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Non-invasive Point-of-Care Device To Diagnose Acute Mesenteric Ischemia

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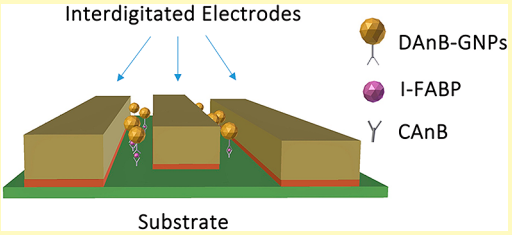
Supporting Information

ABSTRACT: Inadequate blood supply to the intestine can lead to acute mesenteric ischemia (AMI), with a mortality rate ranging from 60% to 90%. This high mortality rate is partially due to late detection and the lack of efficient early diagnostic tests. There is an urgent need for a point-of-care tool for immediate bedside diagnosis. Here we present for the first time a rapid and non-invasive electrochemical biosensor device based on non-faradic impedance spectroscopy to detect intestinal fatty-acid binding protein (I-FABP) as an indication of AMI. The electrochemical biosensors consist of gold interdigitated electrodes that were fabricated using photolithographic techniques on top of silicon dioxide substrates. The electrode surfaces were functionalized with an I-FABP capture antibody (CAnB) to entice the target protein, while gold nanoparticles (GNPs) functionalized with detection antibodies (DAnB-GNPs) were utilized as a novel mechanism to enhance the detection signal. Quantification of the I-FABP concentration in the medium depended on its attachment to CAnB and DAnB-GNPs in a sandwich manner, where the latter boosts the impedance signal through its binding to the I-FABP. This non-invasive non-faradic electric biosensor device demonstrates the potential for bench-to-bedside translation with the goal of decreasing morbidity and mortality from AMI.



KEYWORDS: *point-of-care device, non-invasive urine test, acute mesenteric ischemia, impedance-based biosensor, bedside monitoring*

Acute mesenteric ischemia (AMI) is characterized by critical sickness, and its onset can progress to intestinal necrosis (dead bowel) or even mortality. Broadly, AMI may be classified into non-occlusive mesenteric ischemia and occlusive mesenteric ischemia, where arterial blood supply is completely cut off. AMI is associated with high mortality rates between 60% and 80%, which have not changed in the past 70 years,[1,2] largely due to the severity of the illness once diagnosed. One of the major hurdles for early detection of AMI is the absence of fast and easy sensing instrumentation to accurately quantify its biomarkers. Existing bioanalytical methods usually take a long time to measure and require well-trained personnel to operate. Delayed diagnosis or misdiagnosis could lead to irreversible intestinal necrosis, which is a life-threatening disorder ultimately resulting in death. Other than surgery and biopsy, there are no definitive non-invasive diagnostic techniques currently available. Conventional serum lactate and white blood cell (WBC) count do not provide high enough sensitivity and specificity.[3] CT scans or angiographies, on the other hand, often produce misleading results.[3] In a review article, Powell and Armstrong[4] concluded by stating, "Current traditional and investigative plasma biomarkers are not widely available or standardized for broad-based use outside of clinical trials. Development of a distinctive plasma marker capable of predicting early hypoperfusion to the splanchnic circulation would be of great clinical value in targeting timely diagnosis and directing revascularization and resuscitation."

Intestinal fatty-acid binding protein (I-FABP) in urine or blood has been reported as a promising non-invasive biomarker for identifying patients with AMI. Although others have identified I-FABP as a diagnostic tool, it still has not used in clinical application due to the time requirement of existing technologies to perform this test. We have shown in a preclinical model[5] and in clinical samples[6] that I-FABP has a sensitivity of 92% and a specificity of 80% for detecting AMI. The current clinical testing method uses enzyme-linked

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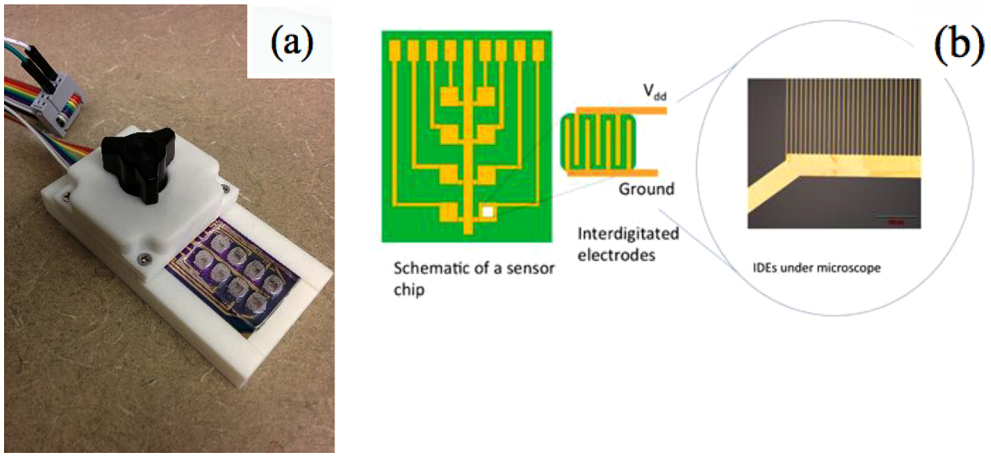

Figure 1. (a) Representation of the device: the array of interdigitated electrodes (IDEs) mounted to the reader device, and the PDMS cover with eight holes aligned with the electrodes of the chip, in which the solution aliquot is placed. (b) Schematics and microscope image of an IDE sensor chip.

immunosorbent assays (ELISA). Funaoka reported that the detection process of the human I-FABP using ELISA took three steps, each taking approximately 2 h to complete.[7] In another study, Shi et al. confirmed that the ELISA test in a clinical research study took them a working time of 4 h.[8] In particular, they stated that "Human FABP enzyme-linked immunosorbent assays kits are solid-phase enzyme-linked immunosorbent assays based on the sandwich principle with a working time of 4 h." Again, these tests can take more than 4 h in the detection stage, not including the samples preparation, and most patients are diagnosed beyond the optimal window needed to effectively treat AMI. As such, developing a point-of-care (POC) tool for immediate bedside assessment is urgently needed.

Interdigitated electrodes (IDEs) were intensively studied as a sensor platform for detecting different bioanalytes such as proteins,[9] fatty acids,[10] viruses,[11,2] DNA,[12] and bacteria,[13] where biomolecules have been employed to enhance the specificity of IDE sensors toward targeted biomarkers.[14] Over the past several years, we have developed an impedance-based POC device and IDE sensing chips.[15,16] We have proved both theoretically and experimentally the role of incorporating gold nanoparticles (GNPs) in enhancing the non-faradaic sensing signal of the IDEs. The double layer generated by the presence of the GNPs can be employed to boost the sensitivity of the IDEs. In this article, we present for the first time the use of IDEs chips as a portable biosensor to fast detect the presence of I-FABP. Measurements were taken at different stages of the sensing process to evaluate the real-time performance. The measurements of the impedance of the I-FABP samples dissolved in human urine determine the sensitivity of the IDEs.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich with analytical purity grade unless otherwise mentioned. Ultrapure Milli-Q water with resistivity above 18 MΩ was utilized during all experiments and in the preparation of all buffers and solutions. CAnB, DAnB, I-FABP and the negative control heart-type (H)-FABP (H-FABP) were purchased from R&D Systems (Minneapolis, USA). Please refer to the Supporting Information for GNP synthesis/characterization and microchip surface functionalization.

Interdigitated Electrode (IDEs) Microfabrication. Gold IDEs arrays were fabricated using standard photolithographic methods on 4-in. silicon wafers as has been reported elsewhere with minor modifications.[15] Each resulting IDE device has eight electrode wells, where each one is 3 mm^2 and consists of 168 digit pairs, each digit having a width of 4 μm. These fingers are recessed by a 2 μm gap between adjacent fingers. Figure 1a displays the shape of the IDEs and how it was connected to the measuring device, and the figure also shows the polydimethylsiloxane (PDMS) cover mounted on the top of the IDEs chip to keep the analyte solutions separate from each other. Figure 1b represents the structure of the IDEs chip and how the gold electrodes are arranged.

The design of the IDE is flexible and mainly depends on the mask used in the photolithography step of fabrication. Here we selected eight electrode wells per IDE chip *as an example* to offer enough space to perform several tests, including control tests, simultaneously. Such design gives reliable analytical test data by including a control sample for comparison. For instance, we did a negative control test at the same time as seven other tests with different concentrations of the I-FABP on a single IDE chip. In realty, such design will give researchers the opportunity to standardize their experiment while carrying out a real test at the same time.

Measuring I-FABP. We performed the detection of the I-FABP using the modified IDEs in two different media, where I-FABP was independently dissolved in PBS buffer 1X and synthetic urine. For example, different concentrations of I-FABP were spiked in PBS buffer 1X. Then, 30 μL of each concentration was added to each CAnB functionalized electrode and incubated for 2 h at 4 °C. After that, the I-FABP solutions were removed, and the IDEs were washed with PBS followed by Milli-Q water. Afterward, 30 μL of DAnB-GNP dispersion was added to each protein-coated electrode, and the IDE chip was incubated for two more hours at 4 °C. Finally, the DAnB-GNP solutions were discarded, and the IDEs were washed with PBS buffer 1X, followed by Milli-Q water. The impedance was measured after each step of the IDE surface functionalization to monitor the change at the electrode surface and ensure quality standardization. We repeated the previous steps using synthetic urine as a dissolving medium for the I-FABP. To test the feasibility of sensing I-FABP in a real situation, we collected human urine samples from three healthy volunteers in a biolaboratory having the biosafety ethical license (ethics number Pro00025429) to collect human liquid samples following the biosafety regulations (all of the collected three urine samples were tested for the I-FABP, and they showed negative results). We separately spiked each urine samples with different concentrations of the I-FABP standard. In the case of using human urine as a medium, the incubation time of the DAnB-GNPs biomarker with the I-FABP trapped IDEs was 1 h at 4 °C. In the case of human urine samples, the samples were freshly collected from the volunteers (three different volunteers, one female and two males), centrifuged, and treated with 10% sodium azide before being spiked with I-FABP (which happened within 3 h upon urine collection). For evaluating

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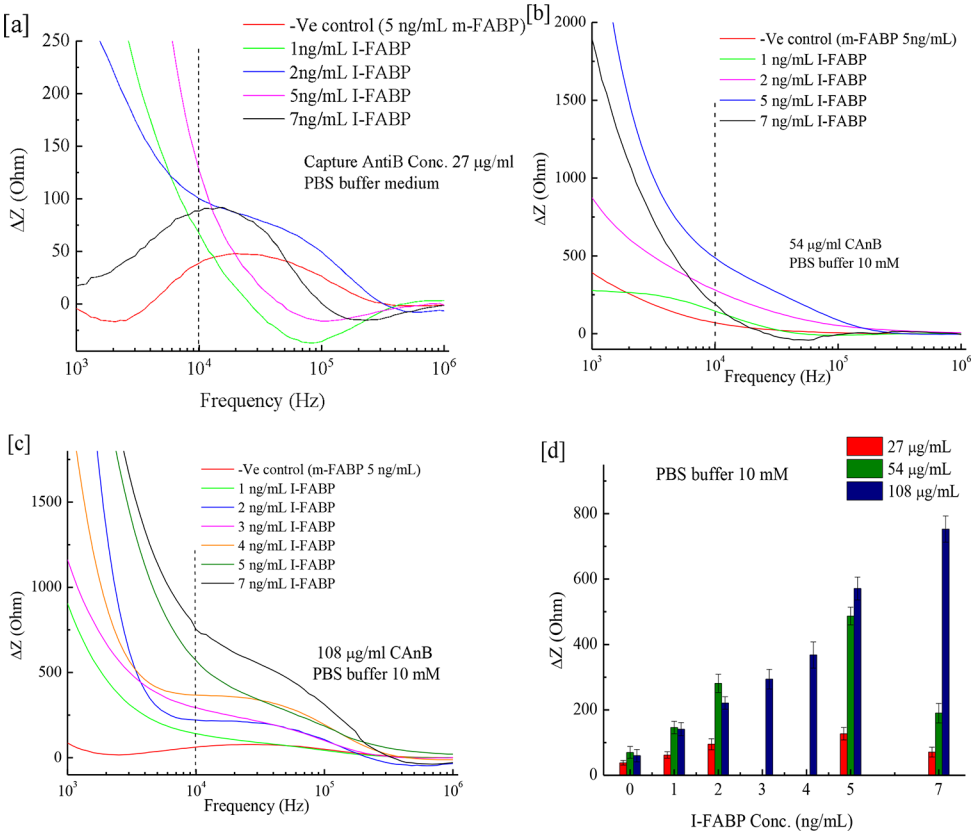

Figure 2. Difference in the impedance magnitude with the addition of the DAnB-GNPs to different concentrations of I-FABP at 10 kHz frequency. The IDEs were treated with (a) 27 μg/mL, (b) 54 μg/mL, and (c) 108 μg/mL concentrations of CAnB. (d) Impedance response at different concentrations of the coated CAnB (27, 54, and 108 μg/mL) for detecting different concentrations of the I-FABP spiked in PBS buffer of 10 mM concentration. The measurements were collected at 10 mV peak-to-peak.

the specificity of the surface functionalized IDEs, we employed H-FABP as a negative control, where we dissolved in the same mediums and used in comparison with I-FABP. H-FABP has been detected in the smooth muscles of the heart, in many striated muscles, parietal cells of the stomach, renal epithelial cells, acinar and ductal cells of the breast, ductal cells of the salivary gland, corpus luteum, Leydig cells of the testis, adipocytes, and vascular endothelial cells.[17]

RESULTS AND DISCUSSION

Please refer to the Supporting Information for the step-by-step validation of IDE surface functionalization, and the role of GNPs in the impedance enhancement.

Sensing I-FABP. To optimize the IDEs to detect the I-FABP, we considered the concentration of the CAnB used in the IDE surface functionalization and working solution in which the I-FABP is dissolved during the sensing process. For the first optimization, three different concentrations of the CAnB (27, 54, and 108 μg/mL) were used to modify the surface of the IDEs, and the sensitivity was estimated by following the impedance response to the alteration of the I-FABP concentration. We prepared a series of I-FABP concentrations for testing and compared with H-FABP (5 ng/mL). We collected the impedance magnitude after modification of the IDEs with CAnB, the addition of the I-FABP, and the addition of the DAnB-GNPs. For each I-FABP concentration, we plotted the difference in the impedance magnitude before and after the addition of the DAnB-GNPs against the frequency range used in the measurements. The same process was repeated by using three different concentrations of CAnB (27, 54, and 108 μg/mL) coated on IDEs, as shown in Figure 2a–c, respectively. The difference in the impedance magnitude at frequency of 10 kHz was quantified at each tested concentration of I-FABP and analyzed to estimate the sensitivity of the IDEs device to the variation in the protein concentration.

After further investigating the test results, we have the following observations. First, the concentration of the anchored CAnB probe has a significant impact on the sensitivity of the IDEs. As the concentration of the anchored CAnB increased, the sensitivity increased. This can be justified from the slope of the difference in the impedance magnitude caused by the addition of the DAnB-GNPs against different concentrations of I-FABP (Figure 2d). By increasing the concentration of the used CAnB in the IDEs coating from 27 to 54 and 108 μg/mL, the slope increased from 6 to 23 and 101 $\Omega\cdot(\mathrm{ng/mL})^{-1}$, respectively. The sensitivity of the functionalized IDEs toward I-FABP increased about 4 times by doubling the concentration of the CAnB probes. Second, the response of the IDEs toward the H-FABP (the negative control) appeared to be insensitive to the concentration of the CAnB used in the IDEs coating. The response of the IDEs coated with 54 μg/mL of CAnB is higher than that of the IDEs coated with either 27 or 108 μg/mL of CAnB. This confirmed the significant specificity of our IDE biosensors to I-FABP. Finally, the detection range of the IDEs significantly depends on the concentration of the CAnB used in the precoating step. In the case of precoating the IDEs with 27 and 54 μg/mL CAnB concentrations, the impedance magnitude difference reading of the 7 ng/mL I-FABP cases showed a considerable

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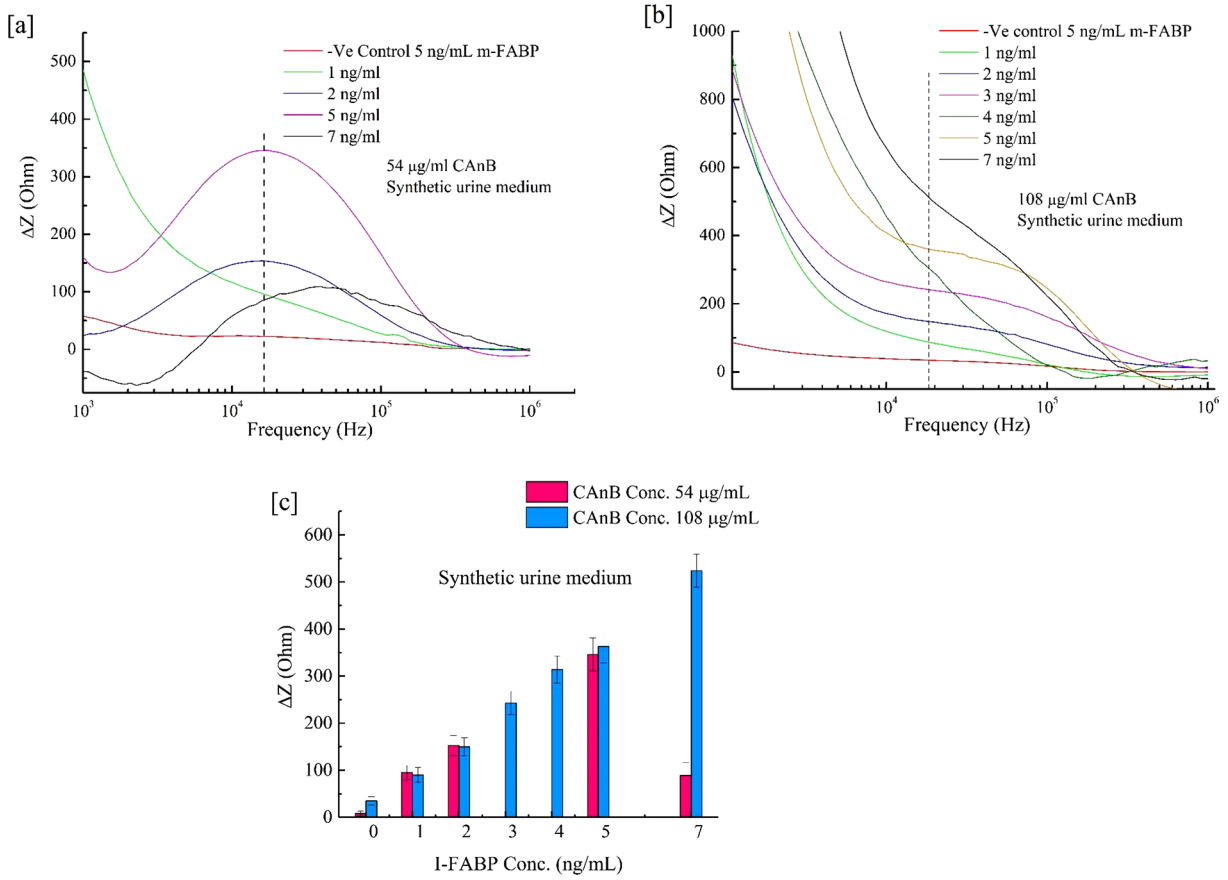

Figure 3. Difference in the impedance magnitude with the addition of the GNPs-DAnB to different concentrations of the I-FABP at 17 kHz. The IDEs were treated with (a) 54 and (b) 108 μg/mL concentrations of CAnB. (c) Impedance response at different concentrations of the coated CAnB (54 and 108 μg/mL) for detecting different concentrations of the I-FABP spiked into a synthetic urine medium. The measurements were collected at 10 mV. Here, the red and blue colors refer to 54 and 108 μg/mL of CAnB used in the IDE surface modification step, respectively.

decline. While, at the lower concentrations of I-FABP, the IDEs response showed a linear trend. On the other hand, by coating the IDEs with 108 μg/mL of CAnB, the IDEs exhibited a linear increase in the impedance magnitude difference reading at I-FABP concentrations up to 7 ng/mL. A possible cause is at lower concentrations of the CAnB the IDEs get over saturated with I-FABP molecules at a concentration as low as 5 ng/mL, and this hindered the detection of higher concentrations of I-FABP. Moreover, the higher concentrations of I-FABP might create a competitive binding environment during the binding process, with the CAnB inhibiting the capture of the I-FABP. This would cause a major decrease in the impedance magnitude after the addition of the DAnB-GNPs, which could limit the usage of the IDEs. Several studies pointed out the importance of saturating the IDE surface with probes to determine the detection limit of the biosensors.[18] As the concentration of CAnB increased on the IDE surface, the modification increased the density of the CAnB probes, and consequently increased the capacity of the IDEs to detect the target I-FABP. This results in the enhancement of the detection limit.

The limit of detection (LOD) of the IDEs coated with CAnB at the concentration of 108 μg/mL is calculated according to the following equation.[19]

$$\text{LOD} = (3 \times \text{standard deviation of the blank}) / \text{slope of the calibration line} \quad (1)$$

As the measured slope of the linear calibration is 101.7, the calculated LOD is 0.56 ng/mL. We estimated the LOD based on eq 1. In each set of sensing experiments, we changed one of the conditions and measured the sensitivity, LOD, and sensing range of the IDEs sensor device, which makes it difficult to correlate the LOD of each experimental set.

Based on the previous protocols, two series of experiments were run in which the IDE chips were divided into two groups: one was modified with 54 μg/mL, and the other was modified with 108 μg/mL of CAnB. This was done to confirm the impact of the CAnB loading density on the sensitivity and the sensing capacity of the IDE chips. Because the 27 μg/mL CAnB's experiments showed low sensitivity and small detection range, we did not repeat the same concentration set here for the synthetic urine study. H-FABP was utilized as a negative control for both series. The difference in the impedance magnitude after the addition of the DAnB-GNPs was measured for each I-FABP concentration and is presented in Figure 3a,b for 54 and 108 μg/mL CAnB modified IDEs, respectively. For IDEs coated with 54 μg/mL CAnB, the impedance magnitude difference shows a linear increase with increasing concentration of the tested I-FABP up to 5 ng/mL. At a higher concentration (7 ng/mL), the impedance magnitude difference dramatically declined, as shown in the red columns of Figure 3c, red columns. This is consistent with the PBS buffer results and strengthens the case for the theory that at high concentrations of I-FABP, the sensitivity of the IDEs chips significantly drops due to either increasing the

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competition of the I-FABP capturing or saturation of binding regardless of the medium. Moreover, the sensitivity of IDE chips can be determined from the slope of the impedance difference against the concentration of the added I-FABP. It was found that by coating the IDEs with 54 μg/mL of CAnB the sensitivity of detecting I-FABP was 20 $\Omega\cdot(\mathrm{ng/mL})^{-1}$. However, by coating the IDE chips with 108 μg/mL CAnB, the sensing capacity extended and the impedance magnitude continued to increase at an I-FABP concentration of 7 ng/mL after the addition of the DAnB-GNPs. Furthermore, the sensitivity of the IDE chips significantly increased to 70.4 $\Omega\cdot(\mathrm{ng/mL})^{-1}$. In both series, by replacing the I-FABP with H-FABP, the IDEs showed an insignificant fluctuation in the impedance magnitude when the DAnB-GNPs were added. In the case of IDEs coated with 108 μg/mL CAnB, the LOD of I-FABP was calculated to be 0.38 ng/mL, where the slope of the linear calibration fitting is 70.4.

We investigate synthetic urine as a different dissolution medium for I-FABP to evaluate its effect on sensitivity robustness of the IDEs biosensor to different ambient conditions. Comparing the sensitivity of the IDEs biosensors to I-FABP dispersed in PBS buffer and synthetic urine mediums, we noticed that they are different by around 30%, where it is 101.7 and 70.4 $\Omega\cdot(\mathrm{ng/mL})^{-1}$, respectively. This was in the case of coating the IDEs with 108 μg/mL of CAnB. This distinction in the sensitivity could be attributed to either the impact of the I-FABP/CAnB binding medium on the efficiency of the protein/antibody interaction or the effect of the binding medium's ionic strength on the value of the measured impedance magnitude. It is worth mentioning that all of the impedance measurements proceeded in 10 μM PBS buffer with all selected media for dissolving the I-FABP in the current study. This was done to eliminate the impact of the measured buffer's ionic strength on the impedance value. This would eliminate the second possibility related to the impact of the binding medium's ionic strength on the value of the measured impedance. Several studies have been carried out to investigate the antibody−antigen interaction and the dominant rules.[20,21] Here, the differences between the PBS and synthetic urine medium are the ionic strength and the ion types. The I-FABP/CAnB interaction is a typical non-covalent protein−protein interaction, where four forces are controlling the binding: (a) hydrogen bonding, (b) electrostatic interaction, (c) dispersion forces, and (d) hydrophobic interaction. All of these forces are significantly affected by the interaction medium's ionic strength and the types of ions present, where the medium defines the charge distribution at the protein-antibody interface. For instance, the ionic strength and the types of ions of the I-FABP/CAnB interaction medium could affect the repulsion of the water molecules with non-polar groups at both molecules, an example of a hydrophobic interaction.[22]

Measuring I-FABP in Human Urine. To evaluate the clinical applicability of our IDEs biosensor chips, we tested their efficiency in an environment that would mimic the proposed application by using human urine spiked with known concentrations of I-FABP. Human urine samples were collected separately from three different healthy volunteers of ages between 20 and 27 years old, one female and two males, and different concentrations of I-FABP were spiked in each of them. Before starting, we test the collected human urine for I-FABP, and all of the samples were negative. In a separate vial, 5 ng of H-FABP was spiked in 1 mL of urine collected from each volunteer and used as a negative control. Two different concentrations of I-FABP were chosen to spike the urine samples, 2 and 5 ng/mL. It has been previously determined that 2.7 ng/mL or higher would be the critical concentration of the I-FABP that, if found in the patient urine, would require immediate surgical intervention.[23] Therefore, we selected these two values as representative for the critical range of I-FABP concentrations. We also changed the incubation time of the DAnB-GNPs addition step to 1 h instead of 2 h as was the case with PBS buffer and synthetic urine to confirm the pace of the detection. In a separate vial, 5 ng of H-FABP was spiked in 1 mL of urine collected from each volunteer and used as a negative control. For all experiments with real urine samples, the IDE chips were coated with 108 μg/mL of CAnB to enhance their sensitivity and maximize the detection range. We repeated each experiment five times to ensure the sensing behavior and to estimate the standard deviation. We noticed by using real human urine to dissolve the I-FABP, the values of the impedance magnitudes significantly increased in comparison with the other mediums. As shown in Figure 4, the IDEs

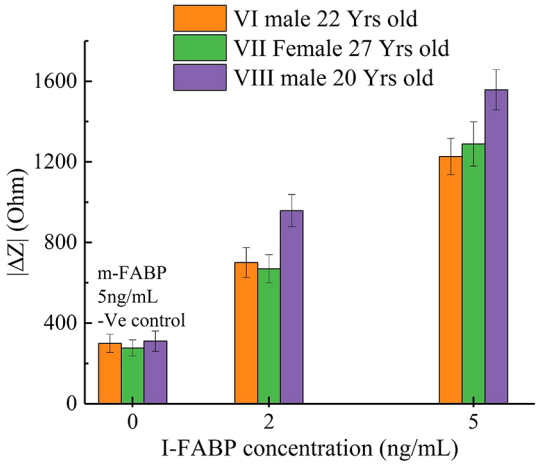

Figure 4. Impedance response at different concentrations of I-FABP spiked in real urine samples from three different volunteers. The measurements were collected at 10 mV, and the IDE chips were coated with 108 μg/mL of CAnB.

chips expressed a significant specificity toward I-FABP in comparison with the H-FABP, where the chips displayed a minimal response to the H-FABP. This can be assessed from the change in the impedance magnitude in the case of the H-FABP before and after the addition of the DAnB-GNPs, where it is less than 310 Ohms. After 1 h of the DAnB-GNP incubation with the IDEs chips, the impedance magnitude exhibited a noticeable change in the case of the I-FABP samples. As the concentration of the spiked I-FABP in the urine increased, the impedance magnitude dramatically increased after the addition of the DAnB-GNPs. This was the trend for all volunteers' samples, but the actual value of the impedance magnitude differed from one volunteer to the other. The sensing of the I-FABP using our biofunctionalized IDEs chips showed a high rate of reproducibility as can be concluded from the small standard deviation for both of the H-FABP and I-FABP measurements, which are between 14 to 16% and 8 to 12%, respectively, depending on the individual volunteer and the concentration of the I-FABP used in the experiment. The difference in the impedance magnitude values in each urine sample might be attributed to the effect of the I-FABP/CAnB interaction medium on the efficiency of the binding. We

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estimated the average sensitivity from the slope of the linear calibration fitting of dosage response for each volunteer and is about 208 $\Omega\cdot(ng/mL)^{-1}$, while the LOD was estimated to be 0.68 ng/mL. As we can see, the magnitude of the impedance is not only sensitive to the binding of the DAnB-GNPs with the captured I-FABP molecules, but also to I-FABP/CAnB interaction efficiency. The estimated value of the LOD is based on the analytical data collected from different experiments, which indicate the sensitivity of the IDE sensor and its capabilities of detecting I-FABP can be achieved below the critical concentration of 2.7 ng/mL.

The detection of the I-FABP usually takes around 6 h using the traditional ELISA bioassay and consists of multiple steps after the addition of the analyte solution (I-FABP). The procedure can include the treatment of biotinylated trace antibody, streptavidin-peroxidase conjugate, and the colorimetric 3,3′,5,5′-tetramethylbenzidine (TMB) steps. Each step takes around 1 h of incubation and multiple washes between steps. Here, we shortened the numerous detection steps and replaced with only one step, that is the biorecognition using DAnB-GNPs. We showed effectiveness after 1 h of binding.

CONCLUSIONS

A non-invasive and reliable POC device was built for rapid detection of I-FABP, a highly specific biomarker for AMI, based on an IDE array. The IDE signal was amplified by using DAnB-GNPs, which bound to immobilized I-FABP in a sandwich configuration. The clinical studies showed that the presence of I-FABP in a patient's urine at a concentration of 2.7 ng/mL or higher is a critical sign of acute intestinal ischemia. Our results proved the dynamic range of the developed biosensor encompasses I-FABP at concentration in urine down to 1 ng/mL and LOD 0.68 ng/mL. Also, compared with conventional enzyme-linked immunosorbent assay (ELISA), our impedance-based POC device showed a faster response time of less than 2 h and higher sensitivity when it was operated using real urine samples. Such a device will make rapid bedside monitoring feasible with the goal of preventing morbidity and mortality from AMI.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.8b00558.

Details of nanoparticle synthesis and characterization as well as IDE surface functionalization (PDF)

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Notes

The authors declare no competing financial interest.

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