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Ultrasonic frequency analysis of antibody-linked hydrogel biosensors for rapid point of care testing

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ABSTRACT

Analyte quantification in highly scattering media such as tissue, blood, and other biological fluids is challenging using conventional spectroscopic methods. Ultrasound easily penetrates these opaque samples, yet currently provides little chemical information. We have developed a general approach for creating hydrogel biosensors based on antibody-linked cellulose polymers. Target recognition induces changes to the sensor stiffness and size, which is accompanied by characteristic changes to a measured ultrasonic frequency profile. Using this technique, nM sensitivity for acetaminophen is demonstrated in a series of biofluids including whole blood, blood plasma, saliva, and urine. Likewise, this methodology is attractive for point of care diagnostics due to the short measurement time, simple methodology which excludes pretreatment of samples, and has minimal chemical or buffer requirements.

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1. Introduction

Subtle biochemical changes have far-reaching physiological impacts on the human body. Changes in metabolite concentrations or the introduction of xenobiotics can lead to responses ranging from minor allergic reactions to complete organ failure and death in a matter of minutes. Biochemical changes are also strong prognosticators of patient health. For instance, measurement of lactate concentrations in blood can provide information about tissue perfusion and oxygenation, which in turn leads to more efficient patient diagnosis [1]. A greater understanding of both the mechanisms of action and the biochemistry of medical conditions has led to a wide growth of specialized diagnostic medicine and treatment in recent years.

Laboratory test results play a key role in diagnostic decisions. In general, fast quantification of medicines and drugs of abuse leads to better patient health outcomes [2,3]. Biomedical analyte quantification is typically done spectrophotometrically. However, due to the highly scattering nature of tissue and biological fluids, optical measurements are often difficult to perform and involve sample pre-treatment such as centrifugation. Although current methodologies are well established, a lead time of several hours for results is common due to testing protocols and administrative overhead [4]. Likewise, operation of a full-scale laboratory to test for a broad range of analytes is often cost-prohibitive outside of primary care facilities. The resulting financial burden is therefore a large impetus to routine patient care and diagnostics.

Point of care (POC) diagnostic techniques have experienced substantial growth as an answer to remote diagnostics. Technologies have been developed using a wide variety of platforms such as lateral flow immunoassays and microfluidic devices [5], all with the aim of providing quick results on-site and minimizing operating costs. These devices have been used in the analysis of a wide variety of analytes including proteins, biomarkers, and viral infections [5,6]. Due to the financial and time savings, the development of POC technologies for small molecules in time-critical environments such as emergency rooms would be highly advantageous.

Acetaminophen (paracetamol) is a widespread medicine used for management of pain and inflammation. This analgesic is available without prescription and under a wide range of trade-names and dosages. However, acetaminophen is also one of the leading causes of liver failure as a result of accidental and deliberate overdose [7,8]. Fortunately, if acetaminophen overdose is detected within the first 12 h, an antidote, N-acetylcysteine, can be administered to a patient and provides nearly 100% protection of liver function. Determination of acetaminophen levels can be done in blood, urine, and saliva, using immunological and chromatographic assays [9–11]. Unfortunately, acetaminophen overdose is accompanied by non-specific diagnostic symptoms such as abdominal pain, nausea, sweating, and diarrhea. Specific indicators of hepatic

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failure such as jaundice and pruritus are typically delayed. As a result, diagnosing an acetaminophen overdose can be challenging without a blood test, often leading to liver damage before a diagnosis can be made. Metabolic processing of acetaminophen also creates a diagnostic challenge. Due to excretion, metabolism, and sequestering processes, the threshold of hazardous acetaminophen levels in blood lowers over time. Acetaminophen concentrations in blood above 1.3 mM represent a health hazard 1 h following ingestion. The threshold for hazardous concentrations decreases exponentially with time, reaching 20 μM after 24 h. As a result, there is a need for a technology with not only a sensitive measurement, but also a wide dynamic range. A quick and inexpensive technology for routine determination of acetaminophen concentrations in blood or other biofluids would therefore be extremely valuable. The design of this technology is the driving force of the work presented here.

Fixed acoustic oscillators for specific analyte determination have seen great interest with the advent of quartz crystal microbalances (QCM). These sensors are based on the linear relationship between the resonant frequency of a vibrating crystal and the mass deposition on the QCM sensor [12]. Mass loading on the QCM surface results in a fractional change to the resonance frequency of the crystal that can be measured. Surface coatings have been applied to crystal surfaces to facilitate specific analyte capture and immobilization. These targeted microbalances have been used for the determination of a wide variety of proteins, biomarkers, and antibiotics [13]. Arrays of microbalances have also been used to develop an "electronic nose," with the aim of simultaneous multi-analyte recognition [14,15]. However, sensor fouling in these applications remains problematic [16]. Device responsivities are dependent on interfacial effects such as molecular motion, surface irregularities, and adsorption of unwanted analytes.

Techniques have been developed to overcome interfacial effects that can limit QCM devices. Ultrasonic frequency analysis examines the attenuation of acoustic waves propagating through a medium. The contribution of molecular vibrations to the change in the ultrasonic intensity is related to the volume fraction of a molecular species [17]. This form of ultrasonic analysis has been employed for numerous rheological studies in colloids and for nano-particle sizing [18,19]. The colloids in these applications can also be designed to resonate at specific ultrasonic frequencies. Specifically tailored colloids have been developed into an image enhancement tool known as contrast agents. Typically used to improve visibility, the application of contrast agent is largely limited to qualitative information [20]. The ultrasonic frequencies and intensities required to make these measurements are non-destructive to biological samples. Likewise, the ultrasonic frequencies easily traverse media that are difficult to probe optically. Quantitative measurements using contrast agents or other acoustic resonators are therefore highly applicable to biofluid analysis.

In this work, we present dispersed, ultrasound-responsive resonators that are sensitive to acetaminophen. The sensors, which are analogous to contrast agents, consist of antibodies embedded in a polymer matrix. Antibody capture of target molecules is advantageous due to both the high selectivity and binding constants of antibodies towards an antigen. We propose that the conformational changes in the antibody that accompany antigen docking will alter the network stiffness of the polymer. The physical change in the polymer network will shift the resonating frequency of the sensor. The application of this sensor technique was investigated using acetaminophen. A simultaneous analysis of several frequencies allowed for the development of multivariate calibration models, which were validated on independent test data sets. Although the sensor could potentially be injected into tissue, there are a wide variety of *in vitro* applications for biological fluid analysis. Analyte assays in biological fluids are routinely performed in biomedical settings. The robustness of the system was examined by performing the analyses in samples of acetaminophen-doped blood, blood plasma, saliva, and urine.

1.1. Principle

Ultrasound-responsive polymer networks have been the focus of extensive treatment and characterization for ultrasonic contrast agent development and characterization. Contrast agents typically consist of a polymeric shell encapsulating an inert gas. Previous work has also shown that with ultrasonic compression and rarefaction, these contrast materials oscillate isotropically at specific resonant and subharmonic frequencies based on the physical properties of the polymer [21–23]. The proposed sensor platform relies on sub-micron hydrogels composed of crosslinked cellulose polymer chains. A number of cellulose hydrogel polymers are known to undergo large volume phase transitions between swollen and condensed states. Phase transition can be induced by changes in environmental forces such as temperature, ionic strength, and hydrostatic pressure [24,25]. The major step towards developing the ultrasonic sensor platform is to create a recognition site in these hydrogel polymers. Antibodies (IgG) were added to the polymer network in order to provide specific analyte targeting. When antigen binding occurs, conformational changes are induced in the antibody structure [26,27]. With the antibody crosslinked into the hydrogel network as described in the methodology, antigen-induced structural changes will also affect the cellulose network. Increased network strain, as well as mass loading from the antigen, results in stiffening of the overall polymer network. This, in turn, changes the fundamental resonance frequency of the polymer when an acoustic field is applied. Likewise, the change in mass loading and conformational change will also alter the effective size of the sensor, further modulating the resonance frequency of the polymer in the ultrasonic field. The result is a change to the ultrasonic frequency response of the solution that is induced by a specific external input (presence of the antigen).

There are many different models that can be generated to describe the resonance effect due to ultrasound. Although hydrogel microsphere have different physical characteristics than gas filled contrast agents, solid polymer particles are known to show a similar resonance effect [28]. The relationship between resonance frequency of hydrogels and the properties of the oscillating polymer can be illustrated using a conceptual model based on the work by Hoff et al. [21]. The resonance frequency (f_0) of the polymer sensors with respect to the network properties network can be approximated as

$$f_0 \propto \frac{1}{2\pi a_e}\sqrt{\frac{3K_p}{\rho_L}} \quad (1)$$

where a_e is the radius of the polymer gel at equilibrium, K_p is the bulk modulus of the polymer gel, and ρ_L is the density of the surrounding medium. Increasing the bulk modulus (stiffness of the polymer) increases the oscillation frequency in an asymptotic manner. In contrast, as the size of the polymer increases, an exponential decrease in the resonance frequency is expected. With analyte binding to the sensor, the resonant frequency is expected to shift to a new value dependent on both size and stiffness. The interrelationship between the two nonlinear parameters would lead to specific resonant frequency shifts based on the concentration of the target analyte. Though the specific frequency change may not be linear, multilinear analysis of the results should allow analytical quantification.

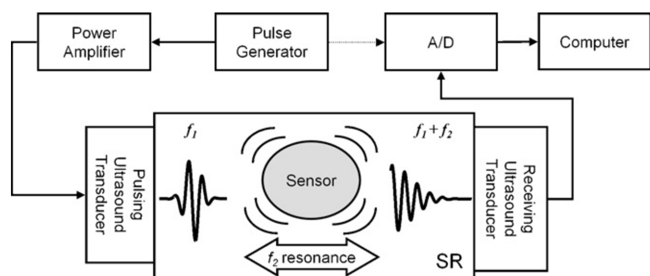

Fig. 1. Schematic diagram of the ultrasound frequency analyzer. Sample reservoir (SR) holds the biofluid sample and the antibody sensor. An ultrasound transducer generates a pulse waveform (f_1), which interacts with the media and sensor. A receiving ultrasound transducer records the waveform that has propagated through the SR and includes the resonance frequency of the sensor (f_2).

2. Materials and methods

2.1. Apparatus

Measurements of acetaminophen concentration were made using the transmission-mode ultrasound configuration depicted in Fig. 1. An ultrasonic Transmitter/Receiver (500PR Panametrics Inc.) was used to generate a <20 ns, 250 V negative impulse with a 0.002% duty cycle. This electrical impulse drove a 5.0 MHz Standard Contact ultrasonic transducer made by Technisonic (Fairfield, CT), which generated an ultrasonic wave train. Reflection of ultrasound waves at interfaces is significant. The wave train resulting from a single impulse reverberates back and forth within a sample cell. The result is an increased background signal and potentially overlapping signals. It is therefore important to allow sufficient time for the attenuation of the ultrasonic reverberations to baseline noise levels. A repetition rate of 1 kHz was used to ensure that any reflections were completely attenuated by the media before the subsequent impulse was generated.

The ultrasonic wave train was transmitted across a 0.9 mL Plexiglas cell with a 1.4 cm pathlength. Ultrasonic waves travel freely through both the liquid sample and the walls of the sample cell. Matching the cell width to the transducer diameter ensures that the measured ultrasound signal interacted only with the fluid media. In order to minimize interfacial ultrasound losses, the ultrasonic transducers were coupled to the sample across two layers. First, the Plexiglas cell wall was removed and replaced with a 60 μm acetate window. Acetate was used due to the lower dampening of the acoustic waves compared to the Plexiglas. The high acoustic impedance of air is a strong attenuator. To reduce the loss between the transducer face and the acetate window, a thin layer of petroleum jelly was used to as a coupling medium. Coupled together, the polyacetate window material and petroleum jelly layers did not significantly attenuate the ultrasound transmission. The transducer used to measure the transmitted acoustic waves was a second Technisonic 5.0 MHz probe. Overlapping bandwidth allows better frequency coverage and an increased sensitivity. The receiver was also coupled using an acetate window and petroleum jelly. Waveforms were digitized using a computer controlled oscilloscope (Handyscope HS3, TiePie Engineering) sampling at 50 MHz with a 12 bit dynamic range.

2.2. Materials

Deionized water was purified using a Millipore (Billerica, MA) Milli-Q OM-154 water purification system, which was used for all experiments. Chemicals were obtained from Sigma–Aldrich (Oakville, ON) unless otherwise noted. Peptide conjugation to the hydrogel network was accomplished using carbodiimide chemistry [29]. Carboxymethylcelluluse (cellulose, 150,000 MW) was dissolved completely into 10 mL of buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) to form a 0.01% solution. The coupling agent, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, 51 μg) was added to the cellulose solution. A protecting agent, N-hydroxysuccinimide (NHS, 15 μg) was added simultaneously to avoid hydrolysis of the EDC activated carboxylic acids, and to prevent the rearrangement of the o-acylisourea to the stable n-acylisourea product. The mixture was stirred for 15 min to allow complete dissolution. Polyclonal anti-acetaminophen (2.66 nmol, American Research Products, MA) was then added and stirred for 4 h to allow the carbodiimide coupling reaction. Divinylsulfone (30 μL) was added in 10 μL aliquots every hour to generate crosslinking within the cellulose chains. The ratio of carbodiimide to antibody in the solution should allow multiple conjugations with the cellulose. Likewise, divinylsulfone may also generate crosslinking with nucleophilic amino acid functional groups, in addition to cellulose crosslinking. The conjugated antibody–polymer hydrogels were dialyzed for 24 h using Spectra/Por regenerated cellulose membrane tubing (3500 MWCO) from Spectrum Laboratories (Rancho Dominguez, CA) to remove any unreacted crosslinking agents. Phosphate buffered saline (0.1 M, 137 mM NaCl, pH 7.4) was used during the dialysis to exchange the reaction buffer to a physiological conditions for better antibody binding.

To characterize the hydrogel sensor, two preparations of the cellulose polymer were made using the above procedure. The first batch was prepared with no antibody present. The resulting hydrogel is a crosslinked cellulose network with no analyte-specific binding sites. The second batch was made with the antibody present. Analyte recognition sites were present in this second batch, which represents the sensor used in subsequent experiments. Particle diameter and polydispersity index (PDI) measurements were made by dynamic light scattering using a ZetaPALS system (Brookhaven Instruments Corporation, NY). The mean particle diameter when no antibody was present in the hydrogel was determined to be 0.39 μm (0.53 PDI). Introduction of the antibody to the hydrogel network resulted in 20% growth of the hydrogel networks, with a mean particle diameter of 0.47 μm (0.45 PDI). The batch-to-batch reproducibility of the particle diameter was determined to be 10%, with a variation in the polydispersity index of 19%. Typical IgG antibody size is less than 20 nm, therefore simple surface coating of the cellulose gels to achieve this size increase is not likely. The increase in size suggests that antibodies may act as bridging sites between cellulose chains during the network formation. As a result, partial to complete embedding of the antibodies into the polymer network is expected. The impact of analyte binding to the sensor was also measured by the addition of acetaminophen to fill 100% of the antibody recognition sites present in a sample. A growth of 4% in the mean diameter (0.49 μm) was measured. An increase in size with the analyte present suggests that binding to the sensor is occurring, and further, that this is inducing strain on the network. As shown in the theory section, an increase in size should decrease the resonance frequency of the hydrogel sensor. Though other mechanisms of analyte adsorption may also be present, multilinear analysis of several ultrasonic frequencies should allow for acetaminophen quantification based on acetaminophen–antibody binding.

2.3. Samples

Whole bovine blood and rat blood plasma were obtained from the Comparative Medicine & Animal Resources Centre at McGill University. Human urine and saliva were collected approximately 2 h before analysis. Trials consisted of 21 independent samples. Aliquots of the antibody sensor were mixed with the acetaminophen-doped biofluids and were incubated for approximately 5 min to allow coupling between the antibody sensor and

acetaminophen present in the sample. Hydrogel sensor concentration in these samples was 0.01% by mass. Following incubation, biofluids containing the antibody sensor were injected into the sample reservoir for ultrasonic frequency measurement. The order of sample analysis was randomized to avoid sampling bias. Ultrasonic frequency spectra were collected after incubation at room temperature ranging between 21 °C and 24 °C.

2.4. Methods of analysis

Total data acquisition time for each sample was 1 min, during which 1500 waveforms were measured and saved. All data processing and analysis was done in Matlab (The MathWorks Inc., 2008a, Massachusetts, U.S.). Analysis consisted of three primary steps: waveform alignment, frequency transform, and multilinear modeling. The velocity of ultrasound, and therefore the phase of the ultrasonic waveform, is related to temperature. To reduce temperature effects in the power spectrum, phase matching of the recorded waveforms was made. Each waveform was aligned relative to the highest amplitude in the first peak. Following this alignment, the 1500 waveforms were averaged to increase the signal to noise ratio in each sample.

Reverberations are present in the data due to reflections at the cell interfaces. Since the impulse wave train is short relative to the path length of the cell, the reverberations are well separated temporally. Only the first transient waveform was retained for further analysis in order to decrease high-frequency ringing and baseline noise. The fast Fourier transform algorithm was then applied to the averaged waveform data to give frequency domain ultrasonic spectra. The frequency data were used for subsequent model generation. The frequency spectrum of each waveform was normalized to the total area. Likewise, water was measured between acetaminophen samples, and the normalized frequency distribution was subtracted to correct for any instrumental drifting. Frequency data between 1 and 10 MHz were retained for further processing.

Triplicate samples were prepared for each measured concentration of acetaminophen. Frequency spectra of the samples were divided into independent calibration and test sets. The calibration set consisted of two replicates of each concentration, representing two thirds of the total data. The test set was made up of the remaining samples not included in the calibration set. The calibration data were used to develop a model for acetaminophen concentration.

A stagewise multilinear regression (MLR) algorithm was used to correlate the intensity of ultrasonic frequencies with the acetaminophen concentration in samples. The algorithm determined the regression of the analyte concentrations with the intensity at each individual frequency. The residual values were then calculated and the process is repeated iteratively with the subset of frequencies that were not yet been incorporated into the model. The linear combination of frequencies with the highest correlation to analyte concentration described the data in the form

$$Y = b_0 + b_1X_1 + b_2X_2 + \ldots + b_nX_n \quad (2)$$

where Y is the dependant variable (here the acetaminophen concentration), $\{X\}$ are independent variables (the intensity at a given ultrasound frequency), and $\{b\}$ are the weighting coefficients determined. This multilinear regression model was then used to estimate and assign a numerical value for acetaminophen concentration in the independent test data set. To reduce overfitting of the regression, the standard error of the model was computed at the addition of each independent variable and weighting coefficient. The most parsimonious model was selected using an F-test ($\alpha = 0.05$) between calibrations so that the addition of any additional parameters would not significantly change the model. Details of the MLR model selection are provided in Draper and Smith [30] and the routine is presented in Arakaki and Burns [31]. Effectiveness of each model is tested by calculating the correlation coefficient and standard error of the estimate for the independent test samples.

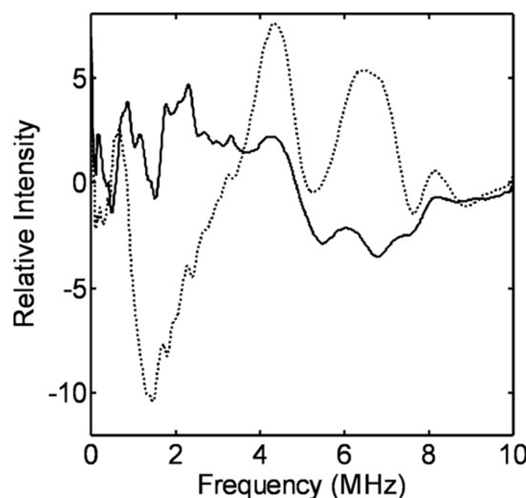

Fig. 2. Frequency profiles of antibody-linked sensor in the presence of low concentration (solid line) and high concentration (dashed line) of acetaminophen where the mean spectral profile has been subtracted.

3. Results and discussion

In order to determine the sensitivity of the method, a trial was designed to investigate acetaminophen quantification in whole blood, which is the most common biofluid analyzed. However, blood is composed of a wide array of proteins, metabolites, nutrients, and other chemicals that could potentially interfere with the ultrasonic detection. Sensitivity determination in this matrix therefore represents realistic conditions in which POC measurements using the antibody sensors would be made.

The relationship between the ultrasonic signal and antigen binding was investigated by varying the concentration of acetaminophen in whole bovine blood. Minimizing the volume of blood required for the measurement is important to lower the burden on a patient. To mirror what would be measured in vivo, acetaminophen was added directly to whole blood at concentrations ranging from 0.35 mM to 1.8 mM. These concentrations correspond to real toxic threshold levels in humans over 12 h following an overdose. The concentration of antibody sensor used was significantly lower, which necessitated dilution of the acetaminophen-doped blood samples. At each acetaminophen concentration, a 50 μL blood aliquot was taken, mimicking a single drop. The acetaminophen-doped blood was then diluted into phosphate buffered saline using serial dilutions. Final acetaminophen concentrations in the diluted samples ranged between 3.5 nM and 18.0 nM, representing a final dilution of $1/10^5$. The antibody sensor was then added to individual samples 5 min before the spectra were collected to allow time for antibody–antigen conjugation.

The interrelationship between the size and stiffness of the sensor polymer results in specific ultrasonic frequency profiles on the analyte concentration. Fig. 2 shows the frequency profiles of the antibody sensor with low (solid line) and high (dashed line) acetaminophen concentrations. The mean of the two profiles has been subtracted, which more clearly illustrates the characteristic differences in the frequency profiles. The principal effect is an exchange from low (<4 MHz) to high (>4 MHz) frequencies, whereby some frequencies increase and others decrease with concentration changes. Likewise, some changes occur in

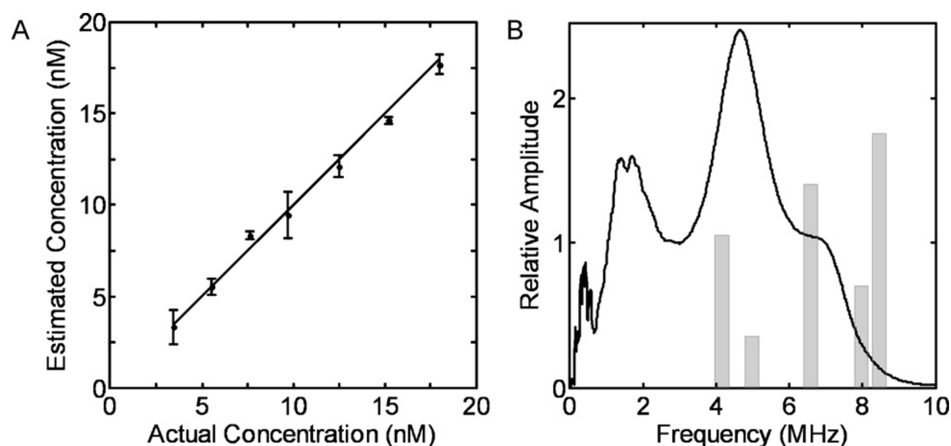

Fig. 3. Quantification of acetaminophen in a dilute whole blood. (A) Known acetaminophen concentration correlated with the concentration of acetaminophen as estimated by the multi-linear model. (B) Typical ultrasonic power spectrum for the antibody sensor in the buffered albumin matrix. Shaded bars illustrate the frequencies selected through MLR for the quantification of acetaminophen, height represents order of selection.

quadrature, where transient frequencies appear only at intermediate concentrations of acetaminophen. The exchange in frequencies is consistent with the model shown in Eq. (1). As the sensor becomes bound to acetaminophen, the stiffness will increase due to conformational changes induced during antibody–antigen binding, and the resonance frequency will increase.

Based on changes in the frequency spectra, a multilinear calibration model was developed for the estimation of acetaminophen concentration in the whole blood samples. A subset of the data (33% of the total samples) was used as an independent evaluation set. The multilinear model revealed a close correlation between known and estimated acetaminophen concentrations in both the calibration and the test data sets ($r^2 = 0.97$, SEE = 0.6 nM) using 5 best fit frequencies. Estimates of analyte concentrations using the multilinear model were plotted against the known values and are shown in Fig. 3(A). This plot demonstrates good linearity over the range of 15 nM with minimal bias.

The frequency response of the ultrasound system for the hydrogel polymer can be estimated by comparison to an existing ultrasound contrast agent. Albunex is a commercial contrast material that consists of air-filled albumin microspheres with a bulk modulus of 5.3 MPa (at radius of 1 μm) [32]. Using Eq. (1), a contrast agent with these properties is expected to resonate at approximately 20 MHz. Whereas the hydrogel sensors are not identical to this type of model, they should be in a similar range, therefore subharmonic frequencies would be measured using the 5 MHz ultrasound transducers. Fig. 3(B) illustrates the frequencies selected for the estimation of acetaminophen concentration using the cellulose sensor. The frequencies used in the multilinear model are clustered above 4 MHz. This range of frequencies would likely be associated with the third subharmonic of the fundamental resonance frequency. The absence of frequencies below 4 MHz in the multilinear model would then be expected because the fourth subharmonics in this range are of a lower magnitude. Ultrasonic analysis nearer to the fundamental harmonic frequency of the polymer sensor should further increase the sensitivity of the technique.

Concentrations of acetaminophen that represent a health concern are high, typically several millimolar. Therefore, monitoring the resonance frequency of the polymer as outlined above is several orders of magnitude more sensitive than needed for measurements in blood. When accounting for the dilution, the concentration range examined corresponds to 0.35 mM to 1.8 mM of acetaminophen in the undiluted blood sample. This range is crucial for POC diagnostic purposes as these acetaminophen concentrations correspond to dangerous levels of acetaminophen found in blood up to 8 h following an overdose. Although concentrations of acetaminophen below the detection limit presented would not be detectable, the dilution factor is easily adjusted. The hazardous threshold after 24 h is approximately 20 μM. Reducing the dilution factor to 1/100 would allow for the quantification of acetaminophen in a blood sample with no other modifications to the procedure.

To determine if ultrasonic frequency analysis is possible in this less dilute range, a trial was designed where whole blood was diluted 1/6. Biofouling and nonspecific adsorption is a common concern in these types of sensors. Reducing the dilution factor for the blood analysis increases the concentrations of a large number of molecules. As a result, any adsorption of the analyte or other molecules into the polymer network would be increased. Additionally, lower dilution will also increase the viscosity of the biofluid analysis sample, which could result in a greater attenuation of the ultrasound signal and shifts in the resonance frequency. For comparison with the highly dilute samples prepared above, the acetaminophen concentrations were in the same range (8.3 nM and 20.8 nM) after 1/6 dilution. Due to the lower dilution factor, these concentrations of acetaminophen in blood prior to dilution (50 nM to 125 nM) represent hazardous biological levels over 24 h after an overdose. Although changes in the frequency baseline profile are apparent with the increased concentration of blood, the addition of a sixth frequency in the multilinear model allows quantification. Multilinear estimation of acetaminophen concentrations showed a close correlation between known and estimated values for both the calibration and the test data sets ($r^2 = 0.95$, SEE = 1.1 nM) using 6 frequencies. Results demonstrate that the dilution factor plays a minimal effect on the quantification of acetaminophen. Despite a 1000 fold in whole blood concentration, similar detection limits are observed, with an increase in error from 0.6 nM to 1.1 nM. This similarity suggests that the resonance frequency of the polymer sensor is minimally affected by the matrix. Likewise, although nonspecific binding to the hydrogel network may increase, multilinear correlation between the sensor resonance frequency and acetaminophen loading is still possible.

A trial using blood plasma was designed to determine if quantification of acetaminophen was more accurate with the removal of blood cells (including red, white, and thrombocytes). Plasma is composed largely of water, and contains a wide array of proteins, dissolved nutrients, and other metabolites. Due to the absence of blood cells, plasma is less viscous than whole blood. The trial was designed to match acetaminophen concentrations with those in the 1/6 dilution whole blood, 8.3 nM to 20.8 nM. Similarly, the

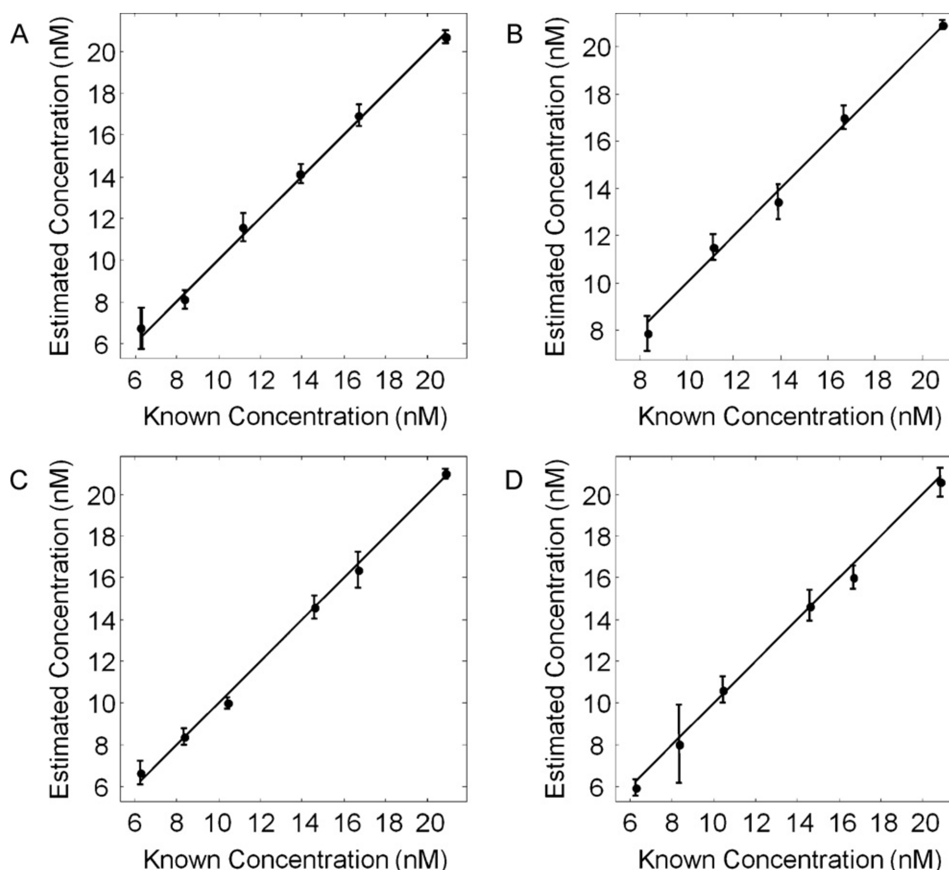

Fig. 4. Quantification of acetaminophen in a series of biological fluids. Known acetaminophen concentration correlated with the concentration of acetaminophen as estimated by the multi-linear model in (A) blood plasma, (B) saliva, (C) undiluted urine, and (D) diluted urine.

plasma samples were diluted 1/10. Multilinear analysis revealed a high correlation between estimated and known concentrations of acetaminophen (r^2 = 0.97, SEE = 0.8 nM). The concentrations of acetaminophen estimated by the multilinear model were plotted against the known values in Fig. 4(A). As with the 1/6 dilution blood, the concentration of acetaminophen in the plasma prior to dilution (83 nM to 208 nM) represents toxic levels over 24 h after an overdose. The error of the acetaminophen quantification in plasma is similar to that in the $1/10^5$ dilute blood (0.6 nM). The multilinear fit of the determination in 1/6 dilution whole blood was lower (r^2 = 0.95), which would be expected because the samples were significantly more viscous than plasma or dilute blood.

To examine if the ultrasonic sensor platform was applicable for other matrices commonly used for acetaminophen measurements, quantification in saliva was also investigated. The trial was designed to compare the same concentrations of acetaminophen as in the blood samples, since both biofluids have similar levels [33]. As outlined in Table 1, good quantification of acetaminophen was shown over the same range as 1/6 dilution whole blood. Prior to a 1/3 dilution, concentrations of acetaminophen in saliva from 25 nM to 62 nM would also be expected over 24 h after an overdose. Fig. 4(B) illustrates the correlation between the estimated and known concentrations of acetaminophen in saliva. Saliva demonstrated the second lowest sensitivity in the biofluids examined, with nearly twice the error of the $1/10^5$ dilution whole blood. This is probably due, in part, to the large number of human and bacterial cells, as well as dissolved gas bubbles, which are present in saliva. Likewise, saliva also contains a glycosylated mucous, which is highly viscous, and attenuates ultrasound propagation similar to results seen for whole blood. Despite the sensitivity limitations, saliva can be collected quickly and non-invasively, making measurement of acetaminophen in this biofluid appealing for point of care applications.

In order to determine the effect of diluting the biofluid matrix while keeping the viscosity relatively constant, acetaminophen quantification was determined in urine. The viscosity of urine is low relative to whole blood and saliva due to the higher water content. Although primarily made up of water, urine also contains a wide variety of small molecules, and can be excreted over a relatively wide pH range. Due to metabolic processing, less than 5% of the acetaminophen dose is excreted in urine without conjugation, though this can increase up to 10% in patients with toxic doses [34]. In clinical practice, the low concentration of biomarkers (including acetaminophen) often necessitates collecting the first urine of the day to take advantage of overnight bioconcentration. To simulate these conditions, acetaminophen quantification was examined in both a morning sample of urine and in a dilute urine sample ($1/10^4$).

The analysis of both undiluted and dilute urine revealed a close correlation between the ultrasonic frequency intensities and

Table 1
Acetaminophen concentration estimation based on ultrasonic measurements in selected biological fluids.

Biofluid	Dilution factor	Acetaminophen concentration range (nM)	Fit (r^2)	Error (SEE) (nM)
Whole blood (bovine)	$1/10^5$	3.5–18.0	0.97	0.6
Whole blood (bovine)	1/6	8.3–20.8	0.95	1.1
Saliva (human)	1/3	8.3–20.8	0.96	1.1
Blood plasma (rat)	1/10	6.2–20.8	0.97	0.8
Urine (human)	0	6.2–20.8	0.97	0.9
Urine (human)	1/104	6.2–20.8	0.97	0.6

acetaminophen concentrations between 6.2 nM and 20.8 nM (see Table 1). Fig. 4C and D shows the concentration estimates plotted against the known values, demonstrating that minimal bias in the calibration is present. For the dilute urine with approximately 5% excretion in urine, these concentrations of acetaminophen in urine prior to dilution would indicate blood concentrations of 62–208 μM. This represents hazardous biological levels between 5 and 10 h following an overdose. For the completely undiluted urine, the nM concentrations would be expected in toxic cases over 24 h following the overdose. The acetaminophen concentration was estimated with a high correlation ($r^2 = 0.97$) in both cases, and with only a 0.3 nM difference in the standard error between concentrations. Results suggest that the low concentration of other constituents in the urine matrix had a minor impact on the sensor resonance frequency when the viscosity is not changed significantly.

As with whole blood, the detection limits of acetaminophen in blood plasma, saliva, and urine are several orders of magnitude better than the diagnostic requirements for the detection of an overdose. Further, the dynamic range of the measurement can be increased by adjusting the biofluid dilution factor. This provides an advantage over current POC systems such as the TOX Drug Screen (Triage, San Diego) which provide qualitative assessment of toxicity based on a fixed acetaminophen concentration. Ultrasonic measurements of the antibody sensor could indicate whether a patient would have a low or high risk of serious health problems due to acetaminophen consumption over wider time window following overdose.

4. Conclusions

Ultrasonic frequency analysis is a promising technique for the determination of specific analytes using antibody-based sensors. The dispersed, sub-micron antibody sensor has a high ratio of surface area to volume, which can improve the sensitivity of the sensor [35]. The sensor platform was shown to be sensitive in blood, blood plasma, saliva, and urine, each of which contains a wide variety of proteins, metabolites, and other small molecules. While biofouling of the hydrogels through nonspecific absorption can be a serious problem for these types of sensors, multilinear analysis revealed systematic variations in frequency response which can be used to correct for these effects. Frequency variations were measured and used to create multilinear models to quantify acetaminophen between 3.5 nM and 20.8 nM. Conventional biomedical blood tests are currently costly and time consuming. The key advantage of the technique outline in this work is that all steps from sample collection to measurement and diagnostic results can be carried out in less than 10 min. With an appropriate sample dilution method, this is comparable in size, analysis time, and complexity with other commercial POC systems on the market. The use of ultrasound allows for direct analysis without significant sample treatment, which is important for fast medical response in a relatively narrow treatment window for acetaminophen overdose. Minimizing procedural steps is important not only for acetaminophen analysis, but also for other analytes which could potentially be measured using this methodology. Additionally, the instrumentation costs are low relative to operating a fulltime laboratory. Likewise, the cellulose-based sensor is non-toxic, reducing the environmental footprint of the device. Because laboratory tests play a key role in the majority of diagnostic decisions, using this technique for analyte determination would decrease time and cost burdens on the health care system and result in better patient outcomes.

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