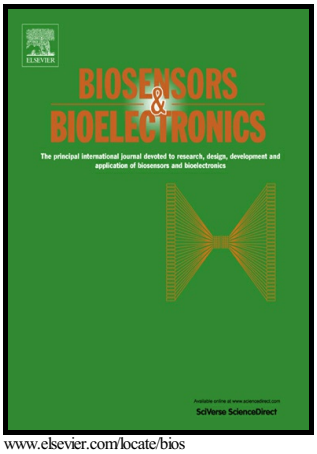

www.elsevier.com/locate/bios

PEP-on-DEP: A competitive peptide-based disposable electrochemical aptasensor for renin diagnostics

Manish Biyani, Keiko Kawai, Koichiro Kitamura, Miyuki Chikae, Madhu Biyani, Hiromi Ushijima, Eiichi Tamiya, Takashi Yoneda, Yuzuru Takamura

PII: S0956-5663(15)30729-6
DOI: http://dx.doi.org/10.1016/j.bios.2015.12.078
Reference: BIOS8308

To appear in: *Biosensors and Bioelectronic*

Received date: 2 October 2015
Revised date: 21 December 2015
Accepted date: 22 December 2015

Cite this article as: Manish Biyani, Keiko Kawai, Koichiro Kitamura, Miyuk Chikae, Madhu Biyani, Hiromi Ushijima, Eiichi Tamiya, Takashi Yoneda and Yuzuru Takamura, PEP-on-DEP: A competitive peptide-based disposable electrochemical aptasensor for renin diagnostics, *Biosensors and Bioelectronic* http://dx.doi.org/10.1016/j.bios.2015.12.078

To be submitted to special issue (BITE2015) *Biosensors and Bioelectronics*

PEP-on-DEP: A competitive peptide-based disposable electrochemical aptasensor for renin diagnostics

Manish Biyani[a, b], Keiko Kawai[b], Koichiro Kitamura[c], Miyuki Chikae[b], Madhu Biyani[d], Hiromi Ushijima[d], Eiichi Tamiya[d, e], Takashi Yoneda[f], Yuzuru Takamura[b]

[a]Green Devices Research Center, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan

[b]School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan

[c]JANUSYS Co. Ltd., Kamiaoki 3-12-18-508, Kawaguchi, Saitama 333-0844, Japan

[d]BioDevice Technology Ltd., 2-13 Asahidai, Nomi, Ishikawa 923-1211, Japan

[e]Department of Applied Physics, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

[f]Program Management Office for Paradigms-Establishing Centers for Fostering Medical Researchers of the Future, Kanazawa University, Takaramachi 13-1, Kanazawa, Ishikawa,920-8640, Japan

Corresponding author:

Manish Biyani, PhD

JAIST, 1-1 Asahi-dai, Nomi, Ishikawa 923-1292, Japan

E-mail: biyani@jaist.ac.jp, Tel: +81-761-51-1665, Fax: +81-761-51-1665

Abstract

Antibody-based immunosensors are relatively less accessible to a wide variety of unreachable targets, such as low-molecular-weight biomarkers that represent a rich untapped source of disease-specific diagnostic information. Here, we present a peptide aptamer-based electrochemical sensor technology called 'PEP-on-DEP' to detect less accessible target molecules, such as renin, and to improve the quality of life. Peptide-based aptamers represent a relatively smart class of affinity binders and show great promise in biosensor development. Renin is involved in the regulation of arterial blood pressure and is an emerging biomarker protein for predicting cardiovascular risk and prognosis. To our knowledge, no studies have described aptamer molecules that can be used as new potent probes for renin. Here, we describe a portable electrochemical biosensor platform based on the newly identified peptide aptamer molecules for renin. We constructed a randomized octapeptide library pool with diversified sequences and selected renin specific peptide aptamers using cDNA display technology. We identified a few peptide aptamer sequences with a K_D in the μM binding affinity range for renin. Next, we grafted the selected peptide aptamers onto gold nanoparticles and detected renin in a one-step competitive assay using our originally developed DEP (Disposable Electrochemical Printed) chip and a USB powered portable potentiostat system. We successfully detected renin in as little as 300 ng mL^{-1} using the PEP-on-DEP method. Thus, the generation and characterization of novel probes for unreachable target molecules by merging a newly identified peptide aptamer with electrochemical transduction allowed for the development of a more practical biosensor that, in principle, can be adapted to develop a portable, low-cost and mass-producible biosensor for point-of-care applications.

Keywords: peptide aptamer, in vitro selection, electrochemical sensor, renin biomarker

Main Text

1. Introduction

Affinity-based biosensing is the fastest growing area in biosensor research and has become increasingly prevalent in clinical diagnostics, environmental monitoring and point-of-care testing (Liu et al., 2012). The most commonly used technique for affinity-based biosensors is the noncompetitive immunoassay, such as the antibody-based sandwich enzyme-linked immunosorbent assay (ELISA) (Leguin, 2005; Findlay et. al., 2000). However, conventional ELISA suffers from two major limitations. First, ELISA is an optical approach and suffers from a sensitivity issue based on the Lambert-Beer law. Therefore, a minimum sample volume and path length is required to achieve an adequate performance. The merging of affinity-based biosensing systems with electrochemistry to produced so called electrochemical immunosensors, where biomolecular reaction monitoring is mainly based on the detection of current or potential changes produced by redox reactions occurring on the transducer electrode surface, is the most promising alternative to optical approaches (Grieshaber et. al., 2008; Ronkainen et. al., 2010). The introduction of screen-printing technology by our group (Ahmed et. al., 2007) and other groups (Metters et al., 2011; [1]Hayat and Marty, 2014) has further widened the application scope of electrochemical immunosensors by miniaturizing and printing/coating solid electrodes in parallel with high speed, reliability and affordability. This technology, termed the disposable screen-printed electrochemical (DEP) chip, made it possible to detect certain analytes that are otherwise challenging to detect.

Second, antibody-based sandwich ELISA has a fundamental limitation in that the target to be measured must be large enough to have at least two epitopes (binding sites) to be captured, and thus, the antibodies-based immunosensors are relatively less accessible (unobtainable issue), especially for low-molecular-weight biomarkers such as peptidome, which has only one epitope, i.e., one available antibody binding site. In addition, a sample matrix, such as that used in environmental testing, may not be compatible with antibody

function. Furthermore, reproducibility is a rather recent issue that has broad and potentially devastating implications for research (Baker, 2015). Only a part of the human proteome has validated antibodies, and thus, reliable antibodies are lacking for many important biomarkers, including G-protein coupled receptors, which are a target superfamily linked to many disorders across all therapeutic areas. Therefore, antibodies have limitations as biosensor probes in the fabrication of robust biosensing devices, which has led researchers to seek alternatives to antibodies (reviewed by Chen and Yang, 2015). In this context, aptamers are a new biorecognition element that are engineered on-demand through an *in vitro* selection procedure to selectively bind with a substrate in the micromolar to picomolar range and have recently emerged as a superior and smart class of versatile building blocks in the construction of novel biosensors (Zhou et. al., 2014). Aptamers offer many advantages compared to antibodies, especially in accurate and reproducible chemical production (Cho et. Al., 2009), and are widely regarded as an ideal recognition element to develop aptamer-based biosensors, so called 'aptasensors,' against a wide range of molecular and therapeutic targets. The first report on the implementation of electrochemical transduction in aptasensors was described in 2004 (Ikebukuro et. Al., 2004), and since then it has received considerable attention that has progressed rapidly ([2]Hayat and Marty, 2014).

Nucleic acid-based aptamers (DNA and RNA) are very popular due to their ease of identification and ease of integration into the electrochemical biosensing platform. Nevertheless, peptide-based aptamers (Colas et. al., 1996), which are comparatively smaller in molecular weight, exhibit a smaller binding footprint allowing for a more thorough and precise interrogation of the target than that afforded by nucleic acid-based aptamers. Therefore, a small surface area of the scaffold peptide aptamer should result in higher molar binding densities and lower background signals arising from nonspecific interactions with regions that are not directly involved in target recognition. Because of these properties, peptide aptamers can recognize smaller target molecules with a selectivity that can compete with antibodies. To date, however, few reports have described the development of peptide aptamer-based electrochemical biosensors, presumably because it is

difficult to identify new peptide-based probes. It is also difficult to integrate peptides onto the electrochemical biosensing surface, as peptides are typically disordered owing to their inherent thermodynamic instability (Gerasimov and Lai, 2010, Puiu et. al., 2014). However, our group recently demonstrated the utility of a peptide aptamer to detect cathepsin E, a cancer biomarker, using an ELISA-like system (Kitamura et. al., 2011).

To further expand the scope of peptide-based electrochemical biosensor technology, we performed *in vitro* screening of a peptide aptamer for renin as a target molecule using advanced evolutionary molecular engineering and then integrated the peptide onto the surface of a screen-printed chip, termed as PEP-on-DEP (Peptide on disposable electrochemical printed) chip concept. Here, we report the first electrochemical peptide aptamer-based sensor for the detection of renin, a biomarker of kidney dysfunction and/or hypertension. Our approach is the first to screen a peptide aptamer for renin and then combine binding-induced changes with the well-established electrochemical biosensing platform. We validated the use of the selected peptide aptamer to detect renin in as little as 300 ng mL^{-1}.

2. Experimental section

2.1. Chemicals and reagents

The human recombinant renin was purchased from BPS Bioscience (Funakoshi, Japan). Peptides were custom synthesized by standard Fmoc/HBTU chemistry (Scrum, Japan) and dissolved in nuclease-free water or dimethyl sulfoxide (DMSO) prior to use. Gold nanoparticles were purchased from BBI Solutions (UK). High-flow nitrocellulose membrane (FH180AK020) and the absorption pad were purchased from Nihon Millipore (Tokyo, Japan). For the fabrication of the lateral flow strips, disodium hydrogen phosphate, sodium dihydrogen phosphate dehydrate, sucrose, polyethylene glycol (PEG) 20,000, boric acid and potassium dihydrogen phosphate were purchased from Wako Pure Chemicals Industries (Tokyo, Japan). Bovine serum albumin (BSA) to block the membrane, and sodium cholate were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Sodium azide was purchased from Nakalai Tesque (Kyoto, Japan) for preserving the proteins in blocking and diluting solutions. Casein was purchased from Kanto (Japan).

2.2. In vitro selection of peptide aptamer by cDNA display

The peptide aptamer pools were prepared by constructing a cDNA library encoding random eight-amino acids peptides with a size of 2.8×10^{14} molecules, as previously reported (Biyani et. al., 2011) . The short-biotin-puromycin (SBP) linker DNA was synthesized using a previously described method (Biyani et. al., 2006). The aptamer candidates for renin were obtained using a previously described cDNA display method with some modifications (Yamaguchi et. al., 2009). For this, first target renin was immobilized on streptavidin-coated magnetic beads with a diameter of 1 μm in size (Dynabeads MyOne^{TM} Streptavidin C1; Life Technologies) using a biotinylation kit (EZ-Link Sulfo-NHS-Biotin, PIERCE) in accordance with the supplier's instructions. The cDNA display library (i.e., cDNA molecule and its coding protein covalently linked by a puromycin linker) of approximately 10 pmol was diluted in 300 μL of selection buffer (100-mM NaCl, 5-mM $MgCl_2$, 0.1% Tween-20 in PBST, pH 7.4) and incubated with 50 μL of renin-immobilized beads

containing 50-pmol renin. The beads were washed with 300 µL of washing buffer-1 (WB-1) (250-mM NaCl, 5-mM $MgCl_2$, 0.1% Tween-20 in PBST, pH 7.4) and/or washing buffer-2 (WB-2) (500-mM NaCl, 5-mM $MgCl_2$, 0.1% Tween-20 in PBST, pH 7.4). To gradually enhance the selection pressure we performed a total of 2 wash steps using WB-1 for the first round, a total of 6 wash steps, 3 each using WB-1 and WB-2, for second round, and a total of 12 wash steps, 6 each using WB-1 and WB-2, for third and final round. After washing, renin-aptamer complexes were eluted with elution buffer. For this, the magnetic beads were suspended in 100 µL of elution buffer-1 (EB-1) (1-M NaCl, 10-mM $MgCl_2$, 1% Tween-20 in PBST, pH 7.4) and incubated at 37 °C for 5 min, and then the supernatant (Sup-1) was collected. For another elution step, the beads were re-suspended in 100 µL of elution buffer-2 (EB-2) (2-M NaCl, 10-mM $MgCl_2$, 1% Tween-20 in PBST, pH 7.4) and incubated at 95 °C for 5 min, and then the supernatant (Sup-2) was collected. Sup-1 and Sup-2 were purified by ethanol precipitation (Dr. GenTLE™ Precipitation Carrier; Takara Bio Inc.). The cDNA portion of the purified sample was then amplified by PCR for the next round of selection. After performing the final round, selected aptamer candidates were subjected to conventional cloning using a TOPO® TA Cloning Kit (Life Technologies), sequencing, sequence (cluster) analysis, and chemical synthesis.

2.3. Post-selection analysis

The binding affinities of the selected peptide aptamers to renin were analyzed by Surface Plasmon Resonance (SPR) using a BIAcore-J Instrument (GE Healthcare, Sweden). We used the CM5 chip, a carboxymethylated 3D dextran matrix. Immobilization of renin on a CM5 chip (GE Healthcare, Sweden) was performed using the amine-coupling method. For this, freshly prepared 400-mM N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and 100-mM N-hydroxysulfosuccinimide (sulfo-NHS) were mixed in a 1:1 ratio and passed over the sensor chip for 6 min, followed by 20 µg mL^{-1} renin in 10-mM sodium acetate at pH 4.0 for 6 min. Unreacted carboxyl groups were then quenched with 1

M ethanolamine at pH 8.5 for 6 min. Binding assays with renin covalently linked to the chip CM5 were performed by passing peptide aptamers at 200, 100, 50, 25, 12.5 μM for 5 min at moderate flow rate (30 μl/min) followed by regeneration buffer Glycine pH 2.0 for 1 min at a similar rate. The K_D values were obtained using the fitting tool of the BIAevaluation software (BIAcore). A 1:1 binding model was assumed in all cases.

2.4. Peptide aptamer analysis by lateral flow assay

The gold nanoparticle (AuNPs) substrate was prepared by diluting renin or peptide aptamer with 5-mM K_2HPO_4 solution (pH 7.5) to a final volume of 60 μL. The diluted renin solution was added to 0.6 mL of AuNPs solution (i.d. 40 nm, 1 OD) with 5-mM K_2HPO_4 solution (pH 7.5) and mixed immediately. The mixture was kept for 10 min at room temperature for the immobilization of renin onto the AuNP's surface by physical adsorption. After immobilization, 30 μL of 1% (w/v) PEG, which was dissolved in 50-mM K_2HPO_4 solution, was added for 5 min followed by the addition of 60 μL of 10% (w/v) BSA for 5 min to block the non-coated AuNP surfaces. After the immobilization and blocking procedures, AuNP-conjugated renin was separated by a centrifugation (8,000 x *g* for 8 min at 4 °C). The AuNP-conjugated renin was mixed with 100 μL of preserving solution (1% (w/v) BSA, 0.05% (w/v) PEG 20,000, 0.1% (w/v) NaN3 and 150-mM NaCl in 20-mM Tris-HCl buffer, pH 8.2) and pulse-sonicated for a few seconds followed by washing twice using 1 mL of preserving solution and stored at 4 °C until use. Peptide aptamers were prepared by conjugating cysteine-modified peptide aptamer to carrier protein (BSA) using a crosslinker (SPDP, Thermo Scientific) reagent in accordance with the supplier's instructions.

The paper strip was prepared by spotting a 0.5 μL drop of BSA conjugated peptide aptamers onto nitrocellulose analytical membrane strips (4 cm in length). The strips were dried at RT for 30 min followed by the blocking procedure to prevent nonspecific adsorption by immersion in 50-mM boric acid buffer containing 0.5% casein (pH 8.5) and incubation for 60 min at RT. The blocked strips were then washed by

immersion in 50-mM Tris buffer (pH 7.5) containing 0.5% sucrose and 0.05% (w/v) cholic acid sodium salt for 30 min at RT. Finally, the strips were dried at RT overnight followed by pasting the absorbent pad and were stored at 4°C until use.

For the lateral flow assay, 5 µL of AuNP-conjugated renin was mixed with 30 µL of running solution (1% (w/v) BSA, 150-mM NaCl in 20-mM Tris-HCl buffer, pH 7.5) and then absorbed to the test strip by capillary force followed by a washing step using the addition of another 30 µL of running solution. The intensity of the red color produced by the accumulation of the AuNP-conjugated renin on the spotted area was measured by a reader.

2.5. Peptide aptamer analysis by electrochemical measurement

A disposable three-electrode screen printed (DEP) chip was obtained from Biodevice Technology, Co. (Ishikawa, Japan). This chip is a three-electrode system with carbon-based working (3 mm in diameter), counter electrodes and a Ag/AgCl reference electrode. Surface characterization of DEP chip has been reported earlier by our group by visualizing Au nanoparticles-labeled immunocomplexes on the surface of DEP chip by scanning electron microscopy (Idegami et. al., 2008). Exactly 2 µl of renin (280 µg mL^{-1}) was dropped on the working electrode and followed by incubating the chip in a water vapor-saturated environment at RT for 1 h to allow the passive adsorption of renin onto the carbon electrode surface. After incubation, excess renin was rinsed with 100-mM PBS, and the chip was dried by gentle air blow. For suppression of unspecific adsorption, the chip was incubated overnight with 3.5 µl of blocking buffer of 100-mM PBS buffer containing 1% BSA at 4 °C. After rinsing thoroughly with 100-mM PBS buffer and drying, the resulting immunosensor chip was used for the assay. A 2-µl sample containing renin (0 to 380 µg mL^{-1}) and 0.4 µl of AuNP conjugated peptide aptamers was used to incubate the renin-modified DEP chip for 15 min at room temperature. After the chip was washed with 100 mM PBS buffer, the chip was connected to an electrochemical analyzer system (Model 650A, CH Instruments, Inc., Austin, USA) and 30 µL of 0.1 M HCl

was dropped on the DEP chip to perform the electrooxidation of AuNPs at a constant potential of +1.4 V for 40 s, immediately followed by DPV (differential pulse voltammetry) detection from +0.6 V to 0 V, with a step potential of 4 mV, a pulse amplitude of 50 mV, and a pulse period of 0.2 s.

Highlights of manuscript:

PEP-on-DEP:A competitive peptide-based disposable electrochemical aptasensor for renin diagnostics

Manish Biyani, Keiko Kawai, Koichiro Kitamura, Miyuki Chikae, Madhu Biyani, Hiromi Ushijima, Eiichi Tamiya, Takashi Yoneda, Yuzuru Takamura

- In vitro selection of Peptide aptamers for renin using cDNA display
- Integration of peptide aptamer technology and screen printed electrode technology
- A portable and disposable peptide-based electrochemical biosensor technology for one-step renin diagnostics as little as 100 ng mL^{-1}
- A simple and robust antibody-free electrochemical biosensor for unreachable biomakers

3. Results

3.1. In vitro selection of a peptide aptamer for renin

Renin, a member of aspartic proteinase with a molecular weight of 38 kDA, plays an important physiological role in the regulation of blood pressure by controlling the first and rate-limiting step of the renin-angiotensin system. Therefore, its role as a biomarker of cardiovascular risk and prognosis is greatly anticipated (Volpe et. al., 2012). ELISA methods based on monoclonal antibodies (mAbs) have been considered as the most practical method to detect renin; however, searching for an optimal pair among 10 commercial available monoclonal and/or polyclonal antibodies for sandwich has been a challenging task (unpublished data). Surprisingly, out of 88 evaluated combination pairs only one set of antibodies, i.e., mAb #411524 and polyclonal Ab sheep, could be used to generate the sandwich-type assay to detect renin on a immunochromatographic strip (see Supplementary Materials, **Fig. S1**). Therefore, the initial goal of this study was to investigate the capacity of the aptamer, in particular the peptide aptamer, to serve as an electrochemical biosensor diagnostic probe for renin. Peptide aptamers were identified by *in vitro* selection using our earlier developed cDNA display method (Yamaguchi et. al., 2009) and then integrated onto our disposable and portable electrochemical biosensor prototype device (Shiohara et. al., 2014). The key steps are schematically illustrated in **Fig. 1**. The initial (primary) cDNA display library which is composed of randomized 8-residue amino acids was used for selection. The weakly-bound cDNA display molecules were washed away, and the remaining cDNA display molecules were used as a starting library for a total of four succeeding rounds of *in vitro* selection. Finally, the enriched peptide aptamer candidates were cloned, sequenced and analyzed for sequence alignment and clustering (see Supplementary Materials, **Fig. S2**). We found that 36 aptamer candidates could be classified into 4 distinct clusters, and at least 2 candidates in each cluster were found to have same sequence. To quantitatively evaluate the binding affinity of peptide aptamers, a SPR experiment was subsequently performed using Biacore, and the dissociation constant (K_D) was calculated using BIA evaluation software. We used the CM5 chip for the immobilization of renin. The

sensograms indicated that peptide aptamers were released in a short time, and thus, the K_D of the selected aptamers could be calculated from the levels of the sensograms at equilibrium in a small micromolar range (see Supplementary Materials, **Fig. S3**). High affinity is not expected because the library used for *in vitro* selection is composed of primary modules (8-residues amino acid), which is consistent with our earlier work (Biyani et. al., 2011). Five potential peptide aptamers were finally considered for further validation experiments.

3.2. Candidate determination by lateral flow assay

Lateral flow (LF) paper strip assay was used to evaluate and select the best available peptide aptamer for the electrochemical biosensor. The principle of the assay is based on the specific binding between the peptide aptamer and the target renin as shown in **Fig. 2**. Briefly, the peptide aptamer is spotted onto the LF paper strip and the target renin that is immobilized on the gold nanoparticles (Au-NPs) is flowed onto the LP strip by capillary action. Target renin interacts with the aptamer probe to form the Au-NP-renin-aptamer complex and can be visualized as a characteristic spot on the strip. The excess of AuNP-renin continues to migrate and is collected in an absorption pad. Quantitative analysis is performed by reading the optical intensity of the spot with a portable strip reader.

The peptide aptamer cannot be functionally adsorbed onto nitrocellulose paper due to its small molecular weight (approximately 1.5 kDa) or because they may wash out with the advancing washing steps during sample wicking. Therefore, cysteine-terminated peptide aptamers were first conjugated with a carrier protein (BSA) via a bifunctional molecular crosslinker (SPDP). Nearly 32 free amino groups are on the surface of BSA (Mao et. al., 2006), and therefore, 20-30 peptide aptamers are expected to covalently link with an individual BSA molecule. A 0.4 microliter 'dot' of conjugated peptide aptamer was typically applied and physically adsorbed onto the strip. **Fig. 2** shows the typical photo images and corresponding optical responses from all of the 5 tested peptide aptamers. Only two aptamers, i.e., peptide III-13 and peptide III-17,

were successfully measured and used for further quantification of renin detection by the electrochemical method.

3.3. Performance of the competitive electrochemical peptasensor for detection of Renin

To evaluate the robustness of the single peptide aptamer-based electrochemical biosensor, we designed a competitive electrochemical assay using a disposable electrochemical printed (DEP) chip with peptide aptamer-conjugated AuNPs as a recognition element. The steps involved in the assay are depicted in **Fig. 1**. The principle of this assay is based on the competition of a binding event between sample renin (unknown concentration) and known renin (immobilized on the DEP chip surface) to the peptide aptamer-conjugated gold nanoparticles. The functionalized immobilization of the peptide aptamer onto the gold nanoparticles is critically dependent on the structure of peptide because AuNPs often aggregates in aqueous solutions when thiol-containing amino acids or peptides are added (Pengo et. al., 2003). Therefore, first we evaluated the type of peptide aptamer able to functionally immobilize onto the surface of the AuNPs via a terminated cysteine. Among all 5 selected candidates, only peptide 7, 13 and 22 could be successfully captured on the AuNPs surface through the SPDP linker and BSA. However, peptides 15 and 17 were severely affected via aggregation behavior (see Supplementary Materials, **Fig. S4**). Therefore, we selected peptide 13 for a one-step competitive assay. The surface of the DEP chip was prepared by immobilizing known renin onto the electrode by electrostatic interaction (electrostatic configuration). Sample renin was then incubated with peptide aptamer-conjugated AuNPs and measured on the DEP chip by detecting the binding event between free (remained) peptide aptamer-conjugated AuNPs and DEP immobilized renin. The system is rinsed, and a current signal is generated by performing a direct redox reaction. Because of the competitive nature of this assay, the generated electrochemical signal (DPV) will be inversely proportional to the amount of renin present in the sample.

Figure 3 shows the DPV measurement for renin in a competitive assay between sample renin (ranging

from 280 μg mL^{-1} to 380 ng mL^{-1}) and known renin (280 μg mL^{-1}), where the presence of known renin is revealed using peptide aptamer-conjugated AuNPs. The signal for each concentration was carried out by performing four independent experiments. Sample renin and known renin, at a fixed known renin concentration, compete for binding with the peptide aptamer-conjugated AuNPs. At a low sample renin concentration, most of the peptide aptamer-conjugated AuNPs on the electrode is bound by known renin, the condition for which the maximum DPV signal is generated. As the sample renin concentration increases, the signal decreases. As shown in **Fig. 3b**, the log[sample renin] is measured in the range from 280 μg mL^{-1} to 380 ng mL^{-1}, and thus, the biosensor was able to detect concentration of renin as low as few 300 ng mL^{-1}. The results obtained with this newly discovered probe for renin, i.e., the peptide aptamer, represent an important step towards the development of non-antibody-based probes for renin measurement.

4. Discussion

The proposed peptide aptamer-based single-step competitive electrochemical sensing strategy is a more practical and simplified approach to access unreachable targets and avoid the cumbersome antibody-based procedure used for the non-competitive method (Supplementary Materials, **Table S1**). This sensor approach is characterized by 2 critically important features: (i) signal specificity using new and specific probe, i.e., the peptide aptamer is specific for a given target on demand using an *in vitro* selection method; (ii) process simplification and signal amplification for real-time analysis by integrating a probe-based biosensing method with an electrochemical sensing platform using a portable and disposable screen-printed electrochemical (DEP) chip. Signal sensitivity can be maximized by selecting high-affinity aptamers (nM-to-pM) using our advanced *in vitro* selection method called the progressive library selection (PLM) method (Kitamura et al., 2012), where information obtained in the preceding selection is exploited in the following step of selection to enrich the library and thus elevate the affinity and function of the aptamer. However, aptamer binding is dependent on target molecule features such as stability, size and the availability of a functional group. In this study, *in vitro* selection is performed using a primary library of 8 randomized amino acids. Our earlier reports mention that the selection of the binding affinity of candidates from the primary library crucially depends on the species of the targeted protein molecule and varies within the μM-to-pM range. Unfortunately, low affinity candidates (μM) were obtained for renin in this study, presumably because of the less stable nature of target renin. To further improve the binding affinity and thus the signal sensitivity, a progressive library selection method will be used. We expect to improve the detection range of renin from ng-to-pg mL^{-1} by performing several progressive library selection rounds using the sequence information obtained in this work.

5. Conclusion

We developed a portable and disposable peptide aptamer-based electrochemical biosensor and we demonstrate its high practical applicability and capability to resolve the challenge of antibody-dependency. We successfully used *in vitro* selected peptide aptamers as electrochemical sensing tags for the quantification of renin with a disposable electrochemical printed chip. The proposed peptide aptamer-based competitive assay provides a simple and rapid method for detecting renin in a serial order ranging from μg-to-ng mL^{-1}, employing *in vitro* selected peptide aptamers with μM binding affinity. This technology has attractive advantages in the application of *in vitro* selection for the construction of versatile electrochemical biosensors for on-demand diagnostics.

Acknowledgements

This work was supported by MEXT Regional Innovation Strategy Support Program Hokuriku Life Science Cluster project.

References

Ahmed MU, Idegami K, Chikae M, Kerman K, Chaumpluk P, Yamamura S, Tamiya E, 2007. Analyst, 132, 431-438.

Baker M., 2015. Nature, 521, 274-276.

Biyani M, Futakami M, Kitamura K, Kawakubo T, Suzuki M, Yamamoto K, Nishigaki K., 2011. Int J Pept., 2011, 834525.

Biyani M, Husimi Y, Nemoto N., 2006. Nucleic Acids Res., 34, e140

Chen A, Yang S, 2015. Biosen Bioelectro 71, 230-242.

Cho EJ, Lee JW, Ellington AD., 2009. Annu Rev Anal Chem (Palo Alto Calif), 2:241-264.

Colas P, Cohen B, Jessen T, Grishina I, McCoy J, Brent R., 1996. Nature 380, 548-550.

Edgami K, Chikae M, Kerman K, Nagatani N, Yuhi T, Endo T, Tamiya E, 2008. Electroanalysis 20, 14-21.

Findlay JW, Smith WC, Lee JW, Nordblom GD, Das I, DeSilva BS, Khan MN, Bowsher RR., 2000. J Pharm Biomed Anal. 21, 1249-1273.

Gerasimov JY, Lai RY, 2010. Chem Commun (Camb)., 46, 395-397.

Grieshaber D, R MacKenzie, JV os1 and E Reimhult 2008. Sensors, 8, 1400-1458.

Hayat A, Marty JL, 2014. Front Chem. 2, 1-9.

Hayat A, Marty JL, 2014. Sensors (Basel), 14, 10432-10453.

Ikebukuro K, Kiyohara C, Sode K, 2004. Anal Lett 37, 2901-2909.

Kitamura K, Biyani M, Futakami M, Ueno-Tsuji S, Suzuki M, Kawakubo T, Yamamoto K, Nishigkai K, 2011. J Mol Biomark Diagn, 2, 1-4.

Kitamura K, Komatsu M, Biyani M, Futakami M, Kawakubo T, Yamamoto K, Nishigkai K, 2012. J Pept Sci, 18, 711-719.

Leguin RM, 2005. Clin Chem 51, 2415-2418.

Liu Y, Matharu Z, Howland MC, Revzin A, Simonian AL, 2012. Anal Bioanal Chem 404, 1181-1196.

MAO Wen-Xue, SU Yong, KANG Jing-Wu., 2006. Chin. J. Org. Chem., 26, 707-710.

Metters JP, Kadara RO, Banks CE, 2011. Analyst, 136, 1067-1076.

Pengo P, Broxterman QB, Kaptein B, Pasquato L, Scrimin P, 2003. Langmuir 19, 2521-2524.

Puiu M, Idili A, Moscone D, Ricci F, Bala C., 2014. Chem Commun (Camb), 50, 8962-8965.

Ronkainen NJ, H.B. Halsall, W.R. Heineman, 2010. Chem. Soc. Rev., 39, 1747-1763.

Shiohara S, M Chikae, Y Ukita, H Ushijima, Y Fukumura, T Takamura, Y Takamura, 2014. Microsystem Technologies, 20, 273-279.

Volpe M, Battistoni A, Chin D, Rubattu S, Tocci G., 2012. Nutr Metab Cardiovasc Dis., 22, 312-317.

Yamaguchi J, Naimuddin M, Biyani M, Sasaki T, Machida M, Kubo T, Funatsu T, Husimi Y, Nemoto N., 2009. Nucleic Acids Res., 37, e108.

Zhou W, Huang PJ, Ding J, Liu J, 2014. Analyst 139, 2627-2640.

Figure Legends

Figure 1. PEP-on-DEP concept. Schematic representation of the electropeptasensor process that begins with a very large library of peptide candidates to select potential peptide aptamers that can specifically bind to a target (renin) using a cDNA display method. The selected peptide aptamers are then grafted onto gold nanoparticles to perform a competitive binding assay using a portable electrochemical analysis system.

Figure 2. Lateral flow assay. (A) Schematic drawing. (B) Typical photo image (top) and corresponding responses (bottom) of renin functionalized gold nanoparticles with selected peptide aptamers.

Figure 3. Electrochemical measurement. (A) DPV curves and (B) corresponding logarithmic calibration curve for the competitive assay with a fixed renin concentration (280 μg mL-1) and variable renin concentrations from 280 μg mL-1 to 380 ng mL-1. The plot of current as a function of concentration of renin with linear trend line is calculated for regression analysis with the value of R-squared 0.953 and correlation coefficient, r -0.77). The data are the average of three independent experiments.

Figure(s)

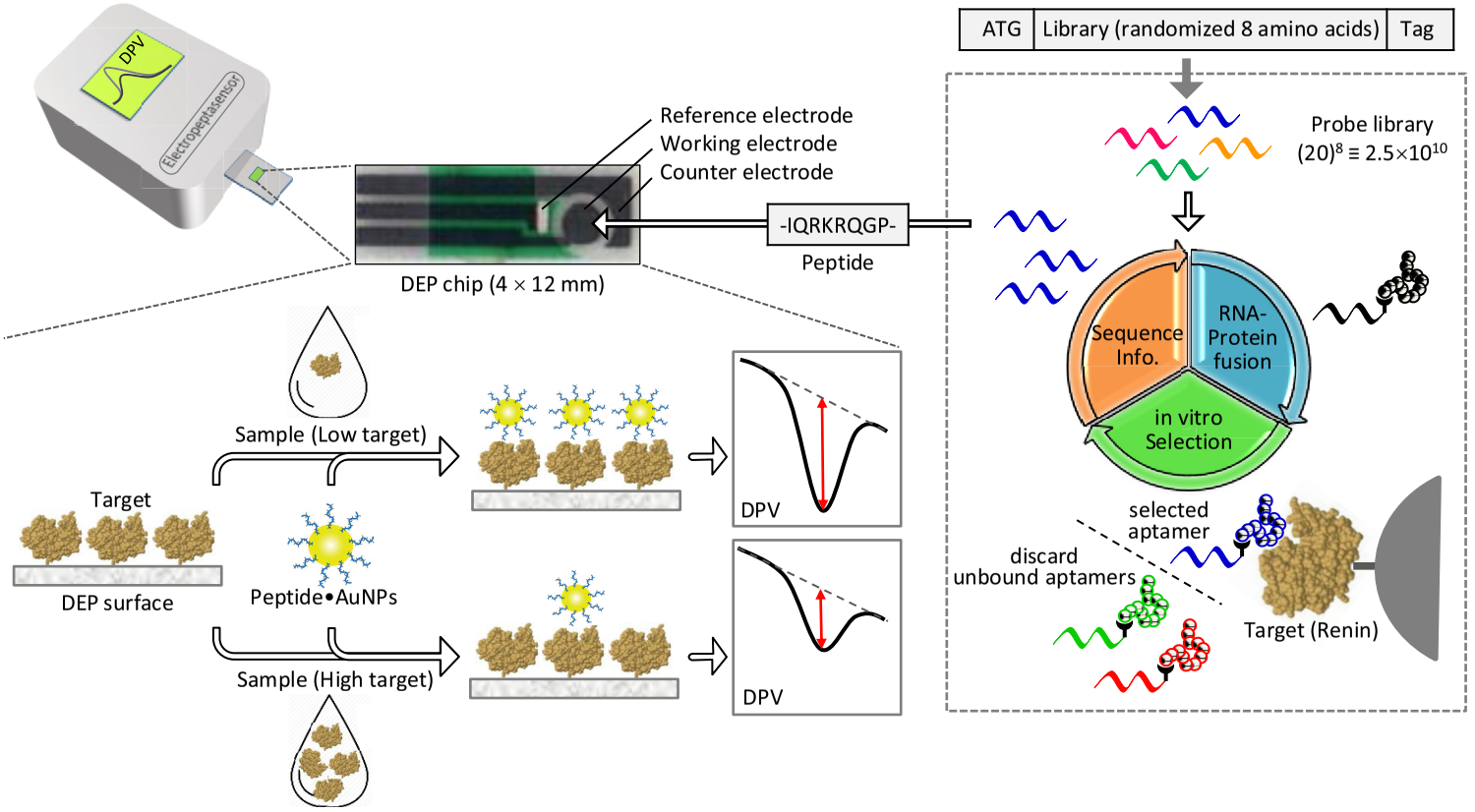

Figure 1, Biyani et. al.

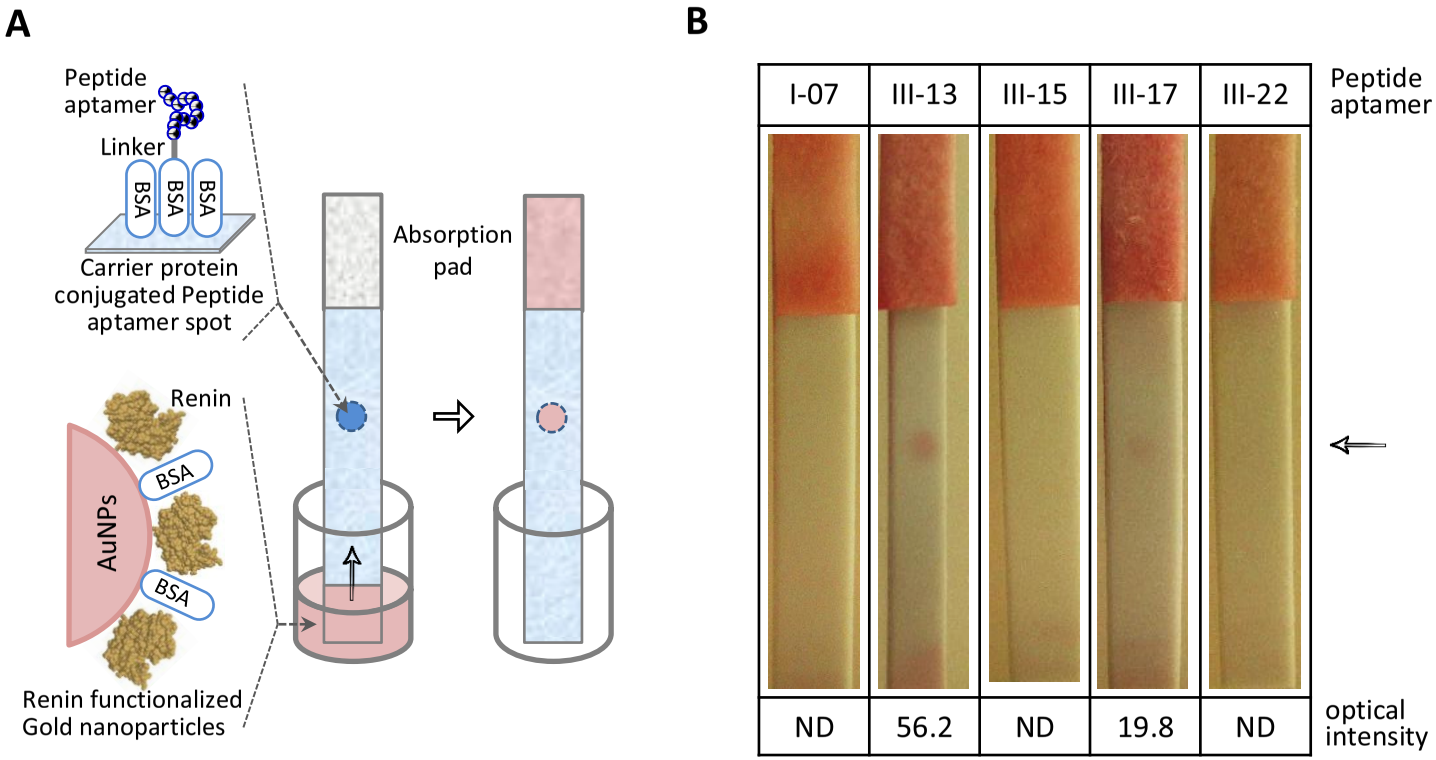

Figure 2, Biyani et. al.

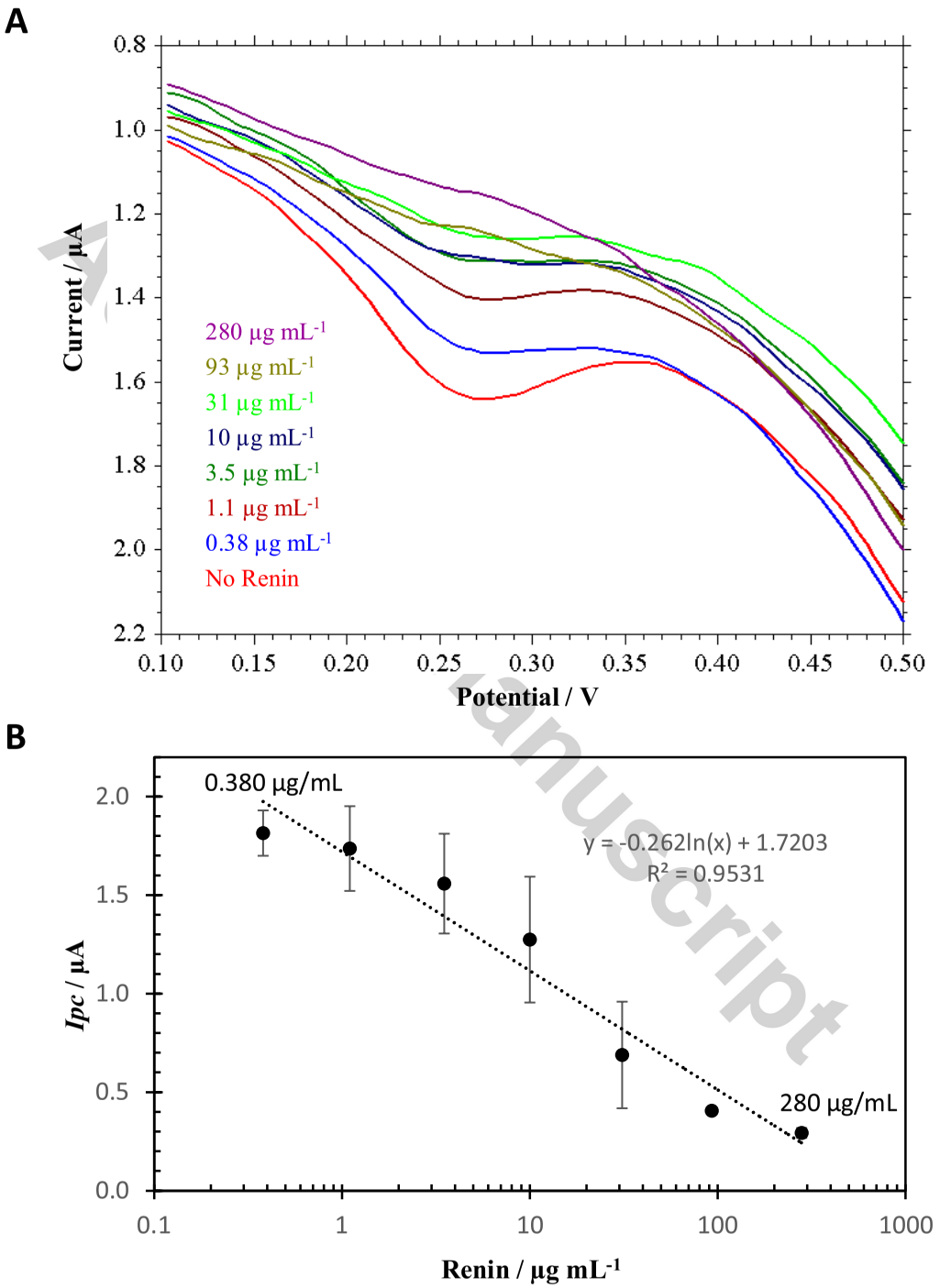

Figure 3, Biyani et. al.