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Detection of \(\alpha\)-Methylacyl-CoA Racemase in Serum and Urine Using a Highly Sensitive Electrochemical Immunodetector

Jiapeng Wang\[a\] and Siu-Tung Yau* \[a, b\]

Because of its lack of specificity in the diagnosis of prostate cancer, prostate specific antigen (PSA) is deemed unsatisfactory as a biomarker for the diagnosis of the disease [1]. New biomarkers with improved performance factors are needed for the early diagnosis of prostate cancer [2]. One potential biomarker is \(\alpha\)-methylacyl-CoA racemase (AMACR), an enzyme that is involved in peroxisomal-oxidation of dietary branched-chain fatty acids. Recent studies have shown that, compared with expression in normal or benign prostate epithelium, AMACR is consistently overexpressed in prostate cancer epithelium, making it a specific marker for cancer cells within the prostate gland [3–5]. The fact that AMACR is present in serum and urine of prostate cancer patients allows it to be used as a convenient biomarker for the diagnosis of prostate cancer.

Previously, AMACR in the urine of prostate cancer patients has been detected in a fluidic system using a macrocantilever-based piezoelectric sensor, whose surface was immobilized with anti-AMACR [6]. Detection of AMACR on the femto-gram/mL level was achieved directly in patient urine without a sample preparation step and without the use of labeled reagents. Another sensor-based detection of AMACR was made using a iridium nanoparticle-based \(\text{H}_2\text{O}_2\) electrochemical sensor [7]. The sensor was used to detect \(\text{H}_2\text{O}_2\) that was released in the AMACR-catalyzed conversion of \((2\text{R})\)-pristanoyl-CoA to \((2\text{S})\)-pristanoyl-CoA. The sample preparation required incubation for 72 hours. The sensor provided detection on the \(\mu\)g/\(\mu\)L level and showed 100% accuracy, which was demonstrated using blood samples of patients.

This paper reports the detection of AMACR on the pico-gram(pg)/mL level, using a novel immunosensing system, which consists of a three-electrode system modified with a gating electrode for applying a gating voltage \(V_G\) to the immune complex immobilized on the working electrode to provide signal amplification. The detection system is realized by integrating gating electrodes with screen-printed electrodes. This detection method does not require involved sample preparation procedures. The detection was demonstrated in serum and urine samples on the nanogram/mL level with \(V_G\) equal to 0.6 V. Detection in serum was also performed on the picogram/mL level with a limit of 100 picogram/mL with \(V_G=0.6\) V being a necessary condition.

Abstract: The detection of \(\alpha\)-methylacyl-CoA racemase (AMACR), a novel biomarker for prostate cancer, is demonstrated in serum and urine using a novel immunodetection method. The detection system consists of a three-electrode conventional electrochemical cell modified with a gating electrode for applying a gating voltage \(V_G\) to the immune complex immobilized on the working electrode to provide signal amplification. The detection system is realized by integrating gating electrodes with screen-printed electrodes. This detection method does not require involved sample preparation procedures. The detection was demonstrated in serum and urine samples on the nanogram/mL level with \(V_G\) equal to 0.6 V. Detection in serum was also performed on the picogram/mL level with a limit of 100 picogram/mL with \(V_G=0.6\) V being a necessary condition.

Keywords: Biomarker detection · Prostate cancer biomarker · Immunoassay · Screen printed electrode

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This paper reports the detection of AMACR on the pico-gram(pg)/mL level, using a novel immunosensing method, which consists of a three-electrode system modified with a gating electrode for applying a gating voltage \(V_G\) to the immune complex immobilized on an electrode. The detection was performed in serum and urine samples with a limit of 100 pg/mL. The detection system was realized by integrating gating electrodes with screen-printed electrodes (SPEs). This emerging detection technique can be potentially used to form a disposable platform for the ultrasensitive and low-cost detection of AMACR for point-of-care applications.

The detection of AMACR was achieved using the detection system described in Supporting Information, the operation of which requires the sandwich structure, Ab-AMACR-Ab(HRP), to be formed on the AMACR-detecting electrode. The detection signal was the measured peak current of the reduction peak of HRP without using \(\text{H}_2\text{O}_2\), the substrate of HRP. A similar reagentless detection approach was also demonstrated previously [8].

Control experiments were performed in order to avoid misinterpretation of experimental results due to non-specific binding. Figure 1a shows a set of cyclic voltammograms (CVs) obtained with a SPE modified with PANI as described above. The modified electrode was first immo-
blished with the primary Ab as described below and sub-
sequently incubated with the labeled secondary
Ab(HRP) for 30 min. The SPE was then washed with de-
imonized water. CV1 was obtained with this electrode in
serum when a $V_G$ of 0.6 V was applied. The reduction peak
at $-0.42$ V appears to be enhanced, illustrating the am-
plification provided by FEED. This effect was essential
for measuring the signals at low AMACR concentra-
tions.

Detection of AMACR was previously carried out in
serum with the lowest concentration at $\mu$g/mL [7]. In
the present work, detection of AMACR in serum was first
demonstrated on the ng/mL level. Figure 2a shows the
amplification curve of AMACR detection in serum obtained
with $V_G=0.6$ V. The detection covers a range from100 ng/
mL to 1 ng/mL. The data points show a nearly linear de-
pendence on the concentration. The straight line is the re-
gression line for the data points, having a correlation co-
efficient of 0.9730. The sensitivity derived using the
straight line is $0.00487 \times 10^{-6}$ A/ng/mL. The small error
bars indicate reproducible device performance. Figure 2b
shows the amplification curve of AMACR detection in
urine obtained with $V_G=0.6$ V. The general features of
the calibration curve are similar to those for the serum
case except that the current level is reduced.

Detection of AMACR in serum was also achieved on
the pg/mL level, where the application of $V_G$ is indispen-
sable since the redox peaks of HRP are extremely weak
on CVs. Figure 2c shows the calibration curve of AMACR
detection in serum on the pg/mL level. The curve was
obtained with $V_G=0.6$ V. The first data point shows the
zero concentration signal. The data points indicate
a nearly linear dependence of the signal on AMACR con-
centration. The straight line is the regression line for the
data points, having a correlation coefficient of 0.9890.
The sensitivity derived using the straight line is
12.9 $\mu$A/mL/ng. The detection limit of this concentration
range is estimated to be 204 pg/mL, using the signal/to
noise $=3$ method. The small error bars indicate reproduc-
ible device performance.

The results presented above show that sensitive detec-
tion of AMACR can be performed in serum and urine
without sample preparation. The detection was made pos-
sible by the amplification provided by the novel detection
system. The specificity provided by the immuno-detection
approach allowed the detection to be carried out in
serum and urine samples. The detection was demonstrat-
ed in serum and urine samples on the nanogram/mL
level. Detection in serum was also performed on the pg/
mL level with a limit of 100 pg/mL.

**Experimental**

The basic principle of the detection is explained and the
detection mechanism is elucidated in previous publica-
tions [11,12]. Briefly, the detection system consists of
a conventional three-electrode electrochemical cell modi-
fied with additional gating electrodes for applying a
gating voltage $V_G$ to the detecting (working) electrode,
on which a redox enzyme, the sensing element, is immobilized. $V_G$ rearranges ions in the sample solution at the electrode-solution interface, inducing an electric field that penetrates the immobilized enzyme. The field reduces the effective height of the tunnel barrier between the active site of the enzyme and the electrode, therefore increasing the tunnel rate of electrons and resulting in intrinsic amplification of the signal current. In the present work, the enzyme was conjugated to the detecting antibody and immobilized on the electrode via the immune-complex. A detailed description of the immunosensing system used in this work is provided in Supporting Information.

Recombinant human AMACR was purchased (Genway Biotech; Cat. No. GWB-CFE200) and used as the positive control. Monoclonal anti-AMACR and polyclonal anti-AMACR (Genway Biotech; Cat. Nos. GWB-8770CE, GWB-AMACRA) were used as the primary/capture antibody and the secondary/detection antibody, respectively. Horseradish peroxidase (HRP) was conjugated to the detection antibody using a conjugation kit. Human serum (Thermo Scientific Pierce; Product # 31876) and synthetic urine (Quick Fix) were purchased.

SPEs were purchased from DropSens. The working electrode of the SPE is a circular carbon electrode with a 4 mm diameter. The working electrode, silver reference electrode and the carbon counter electrode are fabricated on the top side of the SPE. The dimensions of the SPE are $3.4 \text{ cm} \times 1.0 \text{ cm} \times 0.05 \text{ cm}$. The working electrode of the purchased SPE was covered with a layer of polyaniline (PANI). The PANI layer was used to host the capture antibody and therefore the immune complex on electrodes [13] and as electron mediator, shuttling electrons between HRP and the electrode [14]. The PANI layer was synthesized on the electrode using electrochemical polymerization of aniline in a solution of 0.1 M aniline and 1 M HCl at a potential of 1.2 V for 100 s [15]. The PANI prepared using this method was used previously in a HRP-based biosensor to detect H$_2$O$_2$ at pH 6.8 [16] as well as in a pH sensor [17] because the conductivity of PANI persists up to pH 9. Alternatively, sulfonated PANI was prepared by adding poly(vinylsulfonic acid) (Aldrich, cat. # 27,842–4) to aniline and HCl during electrochemical polymerization as described previously [18], to increase the redox activity of PANI at pH 7. The results shown in this work were obtained with PANI prepared using HCl only while the two kinds of PANI led to similar results.

AMACR was spiked into serum and urine samples. The AMACR sample solution did not require treatment. The PANI layer was modified by depositing 2 mL of glutaraldehyde (25% with water, diluted 100 times with water) on PANI until dry. Glutaraldehyde was used as a cross-linker, coupling the capture antibody to the highly porous PANI so that the immune complex was firmly entrapped in PANI. Previous XPS analysis [13] shows that glutaraldehyde binds to the amine groups on PANI to form an imine bond. AMACR-detecting electrodes were formed by incubating the modified SPE with 10 µL of 1 mg/mL

![Fig. 2. AMACR detection calibration curve on the ng/mL level in (a) serum and (b) urine with $V_G=0.6$ V. (c) AMACR detection calibration curve on the pg/mL level in serum with $V_G=0.6$ V. The scan rate of 20 mV/s was used to obtain the CVs.](image-url)
primary antibody dissolved in PBS for 1 h at room temperature. To detect AMACR, an AMACR-detecting electrode was incubated with 5–10 μL of an AMACR sample (a AMACR standard serum solution) for 1 h at 37°C. After rinsing and blocking possible open areas on the electrode with bovine serum albumin (40mg/mL diluted to 10% in Dulbecco’s phosphate-buffered saline), the same electrode was incubated with 5–10 μL of 0.5 μg/mL HRP-labeled secondary antibody for 30 min at 37°C and then rinsed with de-ionized water. The Ab-AMACR-Ab(HRP) sandwich structure now has formed on the electrode. Finally, the electrode was used as the working electrode for electrochemical measurements. A piece of 0.5 mm-diameter copper wire coated with a thin layer of insulator (enamel) was used as the gating electrode for applying $V_G$. The wire was bent to form a U-shaped structure and was attached on the sensing electrode using non-conductive epoxy. All AMACR samples were prepared with undiluted serum sample as received. The measurements of the reduction peak currents of three electrodes were used to determine each data point on the calibration curves.

Electrochemical measurements were made by immersing the detecting electrode in a phosphate saline solution (PBS) contained in a glass beaker. The electrode was driven by a commercial electrochemical controller (CHI 660C Work Station). A potential scan rate of 20 mV/s was used in recording cyclic voltammograms (CV). PBS (0.1 M at pH 7) was prepared using de-ionized water (18.2 Ω-cm). Commercially available hydrogen peroxide (Fisher scientific, 30% concentration) was diluted to the concentrations used in the experiment. All measurements were made with deaerated PBS at room temperature. Reproducible results were obtained by repeating each measurement multiple times.

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