Flexible multielectrode arrays based electrochemical aptasensor for glycated human serum albumin detection

Lei Zhou [a,b], Gabriela Figueroa-Miranda [a], Song Chen [a], Marc Neis [a], Ziheng Hu [a,b], Ruifeng Zhu [a,b], Yaqi Li[a], Michael Prömpers [a], Andreas Offenhäusser [a], Dirk Mayer [a,*]

a *Institute of Biological Information Processing, Bioelectronics (IBI-3), Forschungszentrum Jülich GmbH, Jülich, Germany*
b *RWTH Aachen University, Aachen, Germany*

*Corresponding author: Dirk Mayer

Address: Institute of Biological Information Processing, Bioelectronics (IBI-3), Forschungszentrum Jülich GmbH, Wilhelm-Johnen-Straße 52428, Jülich, Germany

Email: dirk.mayer@fz-juelich.de

Tel.: +49-2461-61-4023

Abstract

Assessment tests for glycemic control of patients with diabetes mellitus commonly use blood glucose and glycated hemoglobin A1C (HbA_{1C}) levels for estimating acute and long-term glycemia, respectively. Diabetes diet, as well as treatment, are adapted accordingly and also the risk of diabetic complications can be estimated. Glycated human serum albumin (GHSA) has been proposed as an alternative to HbA1C since it possesses a four times shorter lifespan and its blood concentration is not affected by hemic diseases. Here, an electrochemical aptasensor is proposed for the simultaneous detection of human serum albumin (HSA) and GHSA utilizing flexible multielectrode arrays. The polymer chip carried two individual sets of gold electrodes, which are separately modified by the corresponding aptamers. The dual-target aptasensors possessed detection limits as low as 13 nM and 25 nM for HSA and GHSA, respectively, together with detection ranges lasting from 40 nM to 10 µM. Moreover, our aptasensor quantified selectively its target in diluted full-blood samples. This low-cost plastic aptasensor facilitates the quantitative determination of HSA and GHSA ratios by analyzing the current signals from several redundant electrodes, which permits a reliable and affordable estimation of long-term glycemia at point-of-care or home.

Keywords: Glycemic control; Flexible multielectrode arrays; Dual-target analysis; Glycated albumin; Electrochemical aptasensor; Blood samples

1. Introduction

Diabetes mellitus, a chronic metabolic disorder of the endocrine system induced by insulin deficiency or/and insulin resistance is characterized under untreated conditions by hyperglycemia as a typical diagnostic criterion [1]. This disease has numerous sequela (pathological conditions) and has been regarded as one of the leading causes of mortality worldwide [2]. Diabetes occurs globally in 537 million people by 2021 and is projected to influence 643 million and 784 million people by 2030 and 2045, respectively [3]. It is estimated that half of the patients with diabetes are even undiagnosed until complications appear [3]. These complications cover macrovascular diseases leading to cardiovascular disease, stroke, and diabetic foot while microvascular diseases causing ocular diseases, nephropathy, and neuropathy. Even the risk of Alzheimer's disease is linked with type 2 diabetes [4–6].

Diabetic complications can be attributed to multiple reasons, among which advanced glycation process through the Maillard reaction plays an important role. The nonenzymatic glycation of proteins due to hyperglycemia involves the formation of reversible early intermediate Schiff bases which can further lead to Amadori products. Following these early stage intermediates formation, further intricate modifications like oxidation, rearrangements, and polymerization lead to irreversible advanced glycation endproducts (AGEs) [7,8]. Intracellular protein modification by AGEs will change cellular functions and extracellular AGEs induce irregular protein cross-linking which may alter the structure and function of a tissue. Besides, circulating AGEs tend to bind to certain receptors of AGEs (RAGEs), which results in enhanced cellular oxidative stress and the release of prosclerotic and proinflammatory cytokines [9–11]. Since a high level of AGEs is correlated with manifold complications and health threats for diabetes patients, glucose level management is fundamental to avoid diabetic secondary diseases [12,13]. The actual blood sugar level is routinely monitored by well-established glucose sensor systems while glycated hemoglobin A_{1C} (HbA_{1C}) can provide, besides an assessment test for glycemic control, also an estimation of the protein glycation. This measure reflects the ratio of the glycated hemoglobin to the gross hemoglobin and provides an estimation of the average blood sugar level during 3 months (lifespan of an erythrocyte) [14,15]. This long period results from the relatively long lifespan of erythrocytes, which is not beneficial to short-term diabetes control, effective prevention of complications, and timely intervention [16]. Besides, several hemoglobin-affecting factors can have an impact on its glycation process, including but not limited to transfusions, anemia, hemolysis, and hemoglobinopathies [17,18]. These factors affect HbA_{1C} levels in different ways and impair the reliability of this biomarker for glycemia. Therefore, it is beneficial to monitor other biomarkers independent of erythrocytes and with a shorter lifespan for diabetes surveillance [1,16,19]. The most abundant plasma protein albumin (HSA) possesses an average lifespan of 3 weeks and has been therefore proposed as an alternative to gain access to the mean blood glucose level for more prompt

intervention strategies [16,19]. Moreover, HSA can interact and transport certain metabolites like bilirubin, metal ions, fatty acids, and drugs. However, those functions can be interfered when the amino groups of lysine residues of HSA are nonenzymatically glycated by high-level blood sugar forming GHSA. In a nutshell, GHSA is not influenced by erythrocyte circulation and exhibits less alteration than direct detection of blood glucose concentration, thus it can be recognized as an alternative biomarker to HbA_{1C} for glycemic control. In analogy to HbA_{1C}, GHSA can be presented by the concentration percentage of GHSA to total HSA. GHSA proportion for healthy individuals ranges from 11 - 16 %, while this value may rise up to 5 times higher for diabetic people. Thus the elevated GHSA level can be applied as an alternative strategy for diabetes monitoring [15,20,21].

Several strategies have been developed for GHSA detection, such as SERS (surface-enhanced Raman scattering), enzymatic sensor, ECL (electrochemiluminescence), and colorimetric sensor [19,22–24]. However, these strategies are commonly time-consuming, labor-intensive, and low performance such as lacking relevant sensitivity and selectivity, thus it is still necessary to establish easy and sensitive means for the selective detection of GHSA. Electrochemical sensors not only possess merits such as fast response, easy operation, cost efficiency, and high sensitivity to fulfill point-of-care diagnosis of certain analytes but also feature considerable miniaturization potentiality for reducing fabrication costs and enabling multi-target detection, all beneficial for chronic disease surveillance [25,26]. For instance, Y. Inoue *et al.* have established a sensing platform for GHSA detection by combining enzymatic and ECL methods [19]. S. E. Son *et al.* proposed a sensor employing 3,5,3',5'-tetramethylbenzidine (TMB) as an electrochemical and colorimetric indicator. APBA (3-aminophenyl boronic acid) modified Prussian blue nanoparticles (PBBA) were involved in a sandwich structure formation facilitating GHSA quantification [24]. Although these electrochemical methods have greatly improved the GHSA detection performance, they are still challenged by the co-occurrence of the GHSA biomarker with other proteins in real samples. Furthermore, the ultrahigh clinical GHSA concentration requires a larger dynamic detection range to permit a versatile biomarker determination.

Recently, aptamer sensors (also aptasensors) have emerged as versatile, robust, and affordable sensor platforms with high target affinity and selectivity for the detection of a huge variety of different analytes, ranging from small neurotransmitters, toxins, oligopeptides, and protein biomarkers, to entire cells [27–31]. As a single-stranded oligonucleotide sequence, which normally contains 15 to 60 nucleotides, aptamers can selectively bind their corresponding targets with high affinity. These synthetic ssDNA molecules have some particular advantages over receptors of biological nature such as versatile chemical modification, easy synthesis/regeneration, high fabrication reproducibility, and considerable thermal stability [32]. Consequently, aptamer receptors have been recently generated also for HSA and GHSA by SELEX (systematic evolution of ligands by exponential enrichment) [21,33]. These aptamers have been utilized to

fabricate HSA sensors but all of them rely on the separate detection of HSA and its glycated analogue. Here, we introduce flexible, polymer-based multielectrode arrays (flex–MEAs) for the combined detection of both isoforms simultaneously.

Generally, multielectrode array sensors combine the capability of multi-target detection with the advantages of increased mass transport rate, redundant signal recording, low device capacitance, and reduced current noise in comparison to conventional macro electrodes [34,35]. Besides, the polymer-based substrate MEAs not only exhibits good cost efficiency, considerable biocompatibility, dielectric strength, insulation resistance, and huge mechanical flexibility but also allows the integration of several sets of individually addressable microelectrodes to be modified by different receptors separately, which is beneficial to eliminate cross-contamination in assembly procedure fundamentally [36,37].

In this work, a multi-target aptamer sensor was established for glycemia monitoring based on the proposed flex–MEAs, which allows the immobilization of aptamers for albumin and glycated albumin on the same chip but on separate and individually addressable electrodes. By this means, our sensor facilitates the simultaneous and quantitative determination of albumin and GHSA from the same blood sample and permits an easy point-of-care assessment test for glycemic control.

2. Experiment section

2.1 Chemicals and apparatus

The diabetes biomarker-specific oligonucleotide aptamers employed in this study were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The malaria 2008s ssDNA aptamers were synthesized on demand by Friz Biochem GmbH (Neuried, Germany). Tris(hydroxy-methyl)-aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), sodium chloride, potassium chloride, magnesium chloride, sodium phosphate dibasic, sodium phosphate monobasic, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol (MCH), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$), hydrochloric acid, sulfuric acid, sodium hydroxide, hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$), GHSA were all purchased from Sigma-Aldrich Chemie GmbH (Germany) and used without further purification. Monofunctional methoxy-polyethylene glycol thiol (PEG, MW 2k) was produced by Creative PEGWorks (Chapel Hill, USA). Alumina micropolish powders (0.3 and 0.05 µm) were purchased from Buehler (Germany). The gold rod electrodes (2 mm diameter) were provided by Gaossunion (Wuhan, China), and Ag/AgCl electrode Dri-Ref-2 was ordered from WPI (Germany). Acetone, isopropanol, and ethanol were acquired from Merck (Darmstadt, Germany). HSA was purchased from

Millipore Merck Chemicals GmbH (Darmstadt, Germany). All these chemical reagents are of analytical grade. Ultra-pure deionized water (18.2 MΩ cm, Milli-Q, Millipore Merck, Darmstadt, Germany) was used throughout the experiments including solutions preparation. High salt phosphate buffer solution (10 mM PBS with 1 M NaCl, 1 mM $MgCl_2$, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4, pH 7.2) was prepared for aptamer assembly, low salt phosphate buffer solution (10 mM PBS with 100 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM NaH_2PO_4, pH 7.4) was prepared for electrochemical measurement, and 10 mM Tris-HCl buffer (100 mM NaCl, 10 mM Tris, 1mM EDTA, pH 8) was used for stocking of aptamers.

HSA aptamer sequence:

5'-ferrocene-$(CH_2)_6$-GTC TCA GCT ACC TTA CCG TAT GTG GCC CAA AGC GTC TGG ATG GCT ATG AA-$(CH_2)_6$-S-S-$(CH_2)_6$-OH-3',

GHSA aptamer sequence:

5'-ferrocene-$(CH_2)_6$-TGC GGT TCG TGC GGT TGT AGT ACT CGT GGC CGA T-$(CH_2)_6$-S-S-$(CH_2)_6$-OH-3'.

Specificity test using malaria 2008s DNA aptamer sequence 5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-CTG GGC GGT AGA ACC ATA GTG ACC CAG CCG TCT AC-3′

2.2 Gold rod electrode and flex–MEA cleaning

Prior to aptamer immobilization, 2 mm diameter gold rod electrodes were sequentially polished using alumina powder slurry with diameters of 0.3 and 0.05 μm, each for 5 min with microcloth polishing pads from BASi Research Products (West Lafayette, USA). Afterwards, the polished electrode was sonicated consecutively in ethanol, isopropanol, and Milli-Q water each for 5 min. After sufficiently purging with argon gas, the electrode was electrochemically cleaned by measuring cyclic voltammograms (CV) in 0.5 M NaOH (scan range from −1.35 to -0.35 V, 500 scans at a scan rate of 2 V s^{-1}) and subsequently 0.5 M H_2SO_4 (scan range from −0.15 V to 1.55 V, 100 scans at a scan rate of 1 V s^{-1}) using a PGSTAT302 potentiostat/galvanostat (Metrohm Autolab, Netherlands). The electro-active surface area of the electrode was determined by electrochemical treatment in 0.5 M fresh prepared H_2SO_4 from −0.15 V to 1.55 V at a scan rate of 1 V s^{-1} until stable CV was recorded [38]. Before further modification, the electrodes were rinsed with Milli-Q water and dried with argon gas.

The fabrication of the flex–MEAs is described elsewhere [37]. For cleaning, the flex–MEAs were sequentially soaked in acetone, isopropanol, and ethanol each for 5 min and rinsed sufficiently with Milli-Q water. After drying with argon gas, it was loaded in the electronic connector and electrochemically

cleaned by recording CVs in 0.5 M NaOH (scan range from −1.35 to -0.35 V, 10 scans at a scan rate of 2 V s^{-1}) and sequentially 0.05 M H_2SO_4 (scan range from −0.35 to 1.5 V, 20 scans at a scan rate of 1 V s^{-1}) with the multi-potentiostat CHI1030B (Austin, USA). Finally, the flex–MEAs were rinsed with Milli-Q water and dried with argon gas for further usage.

2.3 Gold rod electrode and flex-MEAs-based aptasensors preparation

The concentration of aptamers was determined by recording the absorbance at 260 nm with the Perkin Elmer Lambda 900 UV/Vis/NIR spectrometer (USA). The aptamers containing stock solutions were always pretreated by 10 mM TCEP for 1 h to break the disulfide-protecting bonds and diluted to a certain concentration with high salt PBS, which can facilitate aptamer assembly by reducing the DNA electrostatic repulsions. Afterwards, the cleaned gold rod electrodes were immersed in 250 µL of aptamer solutions of various concentrations for 1 h. Then, it was carefully rinsed with low salt PBS and sequentially with Milli-Q water to eliminate non-specifically adsorbed components. After drying with argon gas, the electrode was soaked in 250 µL 1 mM MCH dissolved in ethanol solution for 1 h and carefully rinsed with ethanol, low salt PBS, and Milli-Q water consecutively to remove excess adsorbed MCH. MCH served as both blocking molecule and spacer materials, which can prevent the appearance of pinholes and leaves enough space for proteins binding to aptamer receptors on the gold surface. Both, the thiolated aptamers and MCH self-assemble at the gold surface via the Au-S bond and form a mixed monolayer [39]. When PEG served as backfills, the same procedures as above were used until the aptamer incubation, then electrodes were soaked in 10 mg/mL PEG dissolved in Milli-Q water for 1 h and carefully rinsed with low salt PBS and Milli-Q water afterwards.

In the case of the flex–MEAs chips, those were partially cut into two sections using scissors to generate two sets of electrodes that can be bent individually without fully splitting the electrode array (Scheme 1). Afterwards, the chips were cleaned as mentioned above. Subsequently, the two sets of electrodes of the flex–MEA chips were bent in opposite directions and inserted in separate vials containing TCEP-treated 300 µL of HSA aptamer (20 nM) and 300 µL of GHSA aptamer (20 nM) solutions, respectively, for 1 h. Then the chips were carefully rinsed with low-salt PBS and Milli-Q water. The separate assembly process assured that each of the two electrode sets was incubated only with one type of aptamer receptor and that cross-interferences are avoided. Then, the aptamer-modified flex–MEAs were soaked in backfill solutions (MCH-dissolved ethanol or PEG-dissolved Milli-Q water) for 1 h. Subsequently, the as-prepared aptasensor was rinsed as previously mentioned for the rod electrode.

2.4 AFM investigations

Atomic force microscopy (AFM) was utilized to characterize the variation of the Au surface morphology during electrode modification by means of a Nanoscope Multimode 8 microscope (Bruker, Netherlands). The images were recorded by employing a piezoelectric E-series scanner and aluminum-coated Si cantilever Bruker (RTESPA-3, resonant frequency of 300 kHz). Tapping mode imaging was applied for data acquisition with the following parameters: scan rate 1 Hz, scan size 0.3 to 1 μm, and aspect ratio 1:1. A Au(111) single crystal disk was used for these experiments to maintain atomically flat surfaces and to access morphology changes on a molecular level. The single crystal was cleaned by sequential rinsing with ethanol, isopropanol, and Milli-Q water. Subsequently, the sample was annealed for 10 min in a hydrogen flame and after cooling to room temperature subjected to surface modification treatments like that of the rod electrodes, section 2.3.

2.5 Electrochemical detection of HSA and GHSA

Differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) were both utilized for investigating the sensor preparation and the analyte detection. For EIS measurement, the potentiostat/galvanostat PGSTAT302 was employed in combination with a three-electrode system: the modified gold electrode was used as the working electrode, a platinum wire was served as the counter electrode, and an Ag/AgCl electrode was utilized as a reference electrode. All the EIS tests were recorded by applying a sinusoidal voltage at 0.22 V and an amplitude of 0.01 V covering a frequency ranging from 0.1 Hz to 10 kHz. The software NOVA 2.1 including a frequency response analyzer (FRA) was applied for EIS data fitting based on the Randles equivalent circuit to collect the fitted module values. For DPV tests, multi-potentiostat CHI1030B was utilized and combined with the same three-electrode system. All DPV tests were recorded from -0.2 V to 0.7 V with a modulation amplitude of 0.05 V and a potential step of 0.004 V. Before EIS and DPV measurement, the aptasensors were incubated in a certain concentration of HSA or GHSA contained low salt PBS with the optimized incubation time, then recorded in low salt PBS containing 5 mM $[Fe(CN)_6]^{3-/4-}$. Before DPV tests, the flex–MEAs sensors were heated to 80 °C and cooled to approximately 0 °C through Milli-Q water of the corresponding temperatures to denature the aptamer receptors so that cross-target absorption interference can be eliminated. Directly afterwards, the sensor chips were incubated with HSA and GHSA mixed in low salt PBS or 100 times diluted blood for real sample detection.

2.6 Aptamer density determination

For the determination of the surface density of the aptamer receptors, 250 µL of either 20 nM HSA or 20 nM GHSA aptamer and 1 mM MCH were stepwise applied to the gold rod electrode for 1 h separately. Then the electrolyte cell was filled with 10 mL of 10 mM Tris buffer and the three-electrode system was installed. The buffer was purged with argon gas in the well-sealed cell for 10 min, then a chronocoulometric (CC) measurement was performed every 2 minutes until stable signal was obtained. After this procedure, 50 µL 10 mM RuHex was mixed into the 10 mL of 10 mM Tris buffer forming a 50 µM RuHex / 10 mM Tris buffer mixture. The resultant buffer was also purged for 10 min with argon gas and followed with another 10 min waiting to leave the system enough time for RuHex diffusion to the gold working electrode. Then the CC test was conducted every 2 min to reach a stable value. The following parameters were applied for CC tests: 0.2 V as initial potential, -0.5 V as final potential, 2 as the number of steps, 0.25 s as pulse width, and 0.002 as sample interval.

The RuHex charge (Q_{total}) was acquired at the intercept when t equals 0 by plotting the charge (Q) versus $t^{1/2}$, while the Q_{total} value represents the whole charge measured at the gold electrode including not only Faradaic charges (redox charge) but also non-Faradaic charges (capacitive charge). The charge of the double layer (Q_{dl}, also called capacitive charge) was separately obtained at the intercept of the aptamer-free surface at t = 0. The charge of the ssDNA (HSA/ GHSA aptamer) electrostatically adsorbed RuHex (Q_{ss}) was calculated according to: $Q_{ss} = Q_{total} - Q_{dl}$.

The aptamer surface density (Γ_{ss}) was calculated with the following equation: $\Gamma_{ss} = (Q_{ss}N_A / nFA)\ (z / m)$, where N_A represents Avogadro's number, n equals the electron's number of the reaction, A displays the working electrode area, z indicates the charge on redox molecules, m represents the nucleotide number of the aptamer.

3. Results and discussion

3.1. Characterization of receptor layer

<Figure 1>

The different steps of the sensor preparation were evaluated by measuring the impedance change induced by the adsorption of the respective molecules on the electrode by means of EIS measurement. Therefore, 5 mM ferro/ferricyanide was utilized as a redox probe dissolved in 10 mM PBS. A Randles equivalent circuit was employed to fit the experimental impedance data. R_S and R_{CT} denote the solution and

charge transfer resistance, respectively. *W* stands for the Warburg impedance describing the contribution of the diffusion of the redox probes to and away from the electrode to the interface impedance. The constant phase element CPE is used instead of a simple capacitor to better accommodate the imperfect surface morphology of the gold rod electrode.

The stepwise assembly of the receptor layer led to a gradual increase in the charge transfer resistance, indicating the adsorption of the respective molecules (Fig. 1). The bare electrodes showed R_{CT} of several tens of Ohm, corresponding to a charge transfer between ferro/ferricyanide and gold without molecule blocking. After the immobilization of the aptamer (both HSA and GHSA) R_{CT} increased by several hundreds of Ohms due to the electrostatic repulsion between the negatively charged phosphate groups of the ssDNA and the redox probe. The incubation of albumin on aptamer/MCH/Au electrodes caused a significant increase in R_{CT} which can be attributed to the binding of albumin to the aptamer receptors and a corresponding blocking of the charge transfer. The isoelectric point of albumin is 4.4, thus, lower than the pH value of PBS (7.4), which means a net negative charge of the protein. The corresponding net charge of albumin is reported as -23 / molecule, which explains the R_{CT} increase by simultaneous steric blocking and electrostatic repulsion between negatively charged albumin and redox probes [40]. To evaluate the unspecific adsorption of albumin to MCH, gold rod electrodes modified only with MCH were exposed to HSA and GHSA solutions, Fig. S1. In both cases, the MCH/Au electrodes exhibited ignorable EIS and DPV signal changes, confirming the prevention of unspecific adsorption of albumin and GHSA to MCH proposing that MCH can be utilized as backfill molecules for optimizing the receptor immobilization conditions.

The electrochemical characterization of the SAM formation was complemented by the investigation of the surface morphology change employing AFM analysis during the different steps of surface modification (Fig. 1C). At first, the surface of an atomically flat gold (111) single crystal was imaged as a reference. The topography images exhibit minor fluctuations with amplitudes smaller than the height of a single atom step edge confirming the ultra-smooth surface morphology of this model gold surface with an RMS surface roughness of 0.07±0.01 nm, Fig 1C 1. The immobilization of the aptamer molecule led to the formation of irregular islands with heights of 0.5 nm – 0.8 nm and 0.2 – 0.3 nm for HSA and GHSA aptamers, respectively. Besides, defect structures such as mono-atomically deep holes can be observed for both aptamers on the Au terraces which are characteristic of thiol-based SAM formation [41]. The presence of islands (big and small heights indicating lying molecules), the low aptamer incubation concentration, the low aptamer density (see below), and the fact that features of the gold surface are still clearly visible suggest that a patchy sub-monolayer was formed. The addition of MCH changed the surface morphology considerably characterized by an increase in the number of islands but with smaller size and larger height

than for samples treated with only aptamers. Apparently, the MCH molecules increase the distribution of aptamer molecules on the sample and disrupt some of the weak interactions of the ssDNA strand with the gold surface so that the aptamers stand more upright. The addition of the albumin targets caused a further increase in surface roughness and object heights (1.7 nm – 2.5 nm) while the island distribution remained unaltered, indicating the immobilization of the analyte molecules to the receptor layer. Noteworthy, there is not much difference between the AFM results obtained for HSA and GHSA sensor samples. Actually, the AFM data observed here are well comparable with other aptamer systems for malaria and Alzheimer's disease biomarker previously recorded by us [39,42]. This finding suggests that thiol-tethered aptamer molecules of comparable length possess similar SAM formation characteristics relatively independent from the ssDNA sequence.

The appropriate incubation concentration of aptamers is critical for the performance of the proposed sensing platform on the one hand to maintain a high receptor density and thus large sensor signals and on the other hand to avoid steric hindrance between adjacent receptors during target binding [43]. To obtain the optimum sensing capability of the designed albumin and GHSA aptasensors, the incubation concentrations were optimized for both aptamers by using EIS measurements. Therefore, gold rod electrodes were incubated either with 250 µL of albumin or glycated albumin aptamer at concentrations of 1 pM to 150 nM for 1 h, followed by 1 h incubation in 1 mM MCH. Then each of the aptasensors was exposed to albumin or glycated albumin. EIS measurements of these aptasensors were conducted before and after target incubation, separately. At first, an increase of the R_{CT} signal was observed for rising albumin aptamer concentrations, Fig. S2 A. The signal gain value was calculated as the ratio between signal increase ΔR_{CT} and background signal R_{CT0}. The signal gain enhances gradually along with albumin aptamer concentrations from 1 pM to 20 nM due to the increasing number of aptamer receptors at the gold electrode interface, giving rise to a higher probability for albumin target binding. However, when the aptamer concentration exceeded 20 nM, the signal gain declined asymptotically. This decrease can be ascribed to a steric hindrance effect, which hampers efficacious aptamer folding processes around the albumin target at a larger aptamer loading amount. Thus the analyte binding capability of the receptor layer decreases and the sensor will not exhibit a supreme response. A similar result was obtained for glycated albumin, Fig. S2 C. For both variants of albumin, a 20 nM aptamer concentration facilitated the optimum receptor loading and was employed subsequently for all aptasensor experiments.

Chronocoulometry (CC) was utilized to determine the aptamer densities obtained for the optimal incubation concentration of the respective aptamers [44]. The thiolated aptamers self-assembled at the gold surface via Au-S bond formation, while $[Ru(NH_3)_6]^{3+}$ (RuHex) was added to the system as a redox probe which can "electrochemically stain" the thiolated self-assembled aptamers at the gold surface. The latter

binds stoichiometrically to the anionic phosphates of the ssDNA aptamer molecules through electrostatic interactions. The total redox charge transferred during a potential step is directly correlated to the number of aptamers loaded on the gold electrode surface. As depicted in Fig. S2 B, the surface density for 20 nM HSA aptamer was determined as 2.71×10^{12} molecules/cm^2. The corresponding surface density for 20 nM GHSA aptamer was estimated to be 4.77×10^{12} molecules/cm^2, Fig. S2 D. These low densities indicate that the target binding requires considerable interface space for the binding of HSA as well as GHSA. The corresponding CV measurements before and after RuHex addition further confirms the RuHex-ssDNA binding with very similar redox peak areas for both albumin targets, reflecting almost equal aptamer densities, Fig. S3.

The optimization of the target incubation time was done again by EIS measurements. The EIS signal gain of the albumin aptasensors was recorded at target incubation times lasting from 1 to 45 min, Fig. S4. For HSA, the signal gain exhibited a rising trend until a saturation was reached after 10 min, Fig. S4 A. Likewise, the EIS signal gains of the GHSA aptasensors rose for increasing target incubation times until a saturation was observed after 30 min, Fig. S4 B. Consequently, 10 min and 30 min were utilized as incubation times for HSA and GHSA, respectively, in all following aptasensor experiments. The higher packing density and longer binding time of GHSA in comparison to HSA indicate a different binding mechanism for these two related targets and a considerable impact of the glycation on the DNA albumin interaction [45].

<Figure 2>

Since a competitive detection of both albumin and glycated albumin is intended, a cross-selectivity test of the respective aptamers is crucial for the reliable detection of the ratio of these two analogue targets. First tests indicated that there was a certain binding of albumin to the GHSA aptamer, if the experiment was performed at room temperature, Fig. 2 A. Therefore, the DPV signals of glycated albumin aptasensor were recorded before and after sequentially incubation with 1 nM, 1 µM, 100 µM HSA and 10 µM GHSA each for 30 min. The DPV signals indicated that glycated albumin aptamer can adsorb albumin, later we also found that the HSA aptamer partially binds GHSA. To overcome this cross-interference, heating and cooling treatments were applied before every target incubation step [21]. As was reported before, the aptamer affinity is highly related to temperature, especially for aptamers with secondary structure [46], correspondingly, non-specific binding can be reduced by high-temperature treatment [21,47]. Therefore, we exposed the electrodes incubated with GHSA aptamer to 60 °C 10 mM PBS buffer for 5 min to melt intra- and inter-ssDNA interactions followed by a quenching step in ice water for 1min. Our data suggest that this temperature treatment can diminish the adsorption of albumin to the GHSA aptamer as the

administration of 100 µM albumin caused only a marginal signal change while 10 µM GHSA induced a considerable current drop, Fig. 2 B. Similarly, a comparable treatment was used for the HSA aptasensor applying an 80 °C buffer for 5 min and cooling in ice water for 1min, Fig. 2 C. The higher temperature was required since the 60 °C treatment was not sufficient to eliminate all cross-selectivity for the HSA aptasensor. These heat treatments significantly diminish unspecific interactions between the aptamers and the interfering analog targets improving the corresponding selectivity of the aptasensor and were always applied in the following.

3.2. Characterization of the model sensor performance

To investigate the analytical performance of the albumin aptasensors, their calibration curve was determined using DPV by sequential incubation of the rod electrodes modified with the optimized receptor layer in various concentrations of the respective albumin solutions for 30 min, Fig. 3 A. Since the signal gain is assumed to be proportional to the amount of bound albumin, the signal gain is plotted versus the logarithm of analyte concentrations, which follows a Langmuir-Freundlich adsorption isotherm with the equation of Signal gain (%) = $(97.2\times2.1\times10^{-5}\ (C^{0.8}))/(1+2.1\times10^{-5}\ C^{0.8})$ ($R^2 = 0.998$), (Fig. 3B) [48]. Here, the signal gain value was calculated by the ratio between DPV signal decrease and DPV background signal. The exponent for *C* of 0.8 was determined by fitting the experimental data and describes the inhomogeneity in binding affinity among the surface-tethered aptamer receptors. The dynamic detection range of the system lasted from 45 nM to 10 µM. The detection limit (LOD) of the albumin aptasensor was 15 nM, which was calculated based on the following equation: Signal gain (%) = Mean + 3 SD. The mean represents the mean value of the signal gain at the lowest tested concentration from at least three independent experiments while SD is the standard deviation value of the signal gain as mentioned.

<Figure 3>

Similarly, the GHSA aptasensor was examined by incubating the aptamer-modified gold rod electrode in GHSA solution of different concentrations ranging from 1 pM to 200 µM, Fig. 3 C. The DPV signals were recorded also after 30 min incubation. Plotting the signal gain versus the logarithm value of GHSA concentrations followed a Langmuir-Freundlich adsorption isotherm similar to the HSA aptasensor with the equation: Signal gain (%) = $(92.1\times8\times10^{-6}\ (C^{0.88}))/(1+8\times10^{-6}\ C^{0.88})$ ($R^2 = 0.9981$), (Fig. 3 D). The dynamic detection range covers 18 nM to 10 µM and the LOD was 6 nM (obtained as above). It is noteworthy that both aptasensors show very similar detection characteristics, which is of great advantage for the determination of the HSA glycation based on the ratio of these two analytes. Large differences in

the sensitivity or in the dynamic detection range could have impaired the outcome of such a comparative sensor assay.

HSA accounts for more than half of all proteins in human blood and ranges from 450 µM to 750 µM for healthy people [20]. The concentration of GHSA for healthy individuals covers a range of 50 µM to 120 µM and can be 2 to 5 times higher for diabetes patients [15,20,21]. These high concentrations are almost out of all the dynamic detection ranges of current sensors. However, a 100 times dilution of the blood samples can match the detection range of our sensors with all expectable healthy and pathological (glycated) HSA concentrations.

The detection performance was further consolidated by determining the calibration curve through EIS measurements using the same experimental conditions as for the DPV experiments, Fig. S5 B. The dynamic detection range of the GHSA aptasensor lasted from 18 nM to 100 µM by following Signal gain (%) = $(774\times4.4\times10^{-5}\ (C^{0.64}))/(1+4.4\times10^{-5}\ C^{0.64})$ ($R^2 = 0.9996$). The EIS detection range was one order of magnitude broader but certain instabilities occurred for high concentrations leading to an unexpected signal drop presumably due to $[Fe(CN)_6]^{3-/4-}$ assisted Au etching [49]. The detection limit (LOD) of the GHSA aptasensor was 6 nM and therefore corroborates the DPV characterization.

3.3. Implementation of a flex–MEAs sensor for simultaneous detection of HSA and GHSA

Subsequently, we transferred the developed sensor concepts from Au rod electrodes to flex–MEAs chips, which we recently introduced for the detection of malaria biomarkers [37]. Fig. 4A displays a flex–MEA chip inserted in an electronic connector wiring the individual electrodes with the potentiostat. The chip was cut in the middle, Fig. 4A, to generate two separate sets of electrodes that can be incubated simultaneously in two solutions by bending the flexible polymer chip. Therefore, the left and right electrodes were simultaneously inserted via bending in opposite directions in separate incubation vials containing different aptamer solutions.

<Figure 4>

To eliminate the unspecific adsorption of analyte or matrix molecules on the sensor electrodes and to avoid laborious pretreatments of blood samples, monofunctional methoxy-polyethylene glycol thiol (PEG) was introduced instead of MCH as a blocking agent for real sample experiments [42]. Although we couldn't observe the adsorption of albumin to MCH on the decorated surface, severe unspecific adsorption

of matrix components has been observed previously for real serum samples [42]. Since our sensor is supposed to work even in blood samples, a replacement of the MCH backfill seemed to be advisable. The incubation in the PEG solution was done subsequently by receptor immobilization. To assess the suitability of the PEG backfill modification, the flexible sensor was challenged with blood samples. Therefore, half of the flex–MEAs were stepwise modified with albumin aptamer and 10 mg/mL PEG while the other half of which was only modified with PEG (PEG/Au electrode arrays). Then the sensors were incubated in 100 times diluted human blood samples containing 10 mM PBS and 5 mM ferro/ferricyanide redox couple before (black curves) and after (red curves) 30 min incubation in a mixture with 250 µL 5 µM albumin and 500 nM GHSA, Fig. S6. The corresponding DPV signals were determined subsequently. In comparison with the huge DPV signal change for the albumin aptamer containing electrodes (Fig. S6 A), marginally small signal fluctuations were observed for electrodes modified only with PEG (Fig. S6 B), demonstrating the effective suppression of unspecific adsorption by PEG backfills.

The experimental conditions, optimized employing gold rod electrodes, were applied to the flex–MEA sensors to determine the analyte concentration by DPV measurements. Both sets of electrodes of the same MEA chip were incubated in a mixed standard containing HSA and GHSA solutions at various concentrations (the concentration ratio of HSA and GHSA was always 1:1 up to 100 µM, then larger than 1:1 at very high two concentrations). Thus the concentration of both analytes can be determined simultaneously and the desired glycation ratio can be determined based on the signal gain determined and averaged from several electrodes of the same sensor array. Also for the flex–MEA sensor, we found calibration curves that could be modeled by a Langmuir Freundlich adsorption isotherm with similar characteristics for the detection of albumin as the gold rod electrode sensors. The characteristic values of LOD, detection range, and Langmuir Freundlich fitting parameters are summarized in Table 1. Neither the transfer from rod electrodes to flex–MEAs chips nor the substitution of MCH by PEG affected the sensor performance significantly. This corroborates a similar observation when we replaced MCH with PEG backfill for our malaria sensor that also needs to operate in blood samples [42]. Since the sensor characteristics were very similar for HSA and GHSA, the ratio of [GHSA]/[HSA) can be directly calculated without laborious sample pretreatment or value correction and is already available 30 min after starting the test.

<Table 1>

Some other studies for the quantitative analysis of HSA and GHSA are presented in Table 2. In comparison with those detection concepts, our flex–MEAs-based aptasensor exhibited similar detection limits and wide dynamic detection ranges as other sensors, which however is not the main challenge for the

detection of abundant serum albumin proteins. The capability of detecting both targets simultaneously and sensitively in the same sample distinguishes our sensor from other detection platforms, which holds great promise for point-of-care estimation of protein glycation and to complement future diabetes surveillance.

<Table 2>

To further evaluate the detection of protein glycation, real blood samples after 100 times dilution were analyzed by PEG-blocked flex–MEAs and compared with results obtained for PBS samples of the same analyte concentration. The same concentration ratio of HSA and GHSA was spiked in all sample solutions. The averaged signal gain recovery was calculated from the calibration curve of the signals measured in PBS and diluted blood for 100 nM, 500 nM, and 1 µM, Fig. 5A. The recovery of the measurement for real sample combining 3 different concentrations were 109.3 % and 105.5 % for HSA and GHSA, respectively, compared with the results of the calibration curve recorded in PBS, indicating the PEG-blocked flex–MEAs has high reliability in real sample detection. This was further confirmed by the reproducibility and thus redundancy of the DPV signal gain of the HSA and GHSA flex–MEAs aptasensor toward incubation in 100 nM HSA and 100 nM GHSA mixtures contained in 100 times diluted blood when the analyte was detected by different electrodes of the same chip, Fig. S7.

<Figure 5>

Considering that the concentration of GHSA strongly varies for diabetes and healthy individuals, 100 times diluted blood samples were spiked with a mixture of 5 µM HSA and either 500 nM, 1.5 µM, or 4 µM GHSA, Fig. 5 B. The HSA originally contained in the blood was removed twice in advance by a previously reported chemical extraction method [50,51]. The observed recovery values for the spiked samples were similar compared to that from the calibration curves for HSA and GHSA, respectively. The HSA values were relatively constant while the GHSA values changed according to the spiked GHSA concentrations.

Besides the elimination of unspecific adsorption of matrix components to the electrodes, we aimed to evaluate the specificity of the target binding to the ssDNA aptamer receptors to rule out that HSA tends to bind unspecific to any ssDNA. Therefore, analyte binding to its corresponding albumin aptamer was compared with the binding to a random ssDNA sequence. Specifically, the 2008s aptamer for detecting the malaria biomarker PfLDH was used to exclude those albumin associates with the negatively charged receptor molecules via electrostatic or any other unspecific interaction. Therefore, one set of electrodes of a flex–MEAs chip was modified with a 2008s aptamer / PEG receptor layer while the other set of electrodes was functionalized with HSA aptamer / PEG. Afterwards, all electrodes of the dual aptamer sensor were incubated in the same 100 times diluted blood sample spiked with a 5 µM HSA and 0.5 µM GHSA mixture.

The averaged signal gain of the HSA aptasensors possessed a signal gain of 78% while the malaria part of the sensor exhibited an evidently smaller signal gain than the analyte/aptamer matching system, indicating a very weak coupling of albumins to non-matching ssDNA molecules (Fig. 5 C). This corroborates the specific binding of the albumin targets to their corresponding aptamer receptors (see signal GHSA in Fig. 5 A) for the presented flexible MEA aptasensors for real sample detection.

4. Conclusion

In summary, gold rod electrodes were first employed for optimizing the performance of HSA and GHSA aptamer sensors, and then the optimized preparation conditions were transferred to flex–MEAs chips facilitating the desired simultaneous multi-target detection. The immobilization process was studied by electrochemical impedance spectroscopy, chronocoulometry, as well as atomic force microscopy. Thermal pretreatment of the receptor film was required to provide a selective detection of the respective analyte and to avoid cross-selectivity between HSA and GHSA. The utilization of a polymer substrate for the sensor chip did not only reduce the material costs considerably but also facilitated the easy separation and bending of the electrode arrays in opposite directions so that they can be incubated in separate vials containing for instance different receptor solutions or an analyte solution and a reference sample, respectively. Here, the parallel incubated in different aptamer solutions enabled simultaneous dual-target detection without involving expensive equipment or procedures for local receptor immobilization. The electrochemical aptasensor developed in this work is characterized by high reliability for albumin and GHSA biomarker determination with wide detection ranges, combined with excellent recovery from diluted full-blood samples. Therefore, redundant signals from different electrodes of the same electrode set, all modified with the same receptor, were simultaneously recorded and analyzed reducing the impact of device-specific variations. The usage of polyethylene glycol backfill molecules suppressed effectively unspecific adsorption of blood matrix components and thus supported the highly selective detection of the analyte molecules of interest. Diabetes mellitus is a chronic disease requiring lifetime glycemia surveillance entailing enormous costs. Therefore, affordable point-of-care assessment tests for glycemic control are of great importance not only to permit proper treatment in countries with simple healthcare infrastructure but also to cushion the treatment cost for rising patient numbers in industrial nations. Our flex–MEAs sensor utilizes not only low-cost polymer chips and aptamer, but also immobilizes these receptors on different electrodes without expensive equipment and thus facilitates the quantitative and selective determination of midterm glycemia at point-of-care without complicated sample treatment or expert staff.

Declaration of interests: there are no conflict of interest

Acknowledgement

L. Zhou gratefully acknowledges financial support from the China Scholarship Council (No. 201708320255).

Author contributions

L. Z. conducted experiments, data analysis, writing and editing the original manuscript draft. M. N. participated in the data analysis, figure design, and reviewing the manuscript. G. F.-M contributed with designed of experiments, data analysis, and review and editing of the manuscript. Y. L. contributed with data analysis, discussion, and reviewing the manuscript. Z. H. and R. Z. participated with the preparation of reagents and materials, and reviewing the manuscript. S. C. and M. P. contributed with the fabrication of the flex-MEAs chips and review the manuscript. A. O. contributed to data discussion, reviewing, and editing the manuscript. D. M. participated in design of experiments, data analysis and discussion, writing original draft and editing of the manuscript.

References

[1] M. Roden, G.I. Shulman, The integrative biology of type 2 diabetes, Nature. 576 (2019) 51–60. https://doi.org/10.1038/s41586-019-1797-8.

[2] G. Roglic, World Health Organization, Global report on diabetes, n.d.

[3] IDF Diabetes Atlas 10th edition, n.d. www.diabetesatlas.org.

[4] Y. Zheng, S.H. Ley, F.B. Hu, Global aetiology and epidemiology of type 2 diabetes mellitus and its complications, Nat Rev Endocrinol. 14 (2018) 88–98. https://doi.org/10.1038/nrendo.2017.151.

[5] J.B. Cole, J.C. Florez, Genetics of diabetes mellitus and diabetes complications, Nat Rev Nephrol. 16 (2020) 377–390. https://doi.org/10.1038/s41581-020-0278-5.

[6] D.A. Butterfield, F. di Domenico, E. Barone, Elevated risk of type 2 diabetes for development of alzheimer disease: a key role for oxidative stress in brain, Biochim Biophys Acta Mol Basis Dis. 1842 (2014) 1693–1706. https://doi.org/10.1016/j.bbadis.2014.06.010.

[7] H. Vlassara, M.R. Palace, Diabetes and advanced glycation endproducts, J Intern Med. 251 (2002) 87–101. https://doi.org/10.1046/j.1365-2796.2002.00932.x.

[8] A. Gautieri, A. Redaelli, M.J. Buehler, S. Vesentini, Age- and diabetes-related nonenzymatic crosslinks in collagen fibrils: Candidate amino acids involved in advanced glycation end-products, Matrix Biology. 34 (2014) 89–95. https://doi.org/10.1016/j.matbio.2013.09.004.

[9] A.M. Schmidt, O. Hori, Jing Xian Chen, Jian Feng Li, J. Crandall, J. Zhang, R. Cao, Shi Du Yan, J. Brett, D. Stern, Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice: a potential mechanism for the accelerated vasculopathy of diabetes, Journal of Clinical Investigation. 96 (1995) 1395–1403. https://doi.org/10.1172/JCI118175.

[10] S.Y. Goh, M.E. Cooper, The role of advanced glycation end products in progression and complications of diabetes, Journal of Clinical Endocrinology and Metabolism. 93 (2008) 1143–1152. https://doi.org/10.1210/jc.2007-1817.

[11] J.W. Nin, A. Jorsal, I. Ferreira, C.G. Schalkwijk, M.H. Prins, H.H. Parving, L. Tarnow, P. Rossing, C.D. Stehouwer, Higher plasma levels of advanced glycation end products are associated with incident cardiovascular disease and all-cause mortality in type 1 diabetes: A 12-year follow-up study, Diabetes Care. 34 (2011) 442–447. https://doi.org/10.2337/dc10-1087.

[12] N.M.J. Hanssen, J.W.J. Beulens, S. van Dieren, J.L.J.M. Scheijen, D.L. van der A, A.M.W. Spijkerman, Y.T. van der Schouw, C.D.A. Stehouwer, C.G. Schalkwijk, Plasma advanced glycation end products are associated with incident cardiovascularevents in individuals with type 2 diabetes: a case-cohort study with a median follow-up of 10 years (EPIC-NL), Diabetes. 64 (2015) 257–265. https://doi.org/10.2337/db13-1864.

[13] F.M. Gutierrez-Mariscal, M.P. Cardelo, S. de la Cruz, J.F. Alcala-Diaz, I. Roncero-Ramos, I. Guler, C. Vals-Delgado, A. López-Moreno, R.M. Luque, J. Delgado-Lista, P. Perez-Martinez, E.M. Yubero-Serrano, J. Lopez-Miranda, Reduction in circulating advanced glycation end products by mediterranean diet is associated with increased likelihood of type 2 diabetes remission in patients with coronary heart disease: from the cordioprev study, Mol Nutr Food Res. 65 (2021). https://doi.org/10.1002/mnfr.201901290.

[14] K.H. Gabbay, K. Hasty, J.L. Breslow, R.C. Ellison, H. Franklin Bunn, P.M. Gallop, Glycosylated hemoglobins and long-term blood glucose control in diabetes mellitus, 1977.

[15] H.V. Roohk, A.R. Zaidi, A review of glycated albumin as an intermediate glycation index for controlling diabetes, n.d. www.journalofdst.org.

[16] K.R. Klein, J.B. Buse, The trials and tribulations of determining HbA1c targets for diabetes mellitus, Nat Rev Endocrinol. 16 (2020) 717–730. https://doi.org/10.1038/s41574-020-00425-6.

[17] P.A.C. Freitas, L.R. Ehlert, J.L. Camargo, Glycated albumin: A potential biomarker in diabetes, Arch Endocrinol Metab. 61 (2017) 296–304. https://doi.org/10.1590/2359-3997000000272.

[18] Classification and diagnosis of diabetes: standards of medical care in diabetes-2020, Diabetes Care. 43 (2020) S14–S31. https://doi.org/10.2337/dc20-S002.

[19] Y. Inoue, M. Inoue, M. Saito, H. Yoshikawa, E. Tamiya, Sensitive detection of glycated albumin in human serum albumin using electrochemiluminescence, Anal Chem. 89 (2017) 5909–5915. https://doi.org/10.1021/acs.analchem.7b00280.

[20] J. Anguizola, R. Matsuda, O.S. Barnaby, K.S. Hoy, C. Wa, E. DeBolt, M. Koke, D.S. Hage, Review: glycation of human serum albumin, Clinica Chimica Acta. 425 (2013) 64–76. https://doi.org/10.1016/j.cca.2013.07.013.

[21] C. Apiwat, P. Luksirikul, P. Kankla, P. Pongprayoon, K. Treerattrakoon, K. Paiboonsukwong, S. Fucharoen, T. Dharakul, D. Japrung, Graphene based aptasensor for glycated albumin in diabetes mellitus diagnosis and monitoring, Biosens Bioelectron. 82 (2016) 140–145. https://doi.org/10.1016/j.bios.2016.04.015.

[22] D. Paria, A. Convertino, V. Mussi, L. Maiolo, I. Barman, Silver-coated disordered silicon nanowires provide highly sensitive label-free glycated albumin detection through molecular trapping and plasmonic hotspot formation, Adv Healthc Mater. 10 (2021). https://doi.org/10.1002/adhm.202001110.

[23] M. Kameya, W. Tsugawa, M. Yamada-Tajima, M. Hatada, K. Suzuki, A. Sakaguchi-Mikami, S. Ferri, D.C. Klonoff, K. Sode, Electrochemical sensing system employing fructosamine 6-kinase enables glycated albumin measurement requiring no proteolytic digestion, Biotechnol J. 11 (2016). https://doi.org/10.1002/biot.201500442.

[24] S.E. Son, P.K. Gupta, W. Hur, H. Choi, H.B. Lee, Y. Park, G.H. Seong, Determination of glycated albumin using a prussian blue nanozyme-based boronate affinity sandwich assay, Anal Chim Acta. 1134 (2020). https://doi.org/10.1016/j.aca.2020.08.015.

[25] A. Chen, S. Chatterjee, Nanomaterials based electrochemical sensors for biomedical applications, Chem Soc Rev. 42 (2013) 5425–5438. https://doi.org/10.1039/c3cs35518g.

[26] N. Wongkaew, M. Simsek, C. Griesche, A.J. Baeumner, Functional nanomaterials and nanostructures enhancing electrochemical biosensors and lab-on-a-chip performances: recent progress, applications, and future perspective, Chem Rev. 119 (2019) 120–194. https://doi.org/10.1021/acs.chemrev.8b00172.

[27] Y. Zhang, G. Figueroa-Miranda, Z. Lyu, C. Zafiu, D. Willbold, A. Offenhäusser, D. Mayer, Monitoring amyloid-β proteins aggregation based on label-free aptasensor, Sens Actuators B Chem. 288 (2019) 535–542. https://doi.org/10.1016/J.SNB.2019.03.049.

[28] C. Wu, D. Barkova, N. Komarova, A. Offenhäusser, M. Andrianova, Z. Hu, A. Kuznetsov, D. Mayer, Highly selective and sensitive detection of glutamate by an electrochemical aptasensor, Anal Bioanal Chem. 414 (2022). https://doi.org/10.1007/s00216-021-03783-w.

[29] G. Figueroa-Miranda, L. Feng, S.C.C. Shiu, R.M. Dirkzwager, Y.W. Cheung, J.A. Tanner, M.J. Schöning, A. Offenhäusser, D. Mayer, Aptamer-based electrochemical biosensor for highly sensitive and selective malaria detection with adjustable dynamic response range and reusability, Sens Actuators B Chem. 255 (2018) 235–243. https://doi.org/10.1016/j.snb.2017.07.117.

[30] L. Wang, H. Jin, M. Wei, W. Ren, Y. Zhang, L. Jiang, T. Wei, B. He, A DNAzyme-assisted triple-amplified electrochemical aptasensor for ultra-sensitive detection of T-2 toxin, Sens Actuators B Chem. 328 (2021). https://doi.org/10.1016/j.snb.2020.129063.

[31] J. Yang, X. Li, B. Jiang, R. Yuan, Y. Xiang, In situ-generated multivalent aptamer network for efficient capture and sensitive electrochemical detection of circulating tumor cells in whole blood, Anal Chem. 92 (2020). https://doi.org/10.1021/acs.analchem.0c01195.

[32] M. Labib, E.H. Sargent, S.O. Kelley, Electrochemical methods for the analysis of clinically relevant biomolecules, Chem Rev. 116 (2016) 9001–9090. https://doi.org/10.1021/acs.chemrev.6b00220.

[33] M. Takenaka, Y. Okumura, T. Amino, Y. Miyachi, C. Ogino, A. Kondo, DNA-duplex linker for AFM-SELEX of DNA aptamer against human serum albumin, Bioorg Med Chem Lett. 27 (2017) 954–957. https://doi.org/10.1016/j.bmcl.2016.12.080.

[34] H.Y. Bai, F.J. del Campo, Y.C. Tsai, Sensitive electrochemical thrombin aptasensor based on gold disk microelectrode arrays, Biosens Bioelectron. 42 (2013) 17–22. https://doi.org/10.1016/j.bios.2012.10.063.

[35] K.J. Krause, A. Yakushenko, B. Wolfrum, Stochastic on-chip detection of subpicomolar concentrations of silver nanoparticles, Anal Chem. 87 (2015) 7321–7325. https://doi.org/10.1021/acs.analchem.5b01478.

[36] K.C. Cheung, P. Renaud, H. Tanila, K. Djupsund, Flexible polyimide microelectrode array for in vivo recordings and current source density analysis, Biosens Bioelectron. 22 (2007) 1783–1790. https://doi.org/10.1016/j.bios.2006.08.035.

[37] G. Figueroa-Miranda, S. Chen, M. Neis, L. Zhou, Y. Zhang, Y. Lo, J.A. Tanner, A. Kreidenweiss, A. Offenhäusser, D. Mayer, Multi-target electrochemical malaria aptasensor on flexible multielectrode arrays for detection in malaria parasite blood samples, Sens Actuators B Chem. 349 (2021). https://doi.org/10.1016/j.snb.2021.130812.

[38] F. Schröper, D. Brüggemann, Y. Mourzina, B. Wolfrum, A. Offenhäusser, D. Mayer, Analyzing the electroactive surface of gold nanopillars by electrochemical methods for electrode miniaturization, Electrochim Acta. 53 (2008). https://doi.org/10.1016/j.electacta.2008.03.068.

[39] Y. Zhang, G. Figueroa-Miranda, C. Zafiu, D. Willbold, A. Offenhäusser, D. Mayer, Amperometric aptasensor for amyloid-β oligomer detection by optimized stem-loop structures with an adjustable detection range, ACS Sens. 4 (2019) 3042–3050. https://doi.org/10.1021/acssensors.9b01630.

[40] N. Fogh-Andersen, P.J. Bjerrum, O. Siggaard-Andersen, Ionic binding, net Charge, and Donnan effect of human serum albumin as a Function of pH, 1993.

[41] M. Petri, D.M. Kolb, U. Memmert, H. Meyer, Adsorption of mercaptopropionic acid onto Au(1 1 1): part I. adlayer formation, structure and electrochemistry, in: Electrochim Acta, 2003: pp. 175–182. https://doi.org/10.1016/j.electacta.2003.07.010.

[42] G. Figueroa-Miranda, C. Wu, Y. Zhang, L. Nörbel, Y. Lo, J.A. Tanner, L. Elling, A. Offenhäusser, D. Mayer, Polyethylene glycol-mediated blocking and monolayer morphology of an electrochemical

aptasensor for malaria biomarker detection in human serum, Bioelectrochemistry. 136 (2020). https://doi.org/10.1016/j.bioelechem.2020.107589.

[43] C.T. Fakude, O.A. Arotiba, F. Arduini, N. Mabuba, Flexible polyester screen-printed electrode modified with carbon nanofibers for the electrochemical aptasensing of Cadmium (II), Electroanalysis. 32 (2020) 2650–2658. https://doi.org/10.1002/elan.202060070.

[44] J. Zhang, S. Song, L. Wang, D. Pan, C. Fan, A gold nanoparticle-based chronocoulometric DNA sensor for amplified detection of DNA, Nat Protoc. 2 (2007) 2888–2895. https://doi.org/10.1038/nprot.2007.419.

[45] R.J. White, N. Phares, A.A. Lubin, Y. Xiao, K.W. Plaxco, Optimization of electrochemical aptamer-based sensors via optimization of probe packing density and surface chemistry, Langmuir. 24 (2008). https://doi.org/10.1021/la800801v.

[46] Z.M. Chen, Y. Wang, X.Y. Du, J.J. Sun, S. Yang, Temperature-alternated electrochemical aptamer-based biosensor for calibration-free and sensitive molecular measurements in an unprocessed actual sample, Anal Chem. 93 (2021) 7843–7850. https://doi.org/10.1021/acs.analchem.1c00289.

[47] B.I. Kankia, L.A. Marky, Folding of the thrombin aptamer into a G-quadruplex with Sr^{2+}: stability, heat, and hydration, J Am Chem Soc. 123 (2001) 10799–10804. https://doi.org/10.1021/ja010008o.

[48] W. Ma, L. Liu, Y. Xu, L. Wang, L. Chen, S. Yan, L. Shui, Z. Wang, S. Li, A highly efficient preconcentration route for rapid and sensitive detection of endotoxin based on an electrochemical biosensor, Analyst. 145 (2020) 4204–4211. https://doi.org/10.1039/d0an00315h.

[49] S. Vogt, Q. Su, C. Gutiérrez-Sánchez, G. Nöll, Critical view on electrochemical impedance spectroscopy using the ferri/ferrocyanide redox couple at gold electrodes, Anal Chem. 88 (2016) 4383–4390. https://doi.org/10.1021/acs.analchem.5b04814.

[50] D.A. Colantonio, C. Dunkinson, D.E. Bovenkamp, J.E. van Eyk, Effective removal of albumin from serum, Proteomics. 5 (2005). https://doi.org/10.1002/pmic.200401235.

[51] R.J. Holewinski, Z. Jin, M.J. Powell, M.D. Maust, J.E. van Eyk, A fast and reproducible method for albumin isolation and depletion from serum and cerebrospinal fluid, Proteomics. 13 (2013). https://doi.org/10.1002/pmic.201200192.