrode, the whole mixture was brought under 10 min of cyclic voltammetric scans with proper scanning parameters to induce and regulate the Recognition-induced Covalent Capturing and Labeling (RCCL) process. Then, after violent rinsing with SDS, the slides were ready for measurement.

### 2.4. Experimental measurements

Isothermal titration calorimetry (ITC) measurements were conducted using a MicroCal ITC200 System (GE healthcare life sciences). The titration was conducted at 25 °C. The titration schedule consisted of 38 consecutive injections of 1 μL with at least a 120 s interval between injections. Heats of dilution, measured by titrating beyond saturation, were subtracted from each data set. All solutions were degassed prior to titration. The data were analyzed using Origin 7.0 software. Fluorescence emission spectra of surfaces were measured using a QM-4/2005 fluorescence spectrometer (Photon Technology International, Inc., Birmingham, NJ) equipped with a xenon lamp. This light source and the detector were in the same plane at right angle to each other. The slides were kept in a water-filled cuvette at 60° from the base of the cuvette for fluorescence measurements. The surface with gold film was kept away from the light source and towards the detector. Activating peak wavelength for dityrosine is 325 nm, while that for 3,4-Dihydroxyphenylalanine (DOPA) is around 360 nm. Electrochemical measurements were carried out on a CHI660D Potentiostat (CH Instruments) with a conventional three-