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Ultrasonic quantification using smart hydrogel sensors

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ABSTRACT

Analyte quantification in samples with extensive matrix effects can be challenging using conventional analytical techniques. Ultrasound has been shown to easily penetrate samples that can be difficult to measure optically or electrochemically, though it provides little chemical information. Recent ultrasound contrast agents provide highly localized contrast within a sample based on concentration. We have developed a general approach for creating smart biosensors based on molecularly imprinted hydrogel polymers that recognize and bind a target analyte, changing ultrasonic properties with analyte concentration. Multilinear analyte calibration in hydrogel solutions provided quantification of the chosen analyte, theophylline, from 8.4 μM to 6.1 mM with a high degree of linearity (correlation coefficient exceeding 0.99). Simultaneous quantification of both theophylline and of an interfering species, caffeine, was also carried out, providing an avenue for simultaneous analyte analysis with one smart biosensor that can be dispersed and remotely detected.

1. Introduction

1.1. Background

Detection and quantification of analytes in biomedical applications is typically done by optical spectroscopies or electrochemical techniques. However, many samples containing multiple analytes with overlapping chemical signatures often require chromatographic separation or extensive sample pre-cleaning [1–3]. We have developed a general system for creating smart biosensors based on molecularly imprinted hydrogel polymers. The biosensor exhibits a characteristic ultrasound frequency profile that is dependent on the stiffness and size of the polymer. Target recognition and binding cause changes to these physical parameters, and changes to the ultrasonic frequency profile are measured. We have applied this technique to the analytical determination of theophylline, a therapeutic agent for respiratory diseases. Therapeutic concentrations of theophylline are typically in the 55–110 μM range [4]. Caffeine, a structurally similar molecule, was also investigated by this method as a competitive agent. Using this technique, a high degree of linearity for individual and simultaneous quantification of both chemical species is demonstrated.

Hydroxypropyl cellulose (HPC) and N-isopropylacrylamide (NIPA) hydrogels undergo a reversible, volume phase transition between swollen and condensed states in solution. In the swollen state, hydrogel solutions are clear, owing to a large and diffuse structure. The condensed state is characterized by an increase in turbidity as the hydrogel microspheres contract, becoming stiffer and expelling water from the structure. As a result of these physical changes, ultrasonic properties of hydrogels are highly dependent on the phase of the gel. Hydrogel phase transitions can be prompted by external forces such as fluctuations in ionic strength, temperature, or hydrostatic pressure [5,6]. When the temperature of the hydrogel solution is elevated past the critical threshold temperature (T_c), a broad change to the ultrasound spectrum will be seen. Fig. 1 shows the ultrasound frequency profiles of the HPC and NIPA hydrogels. The mean of the spectra has been subtracted to more clearly illustrate the changes in the frequency power spectrum when the hydrogels undergo a temperature-induced phase transition. It can be seen that frequency exchanges occur over the entire spectrum, including both in-phase and out-of-phase changes.

To promote analyte specificity in the hydrogel biosensors, molecularly-selective binding sites can be created by allowing self-assembly of the hydrogel monomers and analyte prior to polymerization [7,8]. Upon docking of the analyte into an imprinted pocket, the hydrogel will undergo a change in physical properties and in ultrasonic response. Molecular sensitivity of the binding sites is dependent on the affinity of imprinted hydrogels for an analyte. This affinity for the template is based on hydrogen bonding between the polymer network and the template molecule, as well as steric factors due to the shape of the molecularly imprinted pocket. By adjusting the specific chemistries of the hydrogels, both low and high affinity biosensors can be created. With high molecular weight HPC, affinity towards the analyte is based on large,

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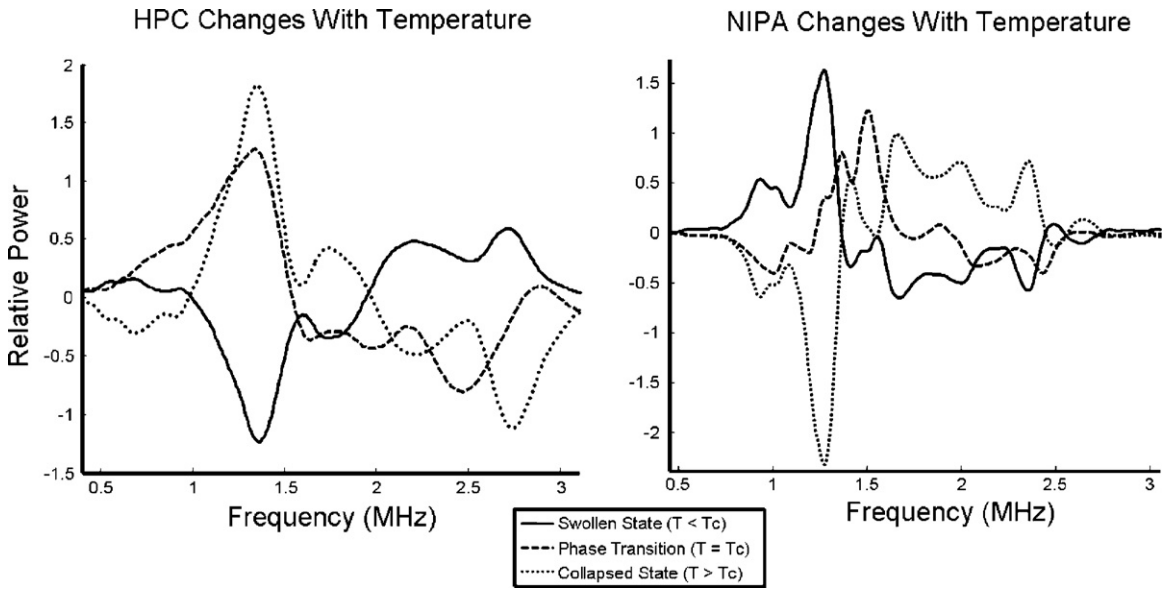

Fig. 1. Frequency profiles of NIPA and HPC hydrogels undergoing phase transition as a result of increasing temperature. Mean ultrasound spectral profile has been subtracted to emphasize the changes before ($T < T_c$) (solid line), during ($T = T_c$) (dashed line), and after ($T > T_c$) phase transition relative to critical threshold temperature T_c.

loose hydrogen bonding pockets once crosslinking is complete [9]. In contrast, high affinity binding is achieved in NIPA hydrogels that are copolymerized with methacrylic acid (MAA), which forms strong hydrogen bonds with the template [10]. Molecularly imprinted NIPA hydrogels have been shown to exhibit turbidity changes with sub-mM concentrations of theophylline [11].

We present a unique method for analyte quantification using imprinted hydrogels in concert with ultrasonic detection. Molecularly imprinting hydrogels with analyte template can provide high degrees of analyte specificity. Non-invasive ultrasonic detection of hydrogels provides potential for rapid, efficient detection of analytes based on contrast enhanced backscatter at select frequencies.

1.2. Principle

A conceptual model can be developed to illustrate the changes to the ultrasonic frequency profile of hydrogel polymers when an analyte binds to the molecularly imprinted pockets. Considering hydrogels to be deformable microspheres in solution with given diameter and modulus, changes in hydrogel microsphere physical properties at equilibrium can alter ultrasonic profile. Each microsphere resonates at a given frequency when perturbed by ultrasound pressure waves. Resonant frequency shifts occur as a result of altering the size and stiffness of the microsphere. Physical properties of molecularly imprinted hydrogels change with docking of an analyte. In particular, hydrogel microspheres have been shown to both increase in stiffness and decrease in size as analyte molecules occupy the binding sites [12,13]. With ultrasonic compression and rarefaction, polymer beads oscillate isotropically at specific resonance frequencies dependent on these physical properties [14]. A model, while not ideal for network hydrogel beads in solution, approximates behavior in ultrasonic fields by assuming hydrogels to be gas-filled microspheres with a thin polymer shell. This thin shell approximation is straightforward to model. Though exact comparisons would not be expected from the shell model for spherical hydrogel networks, the model illustrates relative trends of the resonant frequency with changing hydrogel physical properties. Hydrogel resonance frequency for thin-shelled microspheres in solution can be derived from the resonance frequency of gas-filled contrast agent microbubbles as

$$f_0 = \frac{1}{2\pi a_e}\sqrt{\frac{1}{\rho_L}\left(3kp_0 + 12G_s\frac{d_{Se}}{a_e}\right)}, \quad (1)$$

where a_e is the equilibrium radius of the microbubble, ρ_L is the density of the surrounding liquid, k is the polytropic exponent of the gas, p_0 is atmospheric pressure, G_s is the shell shear modulus, and d_{Se} is the shell thickness [15]. Bulk modulus of an adiabatic gas, K_g, can be written as,

$$K_g = kp_0, \quad (2)$$

while the bulk modulus of a polymeric microbubble, K_p, receives additional contributions from the outer shell,

$$K_p = K_g + 4G_s\frac{d_{Se}}{a_e}. \quad (3)$$

For a deformable hydrogel microbubble with thin shell and no interior gas, the bulk modulus contribution from adiabatic gas can be negated ($K_g = 0$), and using Eq. (3), Eq. (1) can be rewritten as,

$$f_0 = \frac{1}{2\pi a_e}\sqrt{\frac{3K_p}{\rho_L}}, \quad (4)$$

which resembles the Minnaert equation for microbubbles. As the modulus (or stiffness) increases, the resonance frequency of the hydrogel would increase. In contrast, an increase in hydrogel radius would result in the hydrogel resonance frequency decreasing.

An imprinted hydrogel will have a specific resonance frequency based on the size and stiffness. When the template molecule binds to the molecularly imprinted pocket, both the size and stiffness of the gel are expected to change, leading to a more complicated relationship of the resonance frequency. Though a structurally similar molecule with lower affinity may also interact with the molecularly imprinted pocket, the decreased hydrogen bonding would result in different physical changes through the hydrogel network. In a hydrogel with multiple pockets, the relationship between the modulus and radius leads to specific frequency profiles for hydrogels dependent on both the concentration and affinity of the hydrogel analytes bound. In addition, nonlinear propagation of pulsed ultrasound in aqueous samples at high acoustic pressures broadens the frequency content [16]. Therefore, multiple resonance frequencies could be probed with one ultrasonic pulse. The concentration of

analyte absorbed into the hydrogel pockets was estimated based on measurements of multiple frequencies within the ultrasound pulse propagating through the sample cell.

2. Materials and methods

2.1. Synthesis of theophylline imprinted HPC polymer

Low affinity molecularly imprinted HPC was prepared by 0.5 g of HPC powder (100,000 MW) and 0.1 g of theophylline to 49.4 g of dH_2O and stirring for 3 days to form a homogeneous 1 wt% solution of HPC. Fourty μL of divinylsulfone (DVS) and sodium chloride to a concentration of 1 mM were added [17]. After 3 h of stirring, 5 drops of 1 M sodium hydroxide were added to the solution to raise the pH to 12. The cross-linking reaction was allowed to continue for 5 h. The cross-linked polymers were then dialyzed against dH_2O for 3 days to remove the theophylline and any free DVS. The extraction of theophylline was confirmed spectroscopically at 271 nm. All chemicals were purchased from Sigma–Aldrich (Ontario, Canada). The mean HPC hydrogel diameter was determined to be 105 nm (0.39 PDI and batch-to-batch variability of 20%) by dynamic light scattering using a Brookhaven Instruments ZetaPALS particle size analyzer.

2.2. Synthesis of theophylline imprinted NIPA polymer

High affinity molecularly imprinted NIPA was prepared by adding 1.0 g of NIPA monomer, 0.08 g of N,N′-methylene-bis-acrylamide (MBA), 0.08 g methacrylic acid, and 0.18 g theophylline to 99 mL of distilled water (dH_2O) to form a homogeneous 1 wt% NIPA solution with stirring over 4 h to ensure complete dissolution [18]. Oxygen in the solution was purged with nitrogen gas during this time. Following this, 15 mg of ammonium persulfate to initiate the polymerization and 60 μL of tetramethylethylenediamine as an accelerator. The solution was left to polymerize for 4 h with gentle stirring. Once the imprinted hydrogel was formed, the theophylline was removed by successive methylene chloride extractions. The extraction of theophylline was confirmed spectroscopically at 271 nm. All chemicals were purchased from Sigma–Aldrich (Ontario, Canada). The mean NIPA hydrogel diameter was determined to be 396 nm (0.39 PDI and batch-to-batch variability of 5%) by dynamic light scattering using a Brookhaven Instruments ZetaPALS particle size analyzer.

2.3. Hydrogel solutions

HPC and NIPA hydrogels were used to estimate theophylline concentrations based on the ultrasonic response. Solutions containing 1% imprinted hydrogel by weight in water were made and increasing amounts of analyte were added to each solution. In the low affinity-binding imprinting HPC, concentrations of theophylline ranged between 0.2 and 6.1 mM. In order to reflect the higher affinity binding in NIPA, concentrations of theophylline ranged between 8.4 and 167 μM theophylline.

To assess the selectivity of the molecularly imprinted hydrogels, caffeine was used as an interfering species while calibrating for theophylline, due to the chemical structures differing by one methyl group. Matrices of 25 HPC and 30 NIPA 1% by weight solutions were prepared, wherein the concentrations of both theophylline and caffeine were varied. For NIPA, concentrations of caffeine ranged from 4.1 to 21 μM over six solutions and concentrations of theophylline spanned 8.4 to 24 μM over five solutions per caffeine concentration. Likewise, for HPC, the caffeine and theophylline concentrations ranged from 0 to 9.1 mM and 0.2 to 6.1 mM, respectively.

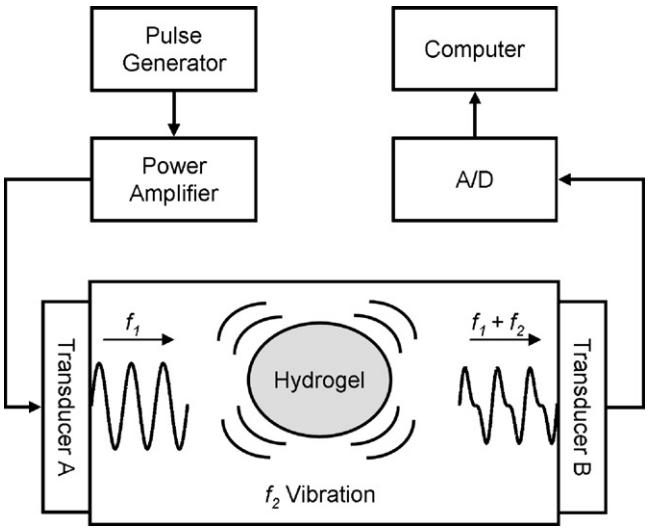

Fig. 2. Schematic for ultrasound data acquisition. A pulse generator and power amplifier are used to drive an ultrasound transducer. Transducer A generates pulses at frequency f_1, which travel through the sample cell and interact with the hydrogel sensors. Transducer B on the parallel end of the sample cell records the ultrasound pulse that includes frequency f_2 cause by the resonating polymer. A computer controlled oscilloscope digitized the signal for further analysis.

2.4. Instrumentation

Ultrasound transducers for sending and receiving pulses were affixed to the sample reservoir as shown in Fig. 2. The sample cells used were a 1.8 cm plexiglas cuvette for the HPC solutions and a 3.8 cm aluminum cell for the NIPA solutions. A 1.9 MHz narrow-band transducer (Advanced Technology Labs Inc., Pennsylvania, U.S.) generated ultrasound pulses and a 10 MHz wideband transducer (Optel Inc., Wrocław, Poland) received the ultrasonic signal. A Panametrics 500PR Pulser/Receiver (Panametrics Inc., Massachusetts, U.S.) as the pulse generator for the transducers. The Panametrics 500PR generated 10 ns 250 V negative impulses to drive the 1.9 MHz narrow-band transducer. A SDS 200 oscilloscope (SoftDSP Co., Seoul, Korea) sampling at 12.5 MHz using 9 bit A/D conversion, and Handyscope HS3 (TiePie Engineering, Sneek, Netherlands) sampling at 50 MHz using 12 bit A/D conversion, collected the ultrasonic data from the 10 MHz wideband transducer for the NIPA and HPC, respectively.

2.5. Data processing

Ultrasound waves propagating through the sample cell were measured by the receiving transducer and digitized by the computer controlled oscilloscope. Data for each sample were acquired over 3 min, for a total number of 4000 waveforms. These waveforms were averaged to increase the signal to noise ratio. A fast Fourier transform algorithm was then applied to the averaged data to allow processing in the frequency domain. Variability due to instrumental and temperature changes was minimized by total area normalization, and random noise fluctuations were removed using a boxcar smoothing function. Frequency spectra were divided into independent calibration and test sets. The test set consisted of a series of samples with varying theophylline levels at one caffeine concentration that was not found in the calibration data set.

Stagewise multilinear regression (SMLR) was used to estimate the concentration of theophylline based on the magnitude of the ultrasound frequencies measured for a given sample. The algorithm determined the regression of the magnitude at each frequency with the analyte concentrations in the calibration set in order to determine the highest correlation. The residual values are then

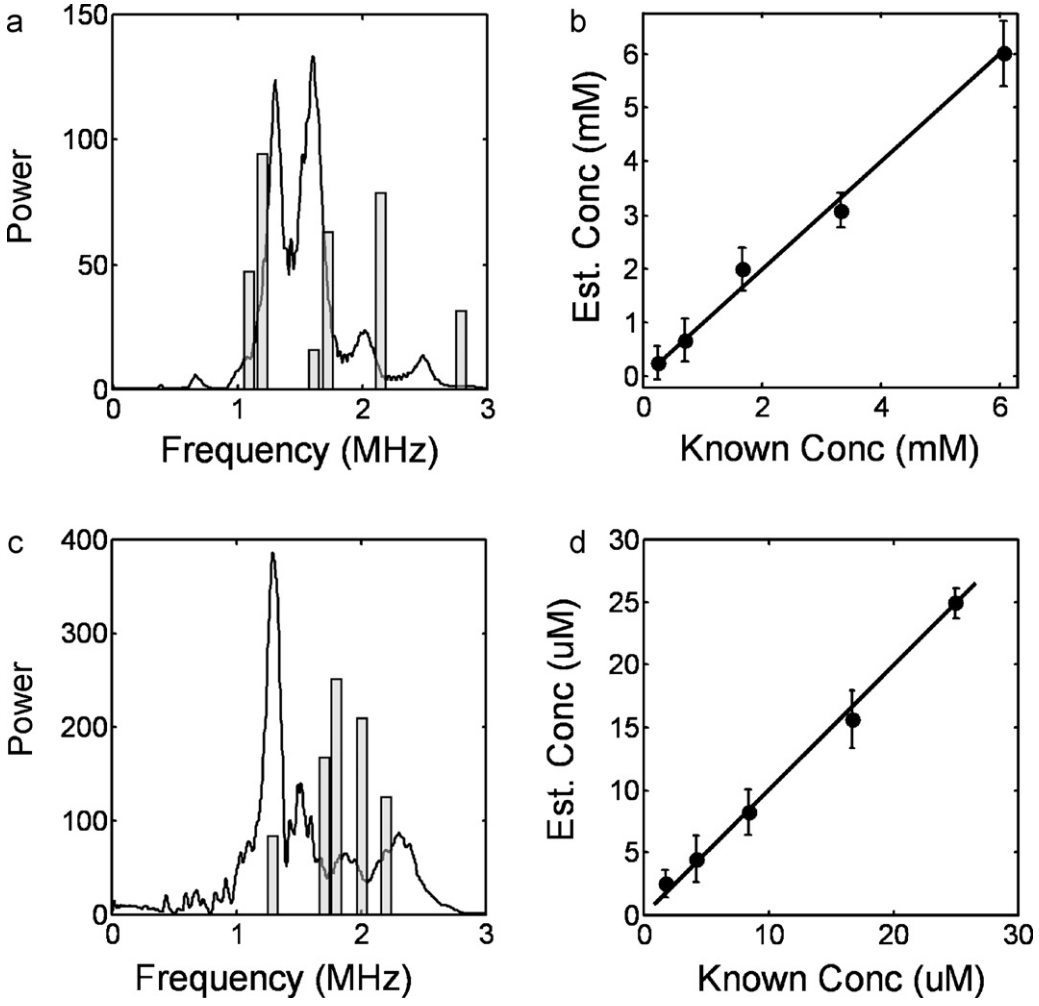

Fig. 3. Theophylline quantification results in presence of caffeine. (a) Ultrasonic frequencies selected to determine analyte concentrations in HPC, (b) estimates of theophylline concentrations with HPC sensors using a multilinear model plotted against known values, (c) ultrasonic frequencies selected to determine analyte concentrations in NIPA, (d) estimates of theophylline concentrations with NIPA sensors using a multilinear model plotted against known values.

calculated and the process is repeated iteratively with the subset of frequencies not yet included in the model. Based on the linear combination of this subset of ultrasonic frequency intensities, the data are described in the form

$$Y = b_0 + b_1X_1 + b_2X_2 + \cdots + b_nX_n \quad (5)$$

where Y is the dependent variable, $\{X\}$ are independent variables, and $\{b\}$ are weighting coefficients. This multilinear model was subsequently used to estimate the concentration of the analyte in the independent test set samples. To avoid overfitting of data by the model, parsimony was determined by F-test ($\alpha = 0.05$) between the standard errors of each model so that the addition of additional parameters would not significantly impact the SMLR model and estimation [19]. All data analysis was done in Matlab (The MathWorks Inc., 2008a, Massachusetts, U.S.).

3. Results and discussion

To examine the relationship of the ultrasound signal with analyte binding in the hydrogels, we have quantified the target analyte sensitivity in a matrix free of interfering species. In both HPC and NIPA hydrogels, the concentration of theophylline was estimated with three frequencies in the derived model. In the low affinity binding HPC, concentrations of theophylline were determined in the millimolar range. A standard error of estimate (SEE) of 0.1 mM with a correlation coefficient (r^2) exceeding 0.99 was obtained in the 0.1–6.1 mM range. With theophylline binding, the mean particle size of the HPC hydrogel increased by 14%. The higher affinity binding of NIPA permitted using a micromolar theophylline concentration range, with a SEE of 2.6 μM and an r^2 exceeding 0.99 in the 8.4–167 μM range. Unlike the HPC sensor, binding to theophylline resulted in a 16% decrease in overall sensor diameter. The decrease in size suggests that the stronger hydrogen bonding between NIPA and theophylline results in a tighter induced fit in the molecularly imprinted binding pocket. The greater structural changes would cause larger changes to the resonance frequency of the NIPA hydrogel, increasing the sensitivity.

An interfering species was used in order to assess the specificity of the molecularly imprinted hydrogels. A commonly used pair of chemicals to determine sensitivity and specificity of imprinted hydrogels is theophylline and caffeine, which are molecularly-similar xanthine derivatives [11,20]. Presence of an additional N-methyl group on caffeine decreases hydrogen bonding potential and increases steric hindrance for molecularly imprinted binding sites resulting in a lower binding constant. Solutions were prepared in which theophylline and caffeine of comparable concentrations were independently varied. Caffeine concentrations ranged from 0 to 9.1 mM in HPC and from 0 to 21.0 μM in NIPA. Likewise, concentrations of theophylline were ranged from 0.2 to 6.1 mM and from 8.4 to 24.0 μM for HPC and NIPA, respectively.

To estimate the concentration of theophylline independently of caffeine, separate calibration models using SMLR were created for HPC and NIPA sensors. Models were tested on an independent evaluation set consisting of one concentration of caffeine excluded from the calibration data. For low affinity HPC, the use of six frequencies, 1.2, 2.2, 1.7, 1.1, 2.8, and 1.6 MHz, was determined to be the most parsimonious for the quantification of theophylline and resulted in a SEE of 0.6 mM with a corresponding r^2 of 0.95. Fig. 3(a) illustrates the typical frequency spectrum of HPC hydrogels, as well as the frequencies used by the multilinear model for theophylline estimation. The concentrations of theophylline estimated by the SMLR model are plotted against the known values in Fig. 3(b), showing linearity of the full range with minimal bias. The most parsimonious estimation of theophylline in high affinity NIPA hydrogels was obtained with five frequencies, 1.8, 2.0, 1.7, 2.2, and 1.3 MHz. A SEE of 1.5 μM with a corresponding r^2 of 0.98 was obtained. Fig. 3(c) illustrates the typical frequency spectrum of NIPA hydrogels, as well as the frequencies used for theophylline estimation. The concentrations of theophylline estimated by the SMLR model are plotted against the known values in Fig. 3(d), showing linearity of the full range with minimal bias. The difference in selected frequencies and correlation coefficients for multilinear quantification in both hydrogels can be partly explained by the different analyte binding strengths and levels of interferant competition. Two nearly identical molecules competing for the same localized hydrogen bonding sites can alter with the hydrogel resonant frequencies. The low affinity HPC hydrogel required an addition frequency component to quantify theophylline with caffeine due to the greater impact an interferant has on a less specific analyte binding environment. Results show that calibration was possible for both hydrogels independent of caffeine interference with an overall linear quantification spanning the micromolar to millimolar range.

To determine if simultaneous analyte quantification was possible using the ultrasound signal, concentration of caffeine in the above theophylline/caffeine mixtures was estimated. Chemical similarity between the two xanthines allows both of the compounds to access the binding sites, albeit with separate binding constants. The decreased interaction between caffeine and the molecularly imprinted pocket as compared to theophylline should induce distinctly different physical changes in the hydrogel. As with theophylline, sensitivity of HPC to caffeine was lower than in NIPA, and for this analyte, proved to be too low for quantification ($r^2 < 0.6$). The mean particle size increased by 23% with caffeine binding, compared to 14% with theophylline. It is possible this greater increase in particle size decreased the resonance frequency of the HPC hydrogels so that the bandwidth of the ultrasonic transducers was not optimal for the determination of caffeine-bound sensors.

Simultaneous caffeine quantification was possible with the NIPA sensor using five frequencies resulting in a SEE of 3.3 μM and an r^2 of 0.87. The mean particle size for caffeine binding with NIPA showed only a 5% decrease, as compared to the 16% decrease with the theophylline molecule. This suggests that while the hydrogen bonding may still have formed strong associations, the presence of the additional methyl group likely resulted in a steric strain on the pocket, preventing certain conformational changes that result when the template molecule occupies the pocket. Lower correlation coefficients were expected when quantifying caffeine in this scenario, as the hydrogels were molecularly imprinted with a theophylline template which is structurally different than caffeine. Results are consistent with the proposed model and provide a mechanism for simultaneous quantification of multiple analytes with one hydrogel sensor. With tuning of the imprinted hydrogel using different binding mechanisms such as, hydrophobicity and π stacking, high sensitivity and specificity should be possible for several analytes.

4. Conclusions

We have successfully quantified theophylline in solutions ranging between 8.4 μM and 6.1 mM using two molecularly imprinted hydrogels. This allows physiological monitoring of theophylline within the therapeutic range (55–110 μM), as well as higher concentrations which are toxic [3]. The model for contrast agent resonant frequency suggests that the change in the ultrasonic signal with increased analyte concentration is likely due to changes in hydrogel physical properties, notably modulus and radius. Furthermore, it was also shown that this molecular quantification is possible in a matrix containing an interfering species with a nearly identical chemical structure. Measurement of multiple frequencies allowed quantification of an imprinted analyte in a matrix containing an interfering analyte with a nearly identical chemical structure. Molecular imprinting of NIPA hydrogels provides a greater analyte selectivity, which is attributed to the presence of the MAA binding group. Likewise, increased specificity due to stronger binding results in a hydrogel more adaptable to analyte molecules and analogous species. These findings are strongly indicative that the molecular imprinting process provided the selectivity required to implement a detection system using ultrasound. It is also possible to quantify the interfering species by looking at different frequencies of the ultrasonic response. This opens up several possibilities for simultaneous detection of multiple compounds with a single templated molecularly imprinted hydrogel. Overall, ultrasonically detected smart hydrogel biosensors appear very promising for a variety of environmental, industrial, and clinical applications.

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