Electro-engineered polymeric films for the development of sensitive aptasensors for prostate cancer marker detection

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Supporting Information Placeholder

ABSTRACT: We report the development of a simple surface chemistry strategy for the construction of sensitive aptasensors on a polypyrrole (PPy) – polyethylene glycol (PEG) platform in order to provide enhanced anti-fouling properties. We report the covalent modification of a PPy film formed on a gold electrode by PEG molecules, without prior chemical functionalization of the pyrrole monomer. This process was mediated by electro-oxidation of amine groups present on the one of the PEG's end chains. Poly-histidine modified aptamers were immobilized to this surface via a Nα,Nα-Bis(carboxymethyl)-L-lysine ANTA/Cu²⁺ redox complex covalently attached to the PPy-PEG adduct. The fabricated aptasensor was then utilized for the detection of α-methylacyl-CoA racemase (AMACR; P504S), an emerging biomarker for prostate cancer. Protein/aptamer interactions were monitored through variation of the copper redox signal, using the square wave voltammetry (SWV) technique. We demonstrate that the PPy-PEG-ANTA/Cu²⁺ hybrid material is characterized by enhanced anti-fouling properties and sensitivity. The aptasensor was able to detect AMACR down to 5 fM both in buffer and spiked human plasma with a limit of detection (LOD) of 0.15 fM and 1.4 fM, respectively. The developed aptasensor can be generalized for use with any type of aptamer-based sensor.

Prostate cancer (PCa) is a leading cause of cancer-related mortality among men worldwide.¹ Whilst prostate specific antigen (PSA) remains the benchmark for prostate cancer diagnosis, its use is considered unreliable due to lack of specificity. In particular, it is difficult to identify malignancy in patients with intermediate levels of PSA (4–10 ng/mL).² Early diagnosis of PCa is critical in defining treatment options, which can only be achieved by looking at panels of established and emerging biomarkers as well as novel detection techniques.³–⁵ One of the potential biomarkers that has been gaining significant attention is α-methylacyl-CoA racemase (AMACR; P504S). AMACR is an isomerase involved in peroxisomal β-oxidation of branched-chain fatty acids. Recent studies have reported that AMACR is overexpressed in the majority of prostate malignancies as compared to normal and benign glands.⁴ Most importantly, AMACR has been shown to have higher sensitivity and specificity than PSA for PCa identification in humoral immune assays, especially those with intermediate levels of PSA.⁵ Although AMACR is a tissue biomarker,⁶ its presence in body fluids of PCa patients has also been reported.²–⁷

Recently, several research groups have tried to develop biosensors for AMACR detection.⁸ In 2014, Yang et al.⁹ first reported an anti-AMACR DNA aptamer (AMC51) with a dissociation constant of 44 nM and demonstrated a fluorescent enzyme-linked aptamer assay (ELAA) for AMACR detection, which featured a low detection limit of 0.44 nM. A DNA aptamer is a ligand binding single stranded DNA that binds to its target with high affinity and specificity by undergoing conformational changes.¹⁰ Compared to antibodies, aptamers have several advantages including ease of synthesis and modification, chemical stability, small size, and low immunogenicity. These features make aptamers promising alternatives to antibodies for biosensor research.³–⁷

In this work, we present the development of an electrochemical AMACR aptasensor based on electro-patterned polyethylene glycol (PEG) on polypyrrole (PPy) film. PEG as a polymer has been widely used to develop biosensors, due to its anti-fouling properties.¹² However, it becomes difficult to employ PEG in an electrochemical platform due to its electri-
cal insulation properties. Nevertheless, several research
groups have demonstrated the use of PEGs on electrochem-
ical platforms. For example, Estrela et al.\textsuperscript{13} developed an
electrochemical biosensor for cyclin-dependent kinase protein
detection using peptide aptamers and PEG, while Kjallman et
al.\textsuperscript{24} reported a DNA-based biosensor using PEG. Akhtar et
al.\textsuperscript{25} demonstrated a design of a PEG-aptamer for detection of
ochratoxin A; however, the strategy employs modification of
aptamers with PEG. We propose a one-step, simple strategy
to deposit PEG by using an organic conducting polymers
such as PPy as a foundation surface.

PPy has attracted considerable attention in the past dec-
dades to those developing biosensors because of its redox
properties\textsuperscript{26} and high electrical conductivity\textsuperscript{27} along with it
being a suitable interface for bio-receptor association.\textsuperscript{28} It
comes with several added advantages including the simplic-
ity of polymerization and the high reproducibility of the re-
sulting films. PPy has been used to immobilize various types
of biomolecules by e.g. adsorption\textsuperscript{29}, entrapment during
polymerization\textsuperscript{30} and covalent linkage.\textsuperscript{31} However, for co-
valent association, pyrrole monomers need prior chemical
modification on their nitrogen or 3-substituted carbon posi-
tions to react with functional chemical groups such as car-
boxylic acids or amines.\textsuperscript{32} Such an approach has been
demonstrated with DNA sensors\textsuperscript{33} and immunosensors.\textsuperscript{34}
However, the chemical modification of the pyrrole monomer
can be time consuming. Miodek et al.\textsuperscript{35} developed a faster
method of functionalization of carbon nanotubes (CNTs)
coated with un-modified PPy using macromolecular den-
drimers PAMAM of forth generation. The electro-oxidation
of the amine groups of dendrimers allow binding to both the
CNTs and the PPy and allows further covalent attachment of
biomolecules.

In this work, we design an aptasensor specific for human
recombinant AMACR 1A protein based on PEG electro-
patterned onto a PPy layer and associated with an
ANTA/Cu\textsuperscript{2+} complex. Modification of a PPy film by PEG
molecules can lead to minimization of non-specific binding.
On the other hand, the association of redox molecules such
as copper complexes with PPy can enhance the electrical
properties of the film and improve the sensitivity of detec-
tion.\textsuperscript{36} Electrochemical patterning was possible by function-
alization of PEG on one terminus with amine groups that
allow linking to PPy via radical cations formed during elec-
tro-oxidation. The second edge of the PEG's chains were
modified with carboxylic acids, which allow further associa-
tion of molecules. Poly-histidine modified DNA aptamers
pecific to AMACR were immobilized onto the PPy-PEG
backbone through the ANTA/Cu\textsuperscript{2+} complex. This complex
was covalently linked to the free carboxylate end of the PEG
chain using EDC/NHS coupling agents.

The resultant biosensor was used for sensitive detection of
AMACR in both buffer and spiked human plasma samples.
The interaction of the target with the aptamer was detected
by measuring variations in the electrochemical signal of
copper. A sensitivity down to 5 fM was obtained using the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of aptasensor manufacture: (1) Electro-deposition of pyrrole on gold electrodes; (2) subsequent electro-patterning of PEG molecules using the amine present on one of its terminus; (3) Attachment of ANTA to the carboxyl end of PEG via covalent bonding with EDC/NHS coupling agents; (4) Immobilization of copper ions; (5) Immobilization of His-tagged DNA aptamers; (6) detection of AMACR; (7) Electrochemical impedance spectroscopy characterization of different layers.}
\end{figure}

square wave voltammetry (SWV) technique. The biosensor
was characterized using scanning electron microscopy
(SEM), Fourier-transform infrared spectroscopy (FTIR),
electrochemical impedance spectroscopy (EIS) and cyclic
voltammetry (CV). We also present a Surface Plasmon Reson-
ance (SPR) study to demonstrate the proof of concept. The
strategy presented can be generalized to other biosensors
using different probes specific for the target analyte.

\section*{RESULTS AND DISCUSSION}
Characterization of electro-generated PPy-PEG material

Figure 1 presents a schematic of the fabrication strategy (1-
6) and a series of impedance spectrum illustrating changes in
conductivity of the electrode during the formation of the differ-
ent layers (7). The optimal conditions of polymerization
and PEG association were obtained by investigating different
parameters such as thickness of PPy layer and potential
range for electro-deposition (see also Figures S2 to S4 in
Supporting Information). Briefly, a clean gold electrode
surface was modified with PPy by scanning the potential from
-0.4 V to 0.9 V vs. Ag/AgCl with a scan rate of 100 mV.s\textsuperscript{-1}
in the presence of 50 mM pyrrole monomer dissolved in H\textsubscript{2}O
containing 0.5 M lithium perchlorate for two cycles. From the
EIS data, a small charge transfer resistance ($R_\text{ct}$) of 40 $\Omega$
was observed (see Figure S5 in Supporting Information). The
film obtained showed a characteristic ‘sickle shaped’ mor-
phology distributed across the electrode surface when ob-
served under a scanning electron microscope (see Figure S5a
in Supporting Information). This shape is characteristic of
PPy when polymerization occurs in aqueous solutions.\textsuperscript{37}
In order to modify the generated PPy film with PEG bearing both amine and carboxylic groups, electrochemical grafting of PEG was performed using the optimized conditions (see Figure S2 in Supporting Information). The modification was realized by the electrochemical oxidation of amine groups of PEG by scanning the potential between 0 V and 1.1 V vs. Ag/AgCl for 5 cycles during which PEG was covalently anchored onto the PPy layer. The mechanism of such a reaction assumes formation of radical cations on nitrogen atoms, which covalently attach to nucleophilic groups such as aromatic rings resulting in the rupture of the double carbon-nitrogen bond and formation of carbon-nitrogen bonds.

From Figure 1 the high Rs of ca. 200 kΩ was obtained after electrochemical PEG deposition. For comparison, electrodes immersed in PEG solution with no electrochemical oxidation showed an insignificant change in impedance (see Figure S5 in Supporting Information); this further validates the electrodeposition approach. Such electrochemical functionalization has been demonstrated earlier in the case of attachment of aliphatic amines to surfaces such as glassy carbon or carbon nanotubes and recently on carbon nanotubes coated with PPy material.

The electrochemical grafting of PEG on PPy film was further characterized with FTIR for PEG adsorbed on the gold surface, PPy film, and finally modified PPy with PEG (Figure 2). From the FTIR spectrum of PEG, one can see characteristic peaks of primary amine bonds at 3566 cm⁻¹ and 1623 cm⁻¹, which can be also assigned to the −OH and C=O groups. For unmodified PPy, peaks at 1623 cm⁻¹, 1539 cm⁻¹ and 1441 cm⁻¹, corresponding to C-N and C=C vibrations, were observed. Modification of PPy by PEG led to appearance of -OH and -C=O vibrations at 3364 cm⁻¹ and 1709 cm⁻¹, respectively, confirming the presence of carboxylic groups on the surface and modification of polymer. The presence of the PEG’s alkyl chain is confirmed by the peak at 2934 cm⁻¹. Also a decrease in intensity of the C=C bond stretching was observed, which could be attributed to the attachment of PEG to double bonds present in the PPy structure, and confirming the mechanism of the reaction.

Association of biomolecules

In order to increase sensitivity of the biosensor, the PPy-PEG material was associated with an ANTA/Cu²⁺ complex as a redox marker. The formation of such a complex has been widely studied and is known to be very stable. First, ANTA was linked by its amine group to the activated carboxylic acid present on the free end of PEG, using EDC/NHS chemistry. This was followed by saturation of the surface with ethanaminol. Modification with ANTA caused a decrease in impedance of the layer and also a decrease in capacitance. Such a decrease can be attributed to stabilization and reorganization of the grafted PEG polymer. This was confirmed using cyclic voltammetry (CV) in the presence of a ferro/ferricyanide [Fe(CN)₆]³⁻/⁴⁻ redox couple, where an increase in redox signal was observed after binding of ANTA to the PEG surface (see Figure S6 and S7 in Supporting Information).

The complex between ANTA and a copper ion was formed by interaction of the metal in sodium acetate buffer, according to the procedure described by Chebil et al. The time used and the concentration of copper ions were crucial for this process and these were thoroughly optimized (see Supporting Information). Finally, histidine-tagged aptamers were immobilized on the surface by coordination of copper with two N-imidazole rings of the modified aptamers. During this process, bivalent metal ions such as Cu²⁺ bond to ANTA anchored on the surface by a tetravalent chelation, leaving two available coordination sites for the histidine modified aptamers. ANTA/Cu²⁺ form a stable complex and the binding of Cu²⁺ to the ANTA-modified self-assembled monolayer was studied by Stora et al., showing a dissociation constant of 5 nM obtained with impedimetric measurements.

Figure 3: SWV characterization of copper ions immobilization followed by immobilization of DNA aptamers measured in PBS pH 7.4 V vs. Ag/AgCl.
The formation of the biolayer was monitored using square wave voltammetry (SWV). Figure 3 shows a characteristic oxidation peak of copper at 0.15 V vs. Ag/AgCl after attachment on ANTA. This peak can be attributed to the Cu\(^{2+}\)/Cu\(^+\) reaction that occurs on the electrode surface. Copper peaks were also investigated by incubating the electrode at different steps of the layer formation, results from which are presented in the Supporting Information. The charge exchanged during the redox process allows calculation of the surface coverage of immobilized copper ions, following the equation:

\[ \Gamma = \frac{Q}{nFA} \tag{1} \]

Where \( Q \) is the charge under the cathodic or anodic peaks, \( n \) is number of electrons involved in the redox process, \( F \) is the Faraday constant, and \( A \) is the area of the electrode. Based on equation (1) we calculated the average coverage of the surface as 3.9 ± 0.4 pmol cm\(^{-2}\).

A reduction in the current corresponding to this peak was observed after attachment of His-tagged DNA aptamers. This could be related to a lower electron transfer or slower diffusion of electrolyte to the surface during the redox process due to the attachment of large molecules such as DNA. Such behavior was previously observed when PPy was associated with redox markers such as ferrocene\(^{25}\) or copper complex.\(^ {26b}\)

The biolayer formation was confirmed by SPR (see Supporting Information).

Anti-fouling properties of PPy-PEG-ANTA/Cu\(^{2+}\) complex

In order to evaluate the selectivity performance of the manufactured sensor, it was important to test its cross-reactivity and nonspecific binding with other prostate cancer biomarkers. The fabricated aptasensor showed a significant variation of oxidation current peak of Cu\(^{2+}\) when it was incubated with 10 nM AMACR in 10 mM PBS pH 7.4. The DNA aptamer being specific to AMACR meant that the sensor showed a decrease in copper peak signal of 37% of relative current changes measured at 0.15 V vs. Ag/AgCl (Figure 3). This decrease is due to the blocking effect of electron transfer after attachment of proteins.\(^ {26}\)

The sensor was then tested with other biomarkers for PCA such as 10 nM prostate specific antigen (PSA), 10 nM PSA-α2-antichymotrypsin (PSA-ACT) and 10 nM human kallikrein 2 (hK2). The aptasensor showed a more efficient selective performance where the signal change was less than 3% in the presence of these other proteins, when compared to PBS alone.

To demonstrate an improved anti-fouling chemistry, the aptasensor was tested with human serum albumin (HSA), which is the most abundant protein present in blood. For the same, the electrodes were dipped in a solution containing 4% w/v HSA in buffer and the biosensor response was recorded. The aptasensor demonstrated an excellent anti-fouling efficiency with a signal change of less than 4% (see Figure 4 inset).

In order to underline the anti-fouling effect of the PEG surface, we investigated another molecule such as β-alanine (H\(_2\)NCH\(_2\)CH\(_2\)COOH) in lieu of PEG. β-Alanine was deposited onto the PPy layer in the same way that PEG was deposited and an aptasensor was manufactured. This β-alanine-based aptasensor was incubated with 4% (w/v) HSA in buffer and the electrochemical signal change was monitored as before. From the Figure 4 inset, it can be seen a significant relative current variation of up to 25%, demonstrating a poor anti-fouling efficiency for this aptasensor. These results confirm that it is the presence of PEG that gives the sensor good anti-fouling properties.

Analytical performance of the biosensor

![Figure 5: Calibration curve in AMACR spiked human plasma samples using SWV delta current. The plot of the relative changes of the current peak vs. concentration of DNA target measured at 0.15 V. Relative current changes ΔI/I₀ where ΔI=(I–I₀), where I₀ is the peak current prior addition of the DNA target and I after incubation of the sensor with certain concentration of target. Data are means from 4 independent electrodes ± standard deviation.](image)

![Figure 4: Selectivity study with other biomarkers for prostate cancer. Inset depicting the efficacy of PEG surface over β-alanine surface. Relative current changes ΔI/I₀, where ΔI=(I–I₀), where I₀ is the peak current prior addition of the DNA target and I after incubation of the sensor with certain concentration of target. Data are means from 4 independent electrodes ± standard deviation.](image)
The formation of the biolayer as well as detection of target were also successfully monitored using SWV by measuring the Cu⁴⁺/Cu⁺ redox signal variation at 0.15 V vs. Ag/AgCl. The aptasensor demonstrated good reproducibility, which was characterized by monitoring the initial copper peaks obtained from each sample. A mean current peak of 4.27 ± 0.26 µA was obtained using 4 independent samples. The PEG aptasensor was initially tested with a wide range of AMACR concentrations from 1 fM to 10 nM in 10 mM PBS pH 7.4, where a decrease in redox signal was observed upon increasing AMACR concentration. The aptasensor demonstrated a sensitive response (Figure S9 in Supporting Information). The sensor could discriminate AMACR concentrations down to 5 fM. A limit of detection (LOD) of 0.15 fM was calculated using 5 independent samples as explained by Armbruster et al. The system was further validated using surface plasmon resonance (SPR) technique, where the chip was fabricated with a similar surface chemistry and a dose response curve was obtained with detection down to 1 nM (see Figure S10c).

The aptasensor was further tested with human plasma samples. The electrodes were initially stabilized in human plasma without AMACR protein. Upon stabilization, spiked human plasma samples supplemented with AMACR were tested. The recorded signal after stabilization was used as a reference to calculate relative changes of current during detection of protein. Again a wide range of AMACR concentrations was tested from 1 fM to 10 nM.

The dose response curve is shown in Figure 5. A reduction in signal change was observed when compared with measurements in buffer, which can be attributed to the blocking effect of the histidine residues present in human plasma proteins. From Figure 5, a redox signal variation of 2.85 ± 0.39% was obtained for 5 fM of AMACR concentration with a saturation at 10 nM corresponding to signal changes of 17.92 ± 1.2%. The electrochemical signal variations expressed in percentage were also compared with the absolute signal change (ΔI). The absolute signal change (ΔI) was obtained for each sample and a calibration curve was obtained using different concentrations of AMACR (Figure S12 in Supplementary Information). A current decrease of 0.14 ± 0.05 µA was observed with 5 fM of AMACR and a maximum response was obtained with 10 nM of AMACR concentration with a variation of 0.84 ± 0.09 µA. Nevertheless, the sensor demonstrated a good response down to concentrations of 5 fM with a LOD of 1.4 fM. The dose response curve exhibits a linear response between fM and 1 nM. The inset of Figure 5 shows the SWV curves obtained with different concentrations of AMACR in human plasma. The sensor starts saturating at around 10 nM. These measurements were highly reproducible with relative standard deviations of 0.3-1.2% for 5 independent repeats. The efficiency of the sensor could be further improved by replacing copper ions with ferrocene as demonstrated in literature.

CONCLUSIONS

We present a simple method of manufacturing a highly sensitive electrochemical aptasensor for detection of AMACR. We demonstrate that by electro-engineering PEG on the surface of a PPy film, anti-fouling properties can be significantly enhanced without loss of sensitivity. The modified PPy film with PEG-NTA/Cu⁺⁺-Aptamer was successfully used to detect AMACR both in spiked buffer and human plasma samples. A low sensitivity value of 5 fM was attained with a LOD of 0.15 fM in buffer and 1.4 fM in human plasma, respectively. The sensor also demonstrated good selectivity when challenged with other prostate cancer biomarkers. This surface chemistry approach can be extended to the detection of a wide range of biomarkers by using suitable aptamers.

EXPERIMENTAL SECTION

Materials and Reagents

Histidine tagged AMACR-specific DNA aptamers [5′(His)₅TTTTTTCTACTCCGCGCTAACCCTGACGAAAT CGTTGTTAACAATAGGCCACCGTGCTACAAAT] were obtained from Eurogentec, U.K. Prostate specific antigen (PSA) from human semen was obtained from Fitzgerald (U.S.A.). Human glandular kallikrein 2 (hK2) was obtained from R&D Systems, U.K. Complexed prostate specific antigen (PSA-CT) was obtained from Lee Biosolutions, U.S.A. Phosphate buffered saline (PBS) tablets, pyrrole, ethanolamine, human serum albumin (HSA), lithium perchlorate, copper acetate, Na₂N₂-Bis(carboxymethyl)-L-lysine hydrate (ANTA), sodium acetate, poly(ethylene glycol) 2-ami... 

Apparatus

The electrochemical measurements were performed using a µAUTOLAB III / FRA2 potentiostat (MetrohmAutolab, The Netherlands) using a three-electrode cell setup with a Ag/AgCl reference electrode (BASI, U.S.A.) and a Pt counter electrode (ALS, Japan). The impedance spectrum was measured in 10 mM PBS measurement buffer (pH 7.4) with a 10 mV a.c. voltage superimposed on a bias d.c. voltage of 0.17 V vs. Ag/AgCl. Cyclic voltammetry was performed in 10 mM PBS (pH 7.4) in the range from -0.2 V to 0.35 V without redox couple and -0.4 V to 0.45 V in 10 mM PBS containing 10 mM ferrocenium [Fe(CN)₆]³⁺/. Square wave voltammetry was performed in 10 mM PBS (pH 7.4) in the potential range from -0.3 V to 0.4 V with a conditioning time of 120 s, modulation amplitude of 20 mV and frequency of 50 Hz.

Gold disk working electrodes with a radius of 1.0 mm (CH Instruments, U.S.A.) were first cleaned by mechanical polishing for 5 min with 50 nm alumina slurry (Buehler, U.K.) on a polishing pad (Buehler, U.K.) followed by 5 min sonication in ethanol and then in water. The electrodes were then subjected to chemical cleaning with piranha solution (3 parts of concentrated H₂SO₄ with 1 part of H₂O₂ for 5 min). The electrodes were then rinsed with Milli-Q water. Thereafter, electrodes were electrochemically cleaned in 0.5 M H₂SO₄ by scanning the potential between the oxidation and reduction of gold, -0.05 V and +1.1 V versus an Hg/Hg₂SO₄ reference electrode, for 50 cycles until no further changes in the volt-
Electro-chemical deposition of PEG

The polypyrrole film (PPy) was grown on the gold surface by cycling the potential from -0.4 V to 0.9 V vs. Ag/AgCl, with a scan rate of 100 mV.s⁻¹ for 2 cycles in Milli-Q water containing 0.5 M LiClO₄. Electro-polymerization was performed using a three-electrode configuration in a small volume cell (BAsi, USA) containing 250 µL of 50 mM pyrrole monomer. After reaction the electrode was rinsed with Milli-Q water.

PEG was immobilized on the PPy film by covalent bonding via electro-oxidation of the amine groups of PEG. The procedure was again performed in the small volume cell containing 250 µL of 1 mM PEG dissolved in Milli-Q water containing 0.5 M LiClO₄ by cycling the potential from 0.0 V to 1.1 V vs. Ag/AgCl during 5 cycles with a scan rate of 50 mV.s⁻¹.

Immmobilization of ANTA/Cu²⁺ complex and His-tagged aptamers

Immmobilization of ANTA/Cu²⁺ complex and the His-tagged aptamers was performed using the protocol reported by Chebell et al. The carboxylic acid groups on the free end of the deposited PEG was used to covalently attach N-(5-Amino-1-carboxypentyl)iminodiacetic acid (ANTA). First, electrodes were subjected to a 2:1 ratio of EDC/NHS (160 mM/80 mM) in water for 20 minutes. Electrodes were then rinsed with Milli-Q water and immersed in 7 mM ANTA dissolved in 10 mM PBS (pH 8.5) for 30 minutes. The electrodes were then rinsed with Milli-Q water and the unreacted activated carboxylate groups were blocked with 10 mM ethanolamine (pH 8.5) for 30 minutes. Finally the electrodes were immersed in a solution of 3.5 mM copper acetate dissolved in 20 mM acetate buffer (NaOAc/HCl, pH 4.6) for 25 minutes. During the process, the copper molecules associated with the ANTA attached to the surface via coordination chemistry. Anti-AMACR aptamers (0.5 µM in 10 mM PBS, pH 7.4) were preheated at 95°C for 5 min and gradually cooled down to room temperature over 25 min. Activated His-tagged anti-AMACR aptamers were immobilized to the copper complexes via coordination of two of the N-imidazole rings of the His-tag with copper.

Detection of AMACR

Detection of AMACR with DNA aptamers immobilized on the surface was achieved by incubating the aptasensor in the solution containing AMACR for 30 min at ambient room temperature. A wide range of AMACR concentrations were used: 1, 10, 100 fM; 1, 10, 100 pM; 1, 10 nM in 10 mM PBS buffer, pH 7.4 and 1 to 10 diluted human plasma (diluted in 10 mM PBS, pH 7.4) spiked samples. The concentration of AMACR stock solutions was determined by UV-visible spectroscopy.

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