Design and Analysis of Hydrogel Sensors for the Quantification of Vitamin D Using Ultrasound Spectroscopy

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Abstract

The role of vitamin D in patient health and well-being has become an area of great interest and concern as studies uncover conditions and issues related to deficiency. It is well established that vitamin D plays a major role in the formation and maintenance of bone structure through the regulation of calcium and phosphate levels in the blood. Recently, studies have shown that vitamin D plays a much larger role in patient health. Recognition sites for vitamin D have been found in soft tissue regions throughout the body, indicating that tissues other than bone are affected by vitamin D status. Recent studies also suggest that long-term deficiency of vitamin D is linked to several autoimmune diseases including type 1 diabetes and multiple sclerosis. Currently, one of the major issues hindering productive research in vitamin D deficiency studies is the lack of a reliable, routine measurement method. International vitamin D measurement comparison studies (such as DEQAS) indicate that measurement variations exists among modern methods for quantifying vitamin D. For these reasons, it is critical to establish a practical method for obtaining reliable vitamin D measurements.

The development of rapid clinical and inline measurement techniques for the analysis of complex chemical systems has become an area of great interest in analytical research. Ideal protocols make quick and accurate measurements while using inexpensive reagents and low-cost instrumentation. There are several measurement challenges that can inhibit many of the current spectroscopic techniques from effectively quantifying certain sample matrices. Highly scattering media such as suspensions, tissue, blood, serum, and other biological fluids are difficult to analyze using conventional optical strategies. Extensive
preparatory and separation techniques are used to reduce the complexity of the matrix. The research illustrated in this thesis is centred on the application of hydrogel sensors together with ultrasound spectroscopy as tools for overcoming these challenges.

In this thesis, specially designed chemical sensors are used to bind target analyte compounds, stimulating a change in the acoustic properties of the sensor. We have developed two sensors based on hydrogel chemistry in order to measure vitamin D status. Frequency analysis of ultrasonic waves that are passed through the sample is made to characterize the acoustic signature of the sensor. A multi-component linear regression model is constructed to correlate the resonance frequency change of the sensor with the addition of analyte.

The first hydrogel sensor uses native vitamin D binding protein (Gc-globulin, VDBP) as the recognition element for vitamin D₃. This novel approach to hydrogel sensor construction consists of multiple protein units chemically bound to chains of cellulose. The cellulose structures are further cross-linked with one another to form a three-dimensional hydrogel network. These sub-micron hydrogels are dispersive in aqueous media, permitting their use in biological samples. Sensor resonance frequency information can be obtained by passing ultrasound through the Gc-globulin hydrogel solution and interpreting the signal changes. When binding events occur between Gc-globulin and vitamin D₃ in solution, a shift in resonance frequency occurs and the response is observed in the transmitted ultrasonic wave. Using this approach, quantification of vitamin D₃ is achieved between 2-10 nM with a 0.87 nM standard error of estimation.

The second protocol described in this work uses dispersive acrylamide-based sub-micron hydrogel molecules. These sensors are synthesized in solution with the target analyte to create molecularly imprinted recognition sites. After polymerization, the target molecule is removed through dialysis and the recognition pockets are retained. These pockets provide a recognition site for the hydrogel sensor, binding to target analyte compounds present in the solution. Aliquots of the analyte are administered into the sensor
solution, which induces physical changes in the hydrogel sensor, resulting in a shift in sensor oscillation frequency. Using this technique, quantification of vitamin D₃ is achieved for 25-150 nM solutions with a 12.3 nM standard error of estimation.

The quantitative analysis of the two aforementioned sensors in complex biological media is demonstrated. Measurements were made in unfiltered human serum to demonstrate the feasibility of the hydrogel sensors in real-world applications. Serum samples were collected from multiple donors to demonstrate the minor impact of the sample matrix on the quantification abilities of the sensors.

The demonstration of ultrasound sensitive hydrogels as an effective method for quantifying chemical compounds in liquid media is presented in this thesis. The ultrasound method is promising for rapid laboratory analysis of patient vitamin D status. Short measurement times, reduced instrumentation cost, and minimal sample preparation makes ultrasound an excellent candidate for point-of-care analysis.
Résumé

Le rôle de la vitamine D dans le domaine de la santé est devenu un enjeu suscitant beaucoup d’intérêt depuis que plusieurs études ont commencé à sa pencher sur les problèmes reliés à sa carence dans l’organisme. Il est bien établi que la vitamine D joue un rôle majeur dans la formation et le maintien de la structure osseuse en effectuant la réglementation du taux de calcium et de phosphate dans le sang. Récemment, un certain nombre d’études ont montré que la vitamine D joue un rôle beaucoup plus important dans la santé des patients. Des récepteurs de vitamine D ont été trouvés dans des régions extra-osseuses à plusieurs endroits dans le corps, ce qui indique que d’autres régions corporelles sont affectées par la vitamine D. Des études ont démontré qu’une carence en vitamine D à long terme est liée à des maladies auto-immunitaires telles que diabète de type 1 et la sclérose en plaques. À l’heure actuelle, le manque de procédures d’analyse et de quantification fiables porte entrave à la recherche sur les effets d’une carence en vitamine D. Des études comparatives internationales (telles que DEQAS) indiquent que des variations de mesures existent entre les méthodes modernes actuellement utilisées pour quantifier la vitamine D. Pour ces raisons, il est essentiel d’établir une méthode pratique pour en obtenir des mesures fiables.

Le développement de techniques d’évaluations cliniques et en ligne associées à l’analyse de systèmes chimiques complexes est devenu un domaine de grand intérêt en recherche analytique. Un protocole idéal pourrait effectuer des mesures rapides et précises en utilisant des réactifs peu dispendieux et à faible coût d’instrumentation. Hors, de nombreux obstacles liés aux techniques de spectroscopie actuelles peuvent nuire à la
quantification efficace de certaines matrices d’échantillons. Par exemple, des milieux diffusants à grande variation tels que du sang, du sérum, des tissus, des suspensions et d’autres liquides biologiques sont difficiles à analyser en utilisant des stratégies optiques conventionnelles. De nombreuses techniques de préparation et de séparation sont utilisées pour réduire la complexité de la matrice. La recherche illustrée dans cette thèse se concentre sur l’application de capteurs hydrogels allant de pair avec la spectroscopie ultrasonore comme outils pour surmonter ces obstacles.

Dans cette thèse, des capteurs chimiques spécialement conçus sont utilisés dans le but de lier des composés faits d’analytes spécifiques. Cela provoque un changement des propriétés acoustiques des capteurs en question. Nous avons développé deux capteurs à base d’hydrogels afin de mesurer les niveaux de vitamine D. Une analyse de fréquence des ondes ultrasonores transmises à travers l’échantillon est effectuée afin de caractériser la signature acoustique des capteurs. Suite à une analyse de régression linéaire multiple, un modèle est construit de façon à corréler le changement de fréquence de résonance du capteur avec l’ajout de l’analyte.

Le premier capteur hydrogel utilise la protéine porteuse de vitamine D (Gc-globulin, VDBP [Vitamin D Binding Protein]) comme composant de reconnaissance de la vitamine D₃. Cette nouvelle méthode de synthèse de capteurs hydrogels se compose de plusieurs unités protéiques liées chimiquement à des chaînes de cellulose. Les structures de cellulose sont réticulées entre elles pour former un réseau d’hydrogels tridimensionnel. Ces hydrogels submicroniques sont dispersifs dans des milieux aqueux, ce qui permet leur utilisation dans des échantillons biologiques. Des informations de fréquence de résonance du capteur peuvent être obtenues par la transmission d’ultrasons à travers la solution d’hydrogels VDBP et l’interprétation des variations du signal. Lors de la liaison entre la VDBP et de la vitamine D₃ en solution, un changement dans la fréquence de résonance se produit et la réponse en fréquence est observée dans l’onde ultrasonore transmise. En
utilisant cette méthode, la quantification de vitamine $D_3$ est atteinte entre 2-10 nM avec une erreur type d’estimation de 0.87 nM.

Le deuxième protocole décrit dans cette thèse utilise des hydrogels submicroniques dispersifs à base d’acrylamide. Ces capteurs sont synthétisés en solution avec l’analyte pour créer des sites de reconnaissance à empreinte moléculaire. Après polymérisation, la molécule cible est éliminé par dialyse et les poches de reconnaissance sont conservées. Ces poches deviennent des sites de reconnaissance pour le capteur hydrogel dont l’empreinte a été prise, et elles peuvent repérer les molécules cibles en solution. Des aliquotes d’analyte sont ajoutées à la solution de capteurs, ce qui provoque des changements physiques dans le capteur hydrogel et un changement dans la fréquence de résonance du capteur. En utilisant cette méthode, la quantification de vitamine $D_3$ est atteinte pour des solutions de 25-150 nM avec une erreur type d’estimation de 12.3 nM.

L’analyse quantitative des deux capteurs mentionnés précédemment est démontrée dans des milieux biologiques complexes. Les mesures ont été effectuées dans du sérum humain non filtré afin de démontrer la possibilité d’utilisation des capteurs hydrogels dans des situations réelles. Les échantillons de sérum utilisés ont été prélevés sur plusieurs donneurs dans le but de démontrer que la matrice de l’échantillon affecte peu les capacités de quantification des capteurs.

La présente thèse traite de l’utilisation d’hydrogels sensibles aux ultrasons comme étant une méthode efficace pour quantifier les composés chimiques en milieux liquides. La méthode ultrasonique est prometteuse en ce qui concerne l’analyse en laboratoire rapide du niveau de vitamine D des patients. Cette méthode est encore plus intéressante du fait qu’elle est associée à de courts délais de mesures, des coûts d’instrumentation réduits et une préparation minimale d’échantillons.
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<td>Point-of-care</td>
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<td>Ergocalciferol</td>
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<td>Cholecalciferol</td>
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<td>25-hydroxyergocalciferol</td>
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<td><strong>25-OHD&lt;sub&gt;3&lt;/sub&gt;</strong></td>
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<td><strong>1α,25-OH&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;2&lt;/sub&gt;</strong></td>
<td>1α,25-dihydroxyergocalciferol</td>
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<td><strong>1α,25-OH&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt;</strong></td>
<td>1α,25-dihydroxycholecalciferol</td>
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<tr>
<td>A.U.</td>
<td>Arbitrary units</td>
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<tr>
<td>VDBP</td>
<td>Vitamin D binding protein (aka. Gc-globulin)</td>
</tr>
<tr>
<td>MLR</td>
<td>Multilinear regression</td>
</tr>
<tr>
<td>SEE</td>
<td>Standard error of estimation</td>
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<tr>
<td>SMA</td>
<td>Simple moving average</td>
</tr>
<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>SMLR</td>
<td>Stage-wise multilinear regression</td>
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<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethyl cellulose</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>NIPA</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>MAA</td>
<td>Methacrylic acid</td>
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<tr>
<td>MBA</td>
<td>N,N’-methylenebisacrylamide</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethlenediamine</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>MI</td>
<td>Molecular imprinting</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet radiation in the 280-315 nm wavelength region</td>
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<tr>
<td>CPB</td>
<td>Competitive protein binding assay</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>CL</td>
<td>Chemiluminescence</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
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<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
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<td>DEQAS</td>
<td>Vitamin D External Quality Assessment Scheme</td>
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<td>SONAR</td>
<td>Sound navigation and ranging</td>
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<tr>
<td>LWR</td>
<td>Long wavelength regime</td>
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<tr>
<td>IWR</td>
<td>Intermediate wavelength regime</td>
</tr>
<tr>
<td>SWR</td>
<td>Short wavelength regime</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>PZT</td>
<td>Lead zirconate titanate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Poly(vinylidene fluoride)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>QCRS</td>
<td>Quartz crystal resonator sensors</td>
</tr>
<tr>
<td>TSMR</td>
<td>Thickness shear mode resonators</td>
</tr>
<tr>
<td>BAW</td>
<td>Bulk acoustic wave</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal micro-balance</td>
</tr>
<tr>
<td>SAW</td>
<td>Surface acoustic wave</td>
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<tr>
<td>FPW</td>
<td>Flexural plate wave</td>
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<tr>
<td>SH-APM</td>
<td>Shear horizontal acoustic plate mode</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>CT</td>
<td>X-ray computed tomography</td>
</tr>
<tr>
<td>$u(x, t)$</td>
<td>Particle displacement ($m$)</td>
</tr>
<tr>
<td>$A$</td>
<td>Maximum wave amplitude ($m$)</td>
</tr>
<tr>
<td>$f$</td>
<td>Frequency (Hz = $s^{-1}$)</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength ($m$)</td>
</tr>
<tr>
<td>$v$</td>
<td>Velocity ($m \cdot s^{-1}$)</td>
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<tr>
<td>$C$</td>
<td>Coefficient of stiffness ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density ($kg \cdot m^3$)</td>
</tr>
<tr>
<td>$K$</td>
<td>Adiabatic bulk modulus ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
</tr>
<tr>
<td>$p/P$</td>
<td>Pressure ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Adiabatic index ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
</tr>
<tr>
<td>$E$</td>
<td>Young’s modulus (or tensile modulus) ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
</tr>
<tr>
<td>$G$</td>
<td>Shear modulus ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
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<td>$M$</td>
<td>P-wave modulus ($kg \cdot m^{-1} \cdot s^{-2}$)</td>
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<td>$I$</td>
<td>Wave intensity ($W \cdot m^{-2}$)</td>
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<td>$t$</td>
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<td>$\alpha$</td>
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<tr>
<td>$\alpha_0$</td>
<td>Media-dependent attenuation coefficient ($m^{-1}$)</td>
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<td>$F_c$</td>
<td>Crystal frequency constant ($m \cdot s^{-1}$)</td>
</tr>
<tr>
<td>$\Delta m$</td>
<td>Loaded mass ($kg$)</td>
</tr>
<tr>
<td>$A_{elec}$</td>
<td>Electrode surface area ($m^2$)</td>
</tr>
<tr>
<td>$d_c$</td>
<td>Crystal thickness ($m$)</td>
</tr>
<tr>
<td>$\rho_c$</td>
<td>Crystal density ($kg \cdot m^{-3}$)</td>
</tr>
<tr>
<td>$Z$</td>
<td>Acoustic impedance ($kg \cdot m^{-4} \cdot s$)</td>
</tr>
<tr>
<td>$s$</td>
<td>Specific entropy ($m^2 \cdot s^{-2} \cdot K^{-1}$)</td>
</tr>
<tr>
<td>$B/A$</td>
<td>Nonlinearity parameter</td>
</tr>
<tr>
<td>$a$</td>
<td>Particle radius ($m$)</td>
</tr>
</tbody>
</table>
Contribution of Authors

This section outlines the contributions of the authors to the article entitled:

Andrew G. Dafoe, Jonathan R. Dion, and David H. Burns, "Ultrasonically Active Hydrogel Sensors for the Quantification of Vitamin D₃".

Mr. Dafoe adapted the NIPA hydrogel procedure from previous work, which was also supervised by Prof. Burns. Dr. Dion originally developed the cellulose-based hydrogel procedure for conjugating cellulose with anti-acetaminophen antibody. Additionally, Dr. Dion assisted Mr. Dafoe in modifying Dr. Dion’s cellulose work for the purpose of coupling the Gc-globulin protein to the cellulose hydrogel sensor. Mr. Dafoe set up the instrument and collected the data for the experiments under the supervision of Prof. Burns. Data analysis was carried out by Mr. Dafoe. Prof. Burns aided in experimental design and suggested methods for processing the data. The manuscript was written and prepared by Mr. Dafoe and edited with Prof. Burns.
CHAPTER 1

Introduction

1.1 Project Summary and Overview

Analytical chemistry is a scientific field of study that focuses on gathering quantitative information about a sample. From routine drinking water testing, to complex metal profiling of alloys, analytical chemistry deals with a wide variety of media and materials. In this day and age, high precision analytical measurements are ubiquitous in areas such as chemistry, medicine, pharmaceuticals, environmental sciences, research, construction, and quality control. Rapid and cost-effective measurement techniques are favorable in these fields. In manufacturing, inline methods are preferential since measurements can be made without inhibiting production. Non-disruptive and minimally invasive measurement techniques are also preferable in medical practices for providing enhanced patient care and comfort. An example of an inline technique that is frequently used in medicine is the rapid measurement of bloodborne waste during dialysis for patients suffering from kidney failure [1, 2]. In clinical settings, it is especially desirable to have minimally invasive, high-throughput techniques that can be carried out at point-of-care. New and innovative measurement methods are actively investigated in order to accommodate today’s analysis requirements.

The research presented in this thesis is centered on the development of a minimally invasive approach to quantifying biological compounds using ultrasonically active hydrogel-based biosensors. The hydrogel approach is demonstrated on specific metabolites
of vitamin D, the quantification of which has become highly sought after in clinical research. Interest in patient vitamin D status has expanded as more studies reveal issues related to deficiency. Recent studies have generated a lot of interest in vitamin D and its role in patient health and well-being. A number of physical ailments, as well as numerous long-term complications arising from vitamin D deficiency are discussed in Section 1.2.

In this thesis, a novel approach to the quantification of vitamin D in serum is described. Using acoustically active hydrogel sensors, the successful quantification of vitamin D in unfiltered human serum is demonstrated. The first sensor discussed in this thesis was synthesized with acrylamide-based hydrogel polymers synthesized in the presence of vitamin D to form molecularly imprinted pockets. The second sensor incorporated high-affinity binding protein inside a 3D cellulose hydrogel network. The binding events were monitored by analysis of an ultrasonic pulse transmitted through the system, which led to successful quantitation of vitamin D in biological media.

The purpose of Chapter 1 is to provide the reader with a comprehensive background on the fundamentals of acoustics and its applications in modern measurement systems. The introduction begins with a background on vitamin D, with an emphasis on the clinical significance and measurement challenges associated with this family of compounds. A historical background, as well as an overview on the science of acoustics is included to provide context for modern research in acoustics. The remaining sections describe modern instrumentation and applications of acoustics towards several areas of science and research. Included among these applications are medical imaging, flaw detection, process quality control, and analyte quantification techniques. Recent hydrogel sensor development and fabrication techniques are summarized in the final sections of Chapter 1. The manuscript presented in Chapter 2 demonstrates the novel application of the two hydrogel sensors mentioned towards the accurate quantification of specific vitamin D metabolites. Finally, the implications of hydrogel research are explored in Chapter 3. A summary with an
emphasis on what the results mean in a broader context is stated. Chapter 3 closes with a brief section on future work and suggestions for experimental improvements.

1.2 Background of Vitamin D₃

Before investigating new medical measurement methods for biological compounds, it is important to understand the physiological role of the chemical. It is beneficial to understand how urgent the measurement information is for the purpose of identifying disorders, deficiencies, and conditions. Early detection of disease markers has been shown to significantly improve patient outcome, thereby allowing doctors to quickly assess patient health and provide rapid medical intervention [3, 4]. Recently, clinical assay development has been focused on creating devices that allow testing and screening at point-of-care locations (POC). These devices can be deployed at or near the site of patient care, thus making diagnostic information readily available and allowing patient treatment to be administered sooner. Currently, many POC devices are used routinely for screening tests such as: blood glucose [5], lactate, cortisol, dissolved gases, and electrolyte levels [6]. POC measurement systems can allow continuous monitoring of these parameters. Devices with small sample requisite are favorable and can be used to make measurements where the resources of a fully equipped analysis laboratory are otherwise not available [7]. Some examples of modern POC devices in use today include disposable testing devices for HIV [8], ovulation, and pregnancy [9]. Novel POC devices for measuring other physiological parameters are currently of great interest in medical research.

In recent years, patient vitamin D status has become a greater clinical concern as new studies have uncovered conditions related to deficiency [10–18]. Further elaboration and discussion of these deficiency consequences are explored in Section 1.2. Currently, quantification of vitamin D requires expensive instrumentation and consumables, as well as skilled personnel. With an increasing interest in the analysis of vitamin D status in patients, there have been a number of technique enhancements through recent years. Although the cost and time associated with monitoring vitamin D levels has been reduced, many
of these modern measurement methods suffer from high variability [19–25]. The clinical
significance of vitamin D, as well as current methods of analysis will be discussed in this
section.

1.2.1 Vitamin D metabolism and circulation status

The term vitamin D describes a group of fat-soluble prohormones that are primarily
responsible for the absorption and regulation of calcium and phosphate levels in the
blood. Unlike other dietary vitamins, mammals can synthesize vitamin D entirely through
exposure to sunlight [26]. Endogenous synthesis begins with the UVB photolysis of
7-dehydrocholesterol that is present in the skin, which results in the intermediate previ-
tamin D$_3$. This intermediate spontaneously isomerizes into cholecalciferol (vitamin D$_3$)
and readily binds to Gc-globulin (vitamin D binding protein, or VDBP). This carrier protein
transports cholecalciferol to the liver, where it is hydroxylated to 25-hydroxycholecalciferol
(25-OHD$_3$), the primary circulating metabolite of vitamin D. Secondary hydroxyla-
tion occurs in the kidneys to form the active hormone 1$\alpha$,25-dihydroxycholecalciferol
(1$\alpha$,25-(OH)$_2$D$_3$). Enzymes necessary to locally hydroxylize 25-OHD$_3$ have also been
observed in other tissues within the body [18]. 1$\alpha$,25-(OH)$_2$D$_3$ then circulates to intestinal
and bone tissues to begin its activity. In these locations, 1$\alpha$,25-(OH)$_2$D$_3$ chemically
interacts with its vitamin D receptor (VDR). As a result, intestinal calcium absorption and
osteoclastic activity are increased.

In order to properly assess serum vitamin D status for patients, it is necessary to
understand which metabolite of vitamin D provides the best indicator for substrate avail-
ability [22]. Cholecalciferol has a relatively short residency period in the circulatory system
(approximate half life of 24 hours) and is thus only indicative of recent sunlight exposure
or supplementation. The lipophilic nature of the molecule also makes quantification
particularly difficult since it is primarily stored in fatty tissues [27]. The enzymatic
generation of 1$\alpha$,25-dihydroxycholecalciferol is tightly regulated with a serum half life
of 4-6 hours. Therefore, its use as a gauge of patient vitamin D status is questionable,
although the compound could be useful in the diagnosis of certain renal diseases [28]. The best indicator of vitamin D level is 25-hydroxycholecalciferol. Its residency time in blood is relatively long (about a 3 week half life) and its production is unregulated. For adults, a 25-hydroxycholecalciferol serum level between 30-150 ng/mL is considered sufficient for proper parathyroid hormone activity, as well as adequate intestinal calcium transport [16]. Rare cases of toxicity have been documented when serum levels exceed 150-200 ng/mL, resulting in a number of symptoms pertaining to vitamin D intoxication including low serum parathyroid hormone levels and hypercalcemia [16, 29].

There are several forms of vitamin D that have very similar metabolic behavior. The two primary variants are vitamin D$_3$ (cholecalciferol) and vitamin D$_2$ (ergocalciferol). The former compound is endogenous to humans, while the latter is synthesized in plants through the UV photolysis of ergosterol. The latter exogenous source is sometimes prescribed as a high dose remedy for cases of extreme deficiencies, although studies indicate that its effectiveness is lesser than that of cholecalciferol [30]. For daily supplementation and in most clinical applications, cholecalciferol is the primary choice for treating vitamin D deficiency.

1.2.2 Clinical significance of vitamin D

Vitamin D plays a major role in the regulation and absorption of dietary calcium and phosphate [16]. Studies have shown that vitamin D is important in bone formation and general health in young children and adults. Vitamin D deficiency has been linked to a number of illnesses including rickets in children, as well as bone related conditions in adults such as osteopenia, osteoporosis, osteomalacia, muscle weakness, and an increased risk of fracture [18]. Insufficient vitamin D has also been associated with occurrences of weakness, irritability, depression, drowsiness, sleep difficulties, inattentiveness, and pain [12].

Recently, other benefits of vitamin D that are unrelated to bone health have been observed. Connections between deficiency and a number of major autoimmune diseases have been documented [15]. Receptors for vitamin D have been discovered in various
tissues including immune cells, pancreatic beta-cells, and cardiac myocytes. Emphasis in recent clinical research has been on the role vitamin D plays in various diseases. Correlations between vitamin D deficiency and the occurrence of certain types of cancers have been observed in epidemiological studies [10]. It has also been suggested that vitamin D deficiency may be linked to several major autoimmune diseases including type I diabetes and multiple sclerosis, although the results are not conclusive [11].

1.2.3 Methods for measuring vitamin D status

The first method for quantifying 25-hydroxycholecalciferol in human serum, introduced in 1971, used a competitive protein binding assay (CPB) after an organic extraction and silicic acid chromatographic separation to isolate the metabolite of interest. The amount of 25-hydroxycholecalciferol present was then determined by CPB, where vitamin D-deficient rat serum was used as a source of vitamin D binding protein and \(^{3}\)H-25-hydroxycholecalciferol acted as a reporter molecule [23, 31]. The CBP procedure provided co-specificity for both 25-hydroxycholecalciferol and 25-hydroxyergocalciferol, thus making it a reasonable candidate for total vitamin D status in patients. However, cumbersome extraction and pre-purification procedures made it difficult to employ in high-throughput routine clinical analysis. The next generation of vitamin D quantification used high performance liquid chromatography (HPLC) coupled with UV detection. This labor intensive process, whose first reported use was in 1977, involved sample preprocessing, solvent extraction, and preliminary chromatographic separation. As well, the costs to own, maintain, and operate HPLC systems are immense, and as such limit this particular procedure to the research laboratory. Time consuming extraction and separation steps also restricted the technique’s potential for routine clinical use.

Practical clinical assays for quantifying vitamin D were not available until 1985, when radioimmunoassay (RIA) was first introduced [32]. RIA was a major step forward in promoting routine vitamin D analysis since quantification time was greatly reduced due to a reduction in sample preprocessing. In addition, system sensitivity was improved with RIA
assay as compared to CPB. Co-specificity was achieved for both 25-hydroxylated species, which meant it provided a good indication of overall vitamin D status. Turnaround times using RIA were low enough to accommodate high throughput clinical laboratories, which in turn led to the inclusion of vitamin D level detection in semi-routine patient screening.

Today, there are a number of assays that can meet and surpass the improved turnaround times achieved by RIA. Fully automated chemiluminescence (CL) assays allow up to 75 samples to be analyzed per hour. These assays, although fast, suffer from higher detection limits on the order of 7 ng/mL. The CL analysis method is co-specific for the two 25-hydroxylated species, but again, high instrumentation costs limited their widespread use. Enzyme-linked immunosorbent assays (ELISAs) also were developed, which demonstrate comparable detection ranges and limits to that of RIA. Since ELISAs involve non-isotopically labeled species, the amount of radioactive waste generated is reduced as compared to other measurement methods. Another technique that has gained popularity in clinical laboratories is liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS can selectively quantify different metabolites in solution, however instrument configuration can produce highly variable results [24].

Absolute determination of serum vitamin D is achieved through gas chromatography-mass spectrometry systems (GC-MS) [33]. Today, GC-MS is the "gold standard" to which all other assays are compared. Although GC-MS is held in high esteem for providing highly accurate and selective measurements, it is impractical for routine analysis [22]. LC-MS/MS is also used as a reference assay for comparison studies [19], however it similarly lacks the simplicity necessary for routine practice.

Difficulties in obtaining reliable total 25-hydroxycholecalciferol content measurements is a direct consequence of highly variable results found between quantification methods [25]. International proficiency surveys have been implemented in order to assess method-to-method variability. One survey that has been monitoring 25-hydroxycholecalciferol assay performance since 1989 is the international vitamin D
Table 1–1: Several figures of merit for common 25-hydroxycholecalciferol assays [23].
*Does not include extraction, counting or microplate reading.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Range (nM)</th>
<th>Intra-assay variance (%)</th>
<th>Inter-assay variance (%)</th>
<th>Time*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>4-400</td>
<td>5.5</td>
<td>7.9</td>
<td>3 hours</td>
</tr>
<tr>
<td>ELISA</td>
<td>6-360</td>
<td>&lt;6</td>
<td>&lt;9</td>
<td>3 hours</td>
</tr>
<tr>
<td>CL</td>
<td>17.5-300</td>
<td>6.6</td>
<td>11.2</td>
<td>75 mins</td>
</tr>
<tr>
<td>CPB</td>
<td>8-312</td>
<td>9.9</td>
<td>14</td>
<td>70 mins</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>15-150</td>
<td>5.2</td>
<td>8.4</td>
<td>20 mins</td>
</tr>
</tbody>
</table>

quality assessment scheme (DEQAS). To date, over 100 participants in 18 countries contributed to this vitamin D standardization survey. From the DEQAS work, it has been found that many assays suffer biases due to matrix effects and interfering species. The variability of measurements can be attributed to the variety of structurally similar compounds present in serum. A summary of the variations, detection ranges, and analysis times of several assays used in routine vitamin D analysis are listed in Table 1–1. Studies in clinical significance and public health regarding serum levels of vitamin D would greatly benefit from a standardized vitamin D assay procedure that could rapidly and accurately determine patient vitamin D status.

1.3 History of Acoustics

Acoustics is the interdisciplinary study of sound. More specifically, it refers to the analysis of how mechanical waves propagate and interact with the media through which they travel. Objects that sound waves encounter along their path can impart changes to the wave properties. Early contributions to the field of acoustics were made by ancient Greek philosophers, whose primary motivation was an understanding of music. As early as the 6th century BC, intellectuals began to develop a basis for sound theory [34]. The
Ionian Greek philosopher, Pythagoras, studied the relationship between vibrating strings and musical sounds. He discovered that dividing the length of a vibrating string into ratios would produce harmonious musical tones. One of the first people to properly understand the connection between these tones and the string’s oscillation frequency was Galileo. He found that by scraping a chisel along a surface at various speeds and forces, he could produce different tones. Galileo determined that the pitch of the tone was closely related to the length of the skips the chisel made along the surface.

Sound propagates through media while displacing a minimal amount of matter [35]. This is best illustrated by the image of waves traveling across the surface of a pond. The wave energy moves outward, while objects floating on the surface remain stationary with respect to the wave propagation. Vibrating objects impart their oscillations to air, thus generating waves of the same vibration frequency. In 1660, Robert Boyle proposed an experiment on sound emanating from a ticking watch encased in a partial vacuum [36]. He observed that the intensity of the sound would diminish as the air was evacuated. In this way, he was able to demonstrate that sound requires a media through which to travel. Mathematical theories for sound propagation arose in 1686 when Isaac Newton published mechanical interpretations of pressure wave propagation in his Principia [37]. His work illustrated the use of thermodynamic considerations in trying to describe acoustic phenomena. Newton incorrectly assumed that the propagation of sound waves was an isothermal process [38]. It wasn’t until later in the late 18th century that Laplace discovered that temperature does not remain constant as air is compressed and rarified [39]. He concluded that propagation was an adiabatic process. Further contributions were made in the 18th century by Euler, Lagrange, and d’Alembert, whose works led to more robust models of acoustic theory that are still highly regarded today.

1.4 Science of Acoustics

In the science of acoustics, time-varying deformations within a material are measured to extract specific properties either of the material itself, or of the source of the acoustic
The following section provides a brief overview of the scientific fundamentals of acoustic waves. The discussion begins with an introduction to wave properties. The dependency of the speed of sound on the propagation media is then introduced. The modes of acoustic wave propagation in different material types are then discussed in Section 1.4.2. The various mechanisms for sound attenuation, signal loss, and noise are discussed with emphasis on ultrasonic applications. After a general overview on the science of acoustics, the benefits, advantages, and applications of ultrasonics are introduced in Section 1.4.4.

1.4.1 Frequency and velocity of sound waves

Sound propagates through media in the form of a mechanical wave. In other words, energy is transmitted through a material by oscillations of the material itself. Figure 1–1 illustrates pressure as a function of time as a wave of a single frequency undergoes one complete cycle, traveling a distance of one wavelength. This simplified illustration demonstrates some of the physical properties and characteristics that belong to acoustic waves. The single-frequency waveform can be modeled by the following function:

\[ u(x,t) = A \sin \left( 2\pi ft - \frac{2\pi x}{\lambda} \right) \]  

(1.1)

where \( u(x,t) \) is the molecular displacement of the particle as a function of equilibrium position \( x \) and time \( t \). The parameter \( A \) is the maximum amplitude of the acoustic wave, i.e. the maximum displacement a single particle will deviate from equilibrium. The frequency \( f \) is the number of full oscillations the particle makes per unit of time \( t \), and \( \lambda \) is the wavelength. When two or more waves from different sources overlap in space, they combine linearly according to the principle of superposition:

\[ u_{\text{total}}(x,t) = u_1(x,t) + u_2(x,t) \]  

(1.2)

The superposition principle is important in acoustic spectroscopy since it allows for the decomposition of any waveform into its individual frequency contributions. By breaking
down a signal into its individual frequency components, it is possible to measure individual frequency contributions to the final waveform.

The human perception of tone is directly tied to frequency, with higher frequencies perceived as higher pitches. The audible frequency range for human beings is generally between 20 and 20,000 Hz, although the range can vary slightly depending upon the individual. Sound waves below 20 Hz are classified as infrasonic. These waves are used for seismology studies, as well as communication between certain animal species. Ultrasonic sound waves are those that exceed 20,000 Hz. Ultrasonic sound waves have a wide variety of applications including SONAR (sound navigation and ranging), medical imaging (sonography), industrial thickness and flaw detection, cleaning, chemistry, and echolocation for bats, insects, and a variety of marine life. The application of ultrasound and its unique properties will be discussed in future sections.

Fundamentally, a simple sound wave can be described by its frequency, wavelength, velocity, and amplitude. Recall that Figure 1–1 illustrates the wavelength and amplitude properties of a wave. Wavelength refers to the distance between identical points of two successive oscillations (e.g. the distance between two consecutive peaks). A wavelength can be determined by the wave frequency and its speed according to the following relationship:

\[ \lambda = \frac{v}{f} \]  

where \( \lambda \) is the wavelength, \( f \) is the frequency, and \( v \) is the velocity of the wave. It is important to note that the velocity of the wave in an ideal gas is independent of its frequency. Rather, the velocity of the wave is determined by the properties of the media. The parameters that govern the speed of sound in different media are given by the Newton-Laplace equation:

\[ v = \sqrt{\frac{C}{\rho}} \]  

where \( C \) is the speed of sound and \( \rho \) is the density of the medium.
Figure 1–1: Two basic properties of an acoustic wave. The wavelength is defined as the distance between two analogous points of two successive waves. The amplitude is pressure difference between average local pressure and the maximum (or minimum) pressure in the sound wave.
where \( v \) is the velocity of the wave, \( C \) is the coefficient of stiffness for the material, and \( \rho \) is the density of the media. This formula is generalized for longitudinal pressure waves in all states of matter, however the modulus term \( C \) must be substituted for the appropriate state-specific modulus term. For liquids, the substitution for the adiabatic bulk modulus \( K \) is necessary:

\[
C_{l,\text{longitudinal}} = K
\]

\[
\therefore v = \sqrt{\frac{K}{\rho}}
\]  

(1.5)

In gases, the bulk modulus is pressure dependent. An approximation of the modulus can be expressed using the adiabatic index \( \gamma \) for the gas:

\[
C_{g,\text{longitudinal}} = \gamma \ast p
\]

\[
\therefore v = \sqrt{\frac{\gamma \ast p}{\rho}}
\]  

(1.6)

The expression must be modified again when considering solid samples. Depending on the physical dimensions of the solid being evaluated, the equation for the speed of sound varies. For thin rods, the expression for the speed of sound is:

\[
C_{s,\text{rod}} = E
\]

\[
\therefore v = \sqrt{\frac{E}{\rho}}
\]  

(1.7)

where \( E \) is Young’s modulus (or tensile modulus) for the material. In cases where the material thickness is much larger than the wavelength of the propagating wave, it is possible to generate sound waves with different velocities. The two primary modes of propagation for sound waves in solids (whose mechanisms for propagation are discussed in the next section) are shear waves and longitudinal pressure waves. For shear waves in solid media,
Table 1–2: Acoustic properties of selected materials [40–43]

<table>
<thead>
<tr>
<th>Material</th>
<th>Density (kg·m$^{-3}$)</th>
<th>Modulus (kg·m$^{-1}$·s$^{-2}$)</th>
<th>Speed of sound (m·s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air (dry, +25°C)</td>
<td>1.1839</td>
<td>1.42 × 10$^5$</td>
<td>346.3</td>
</tr>
<tr>
<td>Water (+4°C)</td>
<td>999.9750</td>
<td>2.2 × 10$^9$</td>
<td>1483.3</td>
</tr>
<tr>
<td>Human tissue</td>
<td>1060</td>
<td>2.5 × 10$^9$</td>
<td>1540</td>
</tr>
<tr>
<td>Stainless Steel 304</td>
<td>8000</td>
<td>2.7 × 10$^{11}$</td>
<td>5810</td>
</tr>
</tbody>
</table>

the substitution is straightforward:

$$C_{s,\text{shear}} = G$$
$$\therefore v = \sqrt{\frac{G}{\rho}}$$

(1.8)

where $G$ is the shear modulus of the material. Longitudinal waves in solids are heavily dependent on the shear modulus property. It must therefore be incorporated into what is called the p-wave modulus for solids, $M$:

$$C_{s,\text{longitudinal}} = M = K + \frac{4}{3}G$$
$$\therefore v = \sqrt{\frac{K + \frac{4}{3}G}{\rho}}$$

(1.9)

Where $K$ is, as before, the bulk modulus of the solid media.

From these equations, it is apparent that the speed of sound, in general, is proportional to the square root of the stiffness of the material, and inversely proportional to the square root of the density of the material. Consequently, sound waves generally travel fastest in solids, followed by liquids, and finally slowest through gases. This trend is evident from the density, modulus, and velocity values listed in Table 1–2.
1.4.2 Modes of propagation

Although the mechanism for acoustic wave generation may be similar in various media types, the modes by which they propagate can be different [44]. For solid samples, there are a wide range of waves types that are present including longitudinal, transverse, surface, and plate waves. In highly viscous liquids, transverse waves can be observed, however, the primary mode of propagation is longitudinal. In gases, the mode of propagation is exclusively longitudinal.

Surface waves and plate waves are surface propagating modes that are useful in solid material analysis for commercial industrial applications. Surface acoustic waves (e.g. Rayleigh waves) propagate along the surface of thick, solid materials. Analyses in which surface waves are used include flaw detection, seismology, and electronics [45]. Plate waves (e.g. Lamb waves) occur in solid materials whose thickness is comparable to the wavelength of the sound wave. Plate waves are capable of propagating several meters along the surface of certain materials, which makes them beneficial for flaw detection in larger structures [46].

Transverse waves are another mode of propagation typically encountered in solids and high viscosity liquids. Particles oscillate through the structure perpendicular to the direction of propagation, as seen in Figure 1–2.a. The transverse waveform is primarily used for internal flaw detection in solid materials such as steel and plastic.

In the longitudinal propagation mode, particle motion is parallel to the direction of propagation, as seen in Figure 1–2.b. The particles are displaced from their equilibrium position and compressed at the interface of the wave source. The result of this compression is a pressure differential between the newly compressed layer of particles and the subsequent, undisturbed layer. As a result, the compressive force is transmitted through the surrounding media, which allows the initial layer to rarefy (or decompress) back to equilibrium. The propagation continues until the pressure front encounters a change in acoustic impedance, or all the wave energy is dissipated.
1.4.3 Signal loss mechanisms

There are several mechanisms though which acoustic intensity deteriorates over time. Heat conduction and shear effects from particle compression and rarefaction are the foremost causes of acoustic dampening through isotropic media. Energy is lost in the form of heat, as particle friction and vibration conduct energy away from the sound wave. The energy dissipation phenomenon is dependent on both the frequency of the acoustic wave, and the specific characteristics of the media, including: temperature, pressure and density. Acoustic intensity diminishes as an exponential function of the propagation distance $d$ such that

$$I(x) = I_0e^{-2\alpha d}$$

(1.10)

where $I_0$ is the initial intensity of the wave, $I(d)$ is the intensity after distance $d$, and $\alpha$ is the amplitude attenuation coefficient [47]. The attenuation coefficient can be approximated
as a function of the wave frequency $f$ according to the following relationship:

$$\alpha = \alpha_0 f^n$$  \hfill (1.11)

where $\alpha_0$ is the media-dependent attenuation coefficient and $n$, also dependent on the media, lies between 1 and 2. The dependency of acoustic penetration on sound wave frequency has important implications in medical diagnosis, where ultrasonic penetration depth is a trade-off for resolution [48]. Higher frequencies allow for distinction of closely spaced interfaces, yet according to Equations 1.10 and 1.11, intensity diminishes exponentially with increased frequency and penetration depth.

In heterogeneous media such as colloids, losses can occur due to interfacial reflection and refraction. This assumes that the diameter of the suspended particulates are smooth-edged and larger than the wavelength $\lambda$ of the ultrasound beam. When an acoustic wave encounters a body with non-equivalent acoustic impedance, a percentage of the energy is reflected off the boundary. The remaining energy continues through the secondary media at an angle dependent on the magnitude of the impedance mismatch. The acoustic impedance parameter $Z$ is a measure of resistance of the medium to the transmission of ultrasound:

$$Z = \rho v$$ \hfill (1.12)

where $\rho$ is the density of the medium and $v$ is the velocity of the wave. The laws that govern the reflection and refraction of light as it encounters a difference in refractive index are the same as those for sound as it confronts a change in acoustic impedance [38]. Therefore, it follows that:

$$\frac{\sin \theta_i}{\sin \theta_t} = \frac{v_i}{v_t}$$ \hfill (1.13)

where $\theta_i$ and $\theta_t$ are the angle of incidence and transmission respectively. The parameters $v_i$ and $v_t$ are the wave velocities of the acoustic signal in the two separate media. Assuming
that the sound wave propagates through the phase border without generating discontinuities in pressure or particle velocity, it follows that the pressure in the transmitted and reflected wave obey the following relationship:

\[
\frac{P_t}{P_i} = \frac{Z_t \cos \theta_i - Z_i \cos \theta_t}{Z_t \cos \theta_i + Z_i \cos \theta_t}
\]

(1.14)

\[
\frac{P_t}{P_i} = \frac{2Z_t \cos \theta_i}{Z_t \cos \theta_i + Z_i \cos \theta_t}
\]

(1.15)

If the pressures of the reflected and transmitted waves are substituted by intensity \(I\), such that:

\[
I = \frac{P^2}{2 \rho v}
\]

(1.16)

and the angle of incidence is assumed to be zero (i.e. the incident wave is normal to the surface of the boundary), the intensity of the acoustic signal reduces to the following expressions:

\[
I_r = \frac{I_i(Z_t - Z_i)^2}{(Z_t + Z_i)^2}
\]

(1.17)

\[
I_t = \frac{I_i(4Z_tZ_i)}{(Z_t + Z_i)^2}
\]

(1.18)

As a consequence of these equations, it is important to consider the implications of making ultrasonic measurements with an external wave generator. Proper probe coupling between the acoustic source and the material boundary interface will help minimize intensity loss due to interfacial reflection. For example, coupling gel, which is used in medical sonography, is designed to match the acoustic impedance of patient tissue while providing an efficient, bubble-free path for the ultrasonic wave to propagate [49]. Fewer mechanisms for intensity loss allows for a greater fraction of signal to pass through the patient’s tissues, which improves the overall image quality of the sonogram.
Figure 1–3: Reflection and refraction of incident acoustic waves. The incident wave $A$ approaches the surface at an angle $\theta_i$ normal to the phase boundary. Part of the wave is reflected at angle $\theta_r$ due to the difference in acoustic impedance of the two phases ($Z_i \neq Z_t$). The remaining energy propagates through to the next phase at the new refracted angle $\theta_t$. 
In cases where ultrasound encounters an object with a diameter less than or equal to the wavelength $\lambda$, or the object surface is rough, the wave will be scattered [50]. Unlike specular reflection, scattering does not redirect sound in a specific direction. Instead, sound is emitted in all directions. Multiple scattering events can occur along the path of the incident wave, which may result in synchronous or asynchronous arrival, thus causing constructive or destructive interference respectively. Synchronous and asynchronous arrival is observed in sonography as speckling, where salt & pepper "noise" will appear across the image. Methods to reduce the effects of speckle noise is an active research field [51–53]. Techniques to reduce speckling include innovations in equipment and technology, as well as additional filtering and post-processing computational steps.

### 1.4.4 Advantages of ultrasound

Ultrasound has several advantageous features for application in industrial, clinical, and biomedical fields. For example, ultrasound propagates easily through highly concentrated media, thereby eliminating the need for preparatory dilution. Without dilution, sample integrity is retained. In concentrated dispersions, sample dilution can lead to changes in aggregation and flocculation. Ultrasound is an excellent characterization tool for many colloid systems [38, 54–57]. The fundamental advantaged of ultrasound over other light-based measurements will be discussed in this subsection.

As with light-based instruments, ultrasound is useful for particle and droplet sizing applications [38, 58–61]. Ultrasound particle sizing involves three molecular radii regimes that each require a different mathematical treatment of the data. These radii regimes are based on the relationship between wavelength and particle size: the long wavelength regime (LWR, $r \ll \lambda$), the intermediate wavelength regime (IWR, $r \approx \lambda$), and the short wavelength regime (SWR, $r \gg \lambda$). Situations that satisfy the LWR requirements are more desirable for particle sizing applications, as this regime is less sensitive to particle shape factors and surface effects. In addition, the mathematical requirements for LWR conditions are much simpler than those of other regimes. The inherent long length of ultrasound
waves ($\sim 300 \mu m$, 5 MHz in water @ 20°C) as compared to light (green $\sim 510$ nm) means that greater sizes of particles can be classified in the LWR. Hence, ultrasound is extremely useful for particle sizing of colloids and dispersions.

An advantage that ultrasound has over light-based measurement methods is its ability to penetrate optically opaque interfaces. This includes materials such as bodily tissue, metals, and plastics. Non-invasive techniques are desirable in many circumstances including prenatal imaging, industrial flaw detection, and food & beverage quality control. At biomedical intensities and frequencies, ultrasound poses little risk to prenatal development other than minor heating of the surrounding tissue [62, 63]. Alternative imaging techniques include forms of ionizing radiation such as x-rays, which have been linked to higher occurrences of cancer in patients with prenatal exposure [64, 65]. Non-invasive ultrasound measurements can be made without inhibiting production in industrial applications such as assembly lines, quality control, and flow systems [66, 67]. Products can be analyzed without disrupting their serviceability due to the non-destructive nature of ultrasonic analysis.

1.5 Instrumentation

In ultrasonics, piezoelectric transducer elements are used to generate pressure waves in materials and media. Transducer modules utilize the piezoelectric effect to create deformities within the active element. The working element in a piezoelectric device consists of a polarized substance with two electrodes attached at opposing ends. When an electric field is applied, the dipoles within the material align and cause a physical deformation in the bulk material. Conversely, an external deformation of the material will generate an electric field. As a result, transducer elements are able to convert electrical energy into acoustic waves and vice-versa. Source-receiver duality behavior of acoustic transducers is incredibly useful in acoustics, since the source of the acoustic signal is also able to act as the receiving element.
At the face of a piezoelectric transducer, a working element oscillates periodically to generate compression and rarefaction within the material. Crystalline materials whose molecules have fixed dipole moments are used in the process of making piezoelectric transducers. Under normal conditions, the dipoles remain fixed and orient randomly within the material. However, when the material is heated beyond its Curie temperature, its molecules are liberated from their fixed orientations. At this point, the crystalline substance is placed between two oppositely charged plates, and the dipoles align themselves roughly along the direction of the electric field in a process called poling. Once the material is cooled, the alignment electrodes are removed and the dipoles remain in their newly oriented state. If the material is now exposed to an external electric field, the molecules will attempt to realign. In doing so, the molecules twist and the thickness of the bulk material changes. Piezoelectric materials behave as such because of their unique molecular properties [68]. Tuned acoustic waves can be generated by the application of an alternating electric field to the crystalline material which will cause a periodic swelling in the material. The ultrasonic "note" that the crystal will play is dependent on its thickness. More specifically, the distance between the two oscillating faces is equivalent to half of the wavelength of the emitted pressure wave.

Early studies in piezoelectricity were done in single crystal materials such as potassium sodium tartrate and quartz [69]. When grown as single crystals, the highly ordered structures of these minerals make them suitable for piezoelectric applications. Depending on the orientation of the crystallographic axis with respect to the external electric field, the deformation can be an expansion or shear distortion [70]. Thus, crystals are selectively sectioned for their intended application as shear-mode or longitudinal wave transducers. Multi-crystal transducer elements made of quartz are not suitable in ultrasonic applications, however, due to the inherent random nature of crystal growth that will result in molecular misalignment. Instead, ferroelectric ceramics such as lead zirconate titanate (PZT) are common transducer construction materials. By heating ceramic materials above their
Figure 1–4: Configurations for a) through-transmission, and b) pulse-echo ultrasound measurements. In configuration a, the ultrasonic pulse is transmitted through the entirety of the material. The signal is received and digitized on the other side using an oscilloscope. In configuration b, the signal is generated and digitized by the same transducer.
Curie temperatures, they can be re-polled in the event of transducer damage. Ferroelectric ceramics can be cast and poled into special shapes for specific applications, such as annular and focused transducers. Doping with different ions to change piezoelectric and physical properties is also common practice in the manufacturing of ceramic transducer elements [71]. Unfortunately, large and complicated transducer shapes are hard to manufacture using ceramic materials due to their brittle structure. Other tunable materials that exhibit piezoelectric behavior include certain polymer compositions. Specifically, poly(vinylidene fluoride) (PVDF) has been found to exhibit comparable piezoelectric properties to that of PZT. Polymer materials show promise for casting unique and complex transducer shapes since they can be made without additional machining or fabrication [70, 72].

Traditionally, ultrasound instruments are configured in one of two configurations depending on the application (see Figure 1–4). Regardless of the specific design, a pulse generator is used to emit specifically timed high-voltage pulses through a transducer element. As a result, the transducer element oscillates with a resonance frequency characteristic of its size and composition. A single pulse of mechanical force on the media in contact with the transducer element is generated, which creates a forward propagating pressure wave. The bandwidth of the frequency pulse can be tuned by adjusting the length of the voltage pulse supplied by the generator. Shorter voltage pulses produce a wider frequency bandwidth, while longer ones narrow the frequency profile of the pressure wave. Signals are then received by another transducer element (or the same one, in the case of pulse-echo ultrasound) and voltages are produced from the reverse piezoelectric effect. The resultant signal is digitized by an oscilloscope and saved for further processing.

A commonly used instrumental configuration for ultrasonic testing consists of two transducer elements facing one another on opposite sides of the testing material. This configuration is illustrated in Figure 1–4.a, where the sound wave is generated and received on the opposite sides of the testing material. One transducer is used to transmit a pulse of ultrasound though the material, while the second transducer "listens" for the pulse.
This measurement method is referred to as through-transmission ultrasound, where the ultrasonic pulse passes through the target media once. Typically, through-transmission configurations are used to characterize solutions. To reduce misalignment issues, the transducer elements are fixed at opposing ends of the sample cell. The drawback to the transmission configuration, however, is that it restricts the sampling field to a single region. In cases where measurements need to be made using a single point of contact, it is more convenient to use a pulse-echo configuration such as the one illustrated in Figure 1–4.b. In pulse-echo configurations, a single transducer is used as both the transmitting and receiving element. There are many advantages to the pulse-echo configuration. Primarily, measurements are no longer limited by fixed transducer elements. The device can be moved along the sample, which will allow for dynamic analysis at multiple contact points across the surface. Additionally, the path length of the material is effectively doubled, since the wave must now traverse to the next available reflector before detection. Time of flight techniques can be used with pulse-echo configurations to detect internal flaws and cracks in the sample material. Time of flight techniques will be discussed in more detail later in this thesis.

1.6 Ultrasound in Industry

Ultrasound has broad application potential as a tool for non-destructive materials testing [44, 73–79]. The growing demand for machine integrity has brought about reliable, cost-effective, and efficient flaw detection techniques.

1.6.1 Flaw detection

One of the more prevalent uses for ultrasound in industry is internal continuity verification. This technique was co-discovered by two researchers in 1929: Solokolov of the Soviet Union, and Mühlhauser of Germany [79]. This rudimentary flaw detection technique employed through-transmission ultrasound to detect discontinuities in materials. Due to the change in acoustic impedance at the interface of the discontinuity, much of the acoustic signal is attenuated. As a result, the flaw screens a portion of the acoustic energy
Figure 1–5: Ultrasound used in nondestructive testing of materials in a pulse-echo configuration.  

a) The ultrasonic pulse sent through the flawless material reflects off the back surface, where a change in acoustic impedance is encountered.  

b) The flaw in the material returns an earlier signal to the transducer, thus providing an estimation of flaw depth based on time of flight and speed of sound. [79]
and a lower power signal is measured at the receiving end. At its time, through-transmission techniques were revolutionary, however it provided little spatial information about the flaw. In 1943, pulse-echo techniques similar to the one illustrated in Figure 1–5 were developed. As an ultrasonic pulse encounters a discontinuity, the pulse undergoes reflection due to the mismatch in acoustic impedance. The resultant signal returns to the transducer element much sooner than if the pulse reflected off the back boundary of the material. Spatial information could be garnered since the time of flight of the wave could be measured, and depth could be calculated based on the speed of sound in the material.

1.6.2 Thickness testing

Another application of pulsed ultrasound is thickness testing of industrial materials. Using the same basic principles as pulse-echo flaw detection, thickness testing instruments use time of flight to measure the distance between two surfaces of a material [80]. The time between the initial pulse and the receipt of the echo multiplied by the speed of sound in the material will give an accurate measure of material thickness. The use of a pulse-echo configuration for thickness testing can be advantageous, since access to both sides of the material is not required. For example, the wall thickness of a hollow sphere, which would otherwise appear solid, could be measured using pulse-echo ultrasound techniques. Ultrasound tools are useful in field analysis, since the instrumentation is low-cost and highly portable, while providing measurements accurate to 0.1 mm and less.

1.6.3 Process applications and quality control

In process and quality control of food and industrial manufacturing, accurate and reliable measurements can often mean the difference between acceptable and poor quality products. Conditions including temperature, pressure, flow rate, volume, physical state, defects, level and distance, density, viscosity, and suspended particle characteristics as well as various other chemical parameters are of important consideration. Recently, ultrasonic sensors have been investigated as non-invasive measurement devices to monitor some of
these properties [81–83]. Low-power ultrasound techniques impart minimal changes to the system apart from negligible sample heating.

1.6.4 High-power applications

High-power ultrasound devices can generate desirable mechanical effects on a system including sonocrystallization [84], emulsification [85], cleaning [86], microbial inactivation [87], and extraction [88]. In addition, an important effect occurs as a result of high amplitude ultrasound known as cavitation, where sufficient rarefaction in the media causes the formation of microscopic vacuum ‘bubbles’. The collapse of these voids results in enormous energy, enough to overcome many intermolecular bonds. Cavitation is useful in applications such as tool cleaning, cell membrane destruction, and localized free radical production [89].

1.7 Ultrasound in Medicine

At low power, ultrasound is used as a safe, non-invasive tool for imaging and diagnosis in a clinical setting. Diagnostic sonography is often the first thing people associate with ultrasound, since it is so routinely used in obstetric examinations. Though application for fetus imaging is the most well-known, ultrasound is used to image many other tissue features and dynamics with real-time tomographic information.

1.7.1 Tissue contrast imaging

Sonogram images are produced using pulse-echo ultrasound instrumentation [68]. An array of piezoelectric transducers, typically hundreds of elements, are arranged either in a linear or curved formation depending on the application. Pulses are generated using small, adjacent groups of elements as opposed to single elements in order to increase the effective aperture and image resolution. Each subsequent pulse utilizes the next group of transducer elements to obtain the next column of information. Once all the elements have been used in an array, a signal is once again emitted from the first set of transducers and the process starts again.
An ultrasonic sound wave pulse is sent through skin surface and the time it takes to receive an echo signal is calculated. The echo information obtained over time from a single pulse from one transducer group is called a scan line, and represents one vertical string of pixels in an image. By recording the intensity and delay of echoes from all array groups, contrast images can be constructed.

When an incident wave encounters a change in acoustic impedance, a portion of the intensity will be reflected according to Equation 1.17. The human body is comprised of many different tissues and structures, several of which are listed in Table 1–4 along with their relevant properties. In cases where the difference in acoustic impedance is high (e.g. traversing muscle to bone), the reflected intensity will be greater. As a result, the echo received will be of greater amplitude and will produce a larger signal in the sonogram.
The primary advantage in using ultrasound as an imaging modality in medicine is that it is safe [62, 63, 91]. Other imaging methods (e.g. diagnostic x-rays) can be harmful in prenatal and prolonged exposure cases. Many ultrasonic probes only require outside contact with the skin, which means that in most applications ultrasound measurements are considered non-invasive. In cases where bypassing certain tissue types is necessary, alternative probes exist that are designed to penetrate the body through the vagina, rectum, esophagus, or blood vessels [50]. Though penetrative probes may be uncomfortable for the patient, these measurement techniques are relatively safe.

1.7.2 Doppler imaging

In medical practices, imaging of the circulatory system is important for cardiovascular diagnosis. In order to measure hemodynamics and blood flow, sonographers exploit phenomena that occur when ultrasound encounters a phase in motion [92].

The Doppler effect is a phenomenon that arises when the ultrasonic source, reflector, or receiver are in motion relative to one another. As a result, the perceived frequency will shift in accordance with the motion. For example, the perceived pitch of a siren on an emergency vehicle will appear to change as it passes an observer. In this case, the source of the acoustic signal is in motion relative to the acoustic receiver. If the vehicle is moving towards the observer, the resulting acoustic wave will be of higher frequency. The same effect is observed when ultrasound encounters an interface in motion, such as blood. If the acoustic impedance interface is moving towards the direction of the transducer, the ultrasonic echo will return at a higher frequency than the original pulse. Likewise, if the flow is directed away from the transducer element, the returning signal will be of a lower frequency.

Real time velocity and flow mapping can be done with modern Doppler scanning systems [93]. Doppler techniques have tremendously important implications in peripheral vascular disease diagnosis, as blood flow for specific regions can be measured. Blood clots in veins can be revealed that could otherwise unknowingly break off and block flow to the
lungs. Stenosis, or the narrowing of a blood vessel, can also be determined with the use of Doppler scanning techniques. Other diagnostic imaging protocols including magnetic resonance imaging (MRI) and X-ray computed tomography scanning (CT) are capable of mapping the circulatory system, however, ultrasound imaging is often used instead due to low operational cost and safe, non-mutagenic emission.

1.7.3 Contrast agents

There are certain regions of the human body that can appear fairly isotropic to an ultrasonic wave. For example, imaging regions in the body predominantly filled with blood is challenging using unadulterated ultrasound imaging techniques. Without large structures to provide impedance interfaces in the media, it is difficult to elicit contrast for imaging. The primary contribution to the returning imaging signal in these regions is from scattering, which is inherently weak. Intravenous injection of ultrasound contrast agents can be used to help alleviate the problem of regions with low signal. Introduced agents serve to increase the number of acoustic impedance interfaces within the media, generating enormous backscattering and thereby greatly increasing the amount of signal received from the targeted area [68, 94–104]. Contrast agents ideally display some preferential tissue uptake as well, since they have functional groups that are tailored for recognition of specific tissue or cellular epitopes. To be useful in a medical setting, contrast agents must demonstrate low toxicity in patients and safe elimination via metabolic or biological pathways. This way, the agent can be deployed with minimal patient risk and without requiring a follow-up procedure for removal. Free gas bubbles are the most rudimentary of contrast agent types. Gramiak and Shah first discovered their utility in providing ultrasonic imaging contrast in 1968 [104], when they noticed that saline injections produced a cloud of echoes during continuous echocardiographic recordings. Free gas bubbles exist for only a short time before removal by the lungs, thus limiting their application in soft tissue regions. Instead, gas bubble contrast agents are mainly used for localized Doppler enhancement of the cardiovascular system.
Table 1–4: Commercially available contrast agents and their compositions [106].

<table>
<thead>
<tr>
<th>Contrast agent</th>
<th>Core gas</th>
<th>Coating material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albunex</td>
<td>air</td>
<td>sonicated serum albumin</td>
</tr>
<tr>
<td>Optison</td>
<td>octafluoropropane</td>
<td>cross-linked serum albumin</td>
</tr>
<tr>
<td>Quantison</td>
<td>air</td>
<td>spray-dried serum albumin</td>
</tr>
<tr>
<td>MP1950</td>
<td>decafluorobutane</td>
<td>phospholipid</td>
</tr>
<tr>
<td>Aerosomes</td>
<td>perfluorocarbon gas</td>
<td>phospholipid</td>
</tr>
<tr>
<td>Filmix</td>
<td>air</td>
<td>phospholipid</td>
</tr>
<tr>
<td>Bisphere</td>
<td>air</td>
<td>polymer</td>
</tr>
<tr>
<td>Sonazoid</td>
<td>perfluorocarbon gas</td>
<td>phospholipid</td>
</tr>
</tbody>
</table>

Other contrast agents have been designed with the aim of extending the resident lifetime within the body, allowing for perfusion studies and targeted contrast of particular regions [104]. Gas bubbles encapsulated with chemical coatings can exhibit longer lifetimes and improved stability in the blood than free gas bubbles. The specific coatings can vary in composition, as well as functional groups in order to prolong stability and improve tissue specificity. Albumin has been used as a coating for gas-filled micro-spheres because of its biocompatibility and primary amines, which can be readily functionalized for targeting [105]. Other shell coating candidates for gas-filled contrast agents include phospholipids [107, 108], polymers [109], and amphiphile-stabilized carbohydrates [68, 110, 111]. In addition to improving circulation lifetimes, functionalized coating agents have allowed for many opportunities to provide targeted contrast to areas of interest.

1.7.4 Targeted contrast agents

Capsule material can be functionalized to impart targeted recognition of tissue and disease markers [112]. Recognition elements are attached to the spheres using a number of different covalent and non-covalent bonding strategies. One of the most routine, non-covalent
methods for sensor functionalization is the incorporation of streptavidin-linkers between biotinylated antibodies and biotin that contain functional groups on the contrast agent. The biotin-avidin interaction is among the strongest non-covalent interactions in nature, and is thus widely used in biomedical and analytical applications [113–116]. In clinical research, however, the introduction of foreign antibodies and proteins can elicit an undesirable immune system response. Covalent coupling, on the other hand, does not require intermediate linking proteins and is thus less likely to trigger an undesirable immune response. Mechanisms for covalent coupling of antibodies take advantage of residues on the protein that contain amine or thiol groups. Amide bond formation is achieved between amine groups on lysine residues within the protein and carboxyl groups on the sensor by using carbodiimide chemistry [117, 118]. Alternatively, in the case of thiol-containing residues, thioether linkages can be made to covalently bind antibody elements to contrast agent membranes [119]. Thiol groups present in cystine residues on the ligand couple with maleimide moieties on the contrast agent, which results in a covalent tether. Spacer arms, such as poly(ethylene glycol) (PEG), are often used when linking antibodies to contrast agents to allow for better coupling with target epitopes [105]. In addition to providing greater specificity in tissue imaging, targeted contrast agents can be designed to couple with specific molecules in solution. Functionalized contrast agent-like biosensors form the basis of the sensors designed and discussed extensively in Chapter 2.

1.8 Nonlinear Ultrasound

When the ratio between the amplitude and the wavelength of a sound wave becomes sufficiently high, the wave exhibits nonlinear propagation behavior. Frequency distortions arise due to velocity inequality between the compression and rarefaction phases of the wave [120]. During the high-density compression phases, the speed of the wave is highest. Likewise, wave speed is lowest during the low-density rarefaction phases. Consequently, the portions of the waveform at positively displaced pressures are pushed forward with respect to propagation direction, while the portions of negatively displaced pressure
lag behind. This progression is illustrated in Figure 1–6, where the nonlinear effect increases with distance propagated. As a result, a single frequency sinusoidal waveform is progressively transformed into a sawtooth waveform and harmonic frequency components arise. The effect is cumulative, meaning that larger distortions are observed as propagation distance increases.

The extent of nonlinear distortion that a waveform will undergo is media dependent. System pressure variations as they are related to material density can be described mathematically. The equation of state describes the compression and expansion of a medium, for which the three physical quantities pressure \( p \), density \( \rho \), and specific entropy \( s \) are needed \([34, 120, 121]\). A fundamental quantity that can be obtained from these three state variables is the velocity of sound \( v \) of the acoustic wave:

\[
v^2 = \left( \frac{\partial p}{\partial \rho} \right)_s
\]

(1.19)

where \( v \) is the velocity of the propagating wave in the medium.

The pressure \( p \) can be expressed as a function of the state variables \( \rho \) and \( s \):

\[
p = p(\rho, s)
\]

(1.20)

For an isentropic system (i.e. the entropy of the system is held constant), an expression can be derived for the pressure as a function of density and entropy using a Taylor series expansion:

\[
p - p_0 = \left( \frac{\partial p}{\partial \rho} \right)_{s, \rho = \rho_0} (\rho - \rho_0) + \frac{1}{2} \left( \frac{\partial^2 p}{\partial \rho^2} \right)_{s, \rho = \rho_0} (\rho - \rho_0)^2 + \ldots
\]

(1.21)

or

\[
p - p_0 = A \frac{\rho - \rho_0}{\rho_0} + B \left( \frac{\rho - \rho_0}{\rho_0} \right)^2 + \ldots
\]

(1.22)

where \( p \) is the instantaneous pressure, \( p_0 \) is the pressure at equilibrium, \( \rho \) is the instantaneous density, \( \rho_0 \) is the density at equilibrium, and \( A \) and \( B \) are the first and second order
Figure 1–6: a) The incident acoustic waveform of frequency $F$ undergoes nonlinear distortion as it propagates through the media. The wave moves quicker through the compression phases than the rarefaction phases, resulting in gradual peak distortions indicated by the gray arrows. b) The waveform approaches a sawtooth shape as it propagates further through the media. As a result, harmonic frequencies are generated, which can be observed in the Fourier transform. c & d) The frequency components of the waveform for initial and distorted stages respectively. [120]
coefficients respectively, where

\[ A = \rho_0 \left( \frac{\partial p}{\partial \rho} \right)_{s, \rho = \rho_0} \]  \hspace{1cm} (1.23)\]

and

\[ B = \rho_0^2 \left( \frac{\partial^2 p}{\partial \rho^2} \right)_{s, \rho = \rho_0} \]  \hspace{1cm} (1.24)\]

Higher order terms in the Taylor series expansion contribute a negligible amount to the expression for pressure and are generally ignored. By substituting Equation 1.19 into Equation 1.24, a simple expression for the first order coefficient is obtained:

\[ A = \rho_0 v_0^2 \]  \hspace{1cm} (1.25)\]

where \( v_0 \) is the small-signal adiabatic speed of sound \([47]\). As well, \( B \) can be expressed as the variation of sound velocity with respect to pressure:

\[ B = 2 \rho_0^2 v_0^3 \left( \frac{\partial v}{\partial p} \right)_{s, p = p_0} \]  \hspace{1cm} (1.26)\]

In order to assess the strength of the nonlinear effect that media imposes on the acoustic wave, the relation of \( B \) to \( A \) is of practical importance. The nonlinearity parameter \( B/A \) is thus defined for specific media as:

\[ \frac{B}{A} = \rho_0 \left( \frac{\partial^2 p}{\partial \rho^2} \right)_{s, \rho = \rho_0} \]  \hspace{1cm} (1.27)\]

or, if Equation 1.25 and 1.26 are used,

\[ \frac{B}{A} = 2 \rho_0^2 v_0^3 \left( \frac{\partial v}{\partial p} \right)_{s, p = p_0} \]  \hspace{1cm} (1.28)\]

In the latter equation, \( B/A \) is expressed as a variation of sound velocity \( v \) with pressure \( p \) in an isentropic system. This is easily measured experimentally provided that the pressure is varied rapidly and smoothly to maintain relatively constant entropy.
Table 1–5: Parameter of nonlinearity ($B/A$) for several different media [55, 122]

<table>
<thead>
<tr>
<th>Material</th>
<th>Temperature ($^\circ$C)</th>
<th>$B/A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoatomic gas (ideal)</td>
<td>20</td>
<td>0.67</td>
</tr>
<tr>
<td>Diatomic gas (ideal)</td>
<td>20</td>
<td>0.40</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>20</td>
<td>5.0</td>
</tr>
<tr>
<td>Methanol</td>
<td>20</td>
<td>9.42</td>
</tr>
<tr>
<td>Porcine whole blood</td>
<td>20</td>
<td>6.2</td>
</tr>
<tr>
<td>Human whole blood</td>
<td>37</td>
<td>6.45</td>
</tr>
<tr>
<td>Bovine whole blood</td>
<td>26</td>
<td>5.5</td>
</tr>
<tr>
<td>Human breast fat</td>
<td>30</td>
<td>9.9</td>
</tr>
<tr>
<td>Levovist contrast agent (6.0 mg/mL in Isoton II saline)</td>
<td>20</td>
<td>597</td>
</tr>
<tr>
<td>Albunex contrast agent (1.61 $\mu$L/mL in Isoton II saline)</td>
<td>20</td>
<td>2172</td>
</tr>
</tbody>
</table>

Table 1–5 shows several nonlinear parameter values for select media. It is clear that the magnitude of the distortion of the wave is significantly dependent on the media. An interesting phenomenon to note is the large difference between homogeneous media such as water, and media that contains contrast agents such as Levovist, or Albunex. This difference in non-linear behavior is due to the nature of the bubble oscillations that occur as a result of incident ultrasonic waves [94]. At low ultrasonic power, the bubble wall will resonate at a characteristic frequency. Bubble and contrast agent resonance can be approximated with the model for single bubble resonance in infinite, isotropic media, which is described by the Minnaert equation [123]:

$$f = \frac{1}{2\pi a} \left( \frac{3\gamma p_A}{\rho} \right)^{1/2}$$

(1.29)

where $f$ is the resonance frequency of the bubble, $\gamma$ is the adiabatic index, $p_A$ is the ambient pressure, $a$ is the radius of the particle, and $\rho$ is the density of the surrounding media. If
the incident acoustic signal is at or near the resonance frequency of the contrast agent, a resonance phenomenon will occur, whereby the contrast agent becomes a secondary ultrasound emitter. In clinical applications, microbubbles obtained commercially are on the order of 1-7 \( \mu \text{m} \) in diameter, which corresponds to the frequencies typically used in ultrasonography (2-10 MHz) [97]. If the power of the incident ultrasonic pulse is sufficient, the rarefaction of the contrast agent will occur quicker than the compression, resulting in the nonlinear emission of harmonics (i.e. integer multiples of the fundamental frequency: \( f, 2f, 3f \ldots \)). The harmonic phenomenon is frequently exploited in harmonic contrast agent imaging [94, 123–125]. If the receiving transducer is tuned to filter out the fundamental frequency and only look at the harmonics, the signal of the contrast agents relative to adjacent tissue is greatly enhanced, as these tissues do not exhibit the same magnitude of nonlinear behavior. Studies that focus on capillary and blood flow perfusion use harmonic contrast imaging techniques, where contrast between blood and neighboring tissues is low using regular pulse-echo imaging methods.

### 1.9 Piezoelectric Acoustic Biosensors

There are numerous devices in use today that exploit the mass-sensitive frequency properties of piezoelectric materials. The use of acoustics as a tool for molecular recognition analysis has become increasingly popular in recent years [126, 127]. Specifically, the application of quartz crystal resonator sensors (QCRS) towards fields relating to biosensing and mass analysis have become more prevalent. QCRS became of great interest when scientists discovered a linear relationship between their resonance properties and total mass. This mass dependent behavior provides a highly accurate mechanism for measuring the mass of very small entities. Further, addition of chemical coating onto QCRS devices has enabled molecular recognition properties to be imprinted on their surfaces. A number of different resonator devices are distinguished by the propagation modes used in their applications. The most popular of which are thickness shear mode resonators (TSMR), bulk acoustic wave resonators (BAW), or quarts crystal micro-balances (QCM). Other devices,
which utilize different propagation modes, include surface acoustic wave resonators (SAW), flexural plate wave resonators (FPW), and shear horizontal acoustic plate mode resonators (SH-APM).

QCM devices are made of a thin piezoelectric wafer inserted between two electrodes. When the device is in use, an RF potential is applied to the electrodes, which induces resonance in the piezoelectric crystal. When an entity becomes docked on the quartz surface, the total mass of the wafer increases, effectively altering the resonance frequency. In 1959, a simple model for the dependence of the resonance frequency on the absorbed mass was first described by Sauerbrey:

\[ \Delta f = -\left( \frac{f_0^2}{F_c \rho_c} \right) \left( \frac{\Delta m}{A_{elec}} \right) \]  \hspace{1cm} (1.30)

where \( f_0 \) is the initial resonance frequency of the quartz resonator, \( \Delta m \) is the loaded mass, \( \rho_c \) is the quartz density, \( A_{elec} \) is the surface area of the electrode, and \( F_c \) is the frequency constant of the crystal defined by:

\[ F_c = f_0 d_c \]  \hspace{1cm} (1.31)

where \( d_c \) is the thickness of the crystal resonator. Classically, these QCM devices were used under vacuum to monitor thin film deposition rates [128]. Later applications extended functionalized QCM devices to employment as gas-phase sensors [129], whereby suspended aerosol or gas content is monitored in real time. More recently, QCM use has been demonstrated in liquid systems to characterize proteins and study cell adhesion [130, 131]. The use of functionalized QCM instruments demonstrates the utility of recognition coatings in quantitative measurement systems. Molecular recognition can be incorporated to induce target-specific binding in solution. The molecular target recognition principle is exploited in numerous biosensing applications, including those demonstrated in this thesis.
1.10 Recent Developments in Ultrasonic Frequency Analysis

Recent developments in the field of ultrasonic frequency analysis have led to the development of a transmission ultrasound device for the characterization of liquid systems. Mixture compositions have been estimated in systems where the volume fractions vary greatly. In particular, Dion et al. have reported the use of ultrasound instrumentation to quantify volume fractions of multicomponent mixtures [132], and to determine alcohol and carbohydrate concentration in commercial beverages [133]. Acoustic measurement of these parameters or constituents can be made by determining the speed of sound in the multicomponent mixture, however this leads to ambiguity for larger volume fractions [134]. The speed of sound increases monotonically as the fraction of ethanol increases from 0-30%. After 30%, the speed of sound begins to decrease as the volume fraction of ethanol increases. It is difficult to determine whether a sample is pure water or a 50-50 mixture of water and ethanol, given that both solutions have a speed of sound of approximately 1500 m/s. By making a frequency analysis of the transmitted acoustic signal, a more robust model of composition can be established. Nonlinear distortions imparted on the acoustic signal by the media are highly dependent on the specific volume fraction of the system, allowing for accurate estimation of water-ethanol composition [132]. Composition measurements have been taken further to quantification of commercial beverages, where alcohol and carbohydrate content of several beverages from various manufacturers were estimated [133].

Low-level molecular quantification using ultrasound is difficult when the target species is very small. Detection limits are lowered by several orders of magnitude through the use of large molecular sensors. When tagged with recognition elements, hydrogel sensors achieve high affinity for the target analyte. Dion et al. have investigated the use of dendrimers [135], antibodies [136], and hydrogels [136, 137] as components for creating ultrasonic sensors. Large, readily functionalizable materials, such as hydrogels or dendrimers, are used as the core of these sensors. One approach for imparting molecular recognition on hydrogel
sensor bodies is to conjugate their structures to antibodies. Using carbodiimide chemistry, carboxyl groups within the hydrogel or dendrimer can be chemically paired to amine groups on lysine residues of the antibody. By controlling the size of the dendrimer or hydrogel, it is possible to tune the acoustic properties of the sensor. The conjugated antibody behaves as a recognition element, undergoing conformational changes when bound to the analyte molecule. Conformational changes are greatly enhanced by the presence of the hydrogel or dendrimer sensor, as these components contort to accommodate the newly arranged antibody. Numerous antibodies can be attached a single hydrogel or dendrimer entity, which greatly enhances signal differences as multiple binding events incur greater changes in the physical shape of the sensor.

Another method for creating recognition sites in the sensor is molecular imprinting [137]. Acrylamide hydrogel polymers can be synthesized in the presence of the analyte molecules. The monomers self assemble and organize around the target compound via intermolecular forces such as hydrogen bonding and pi-stacking. When polymerized in solution, the monomers retain their position in space, forming an analyte pocket of high molecular recognition. The molecular imprinting technique has the added advantage of being tunable for a multitude of target species, while antibodies need to be obtained specifically for their application. Without the need for expensive antibodies, the cost of sensor synthesis is greatly reduced. Acrylamide polymers can also be lyophilized and stored for extended periods without degradation of their molecular recognition properties. A wide variety of polymers can be prepared well in advance, meaning specific sensors can be readily available for their application.

1.11 Research Objectives

Ultrasound has a variety of uses in many diverse fields of science, medicine, and technology. Recent progress in these areas has led to improved means of quantifying chemical systems using acoustic waves. The use of ultrasound allows for non-destructive and non-invasive measurements to be made in real time, thus making it a viable tool for
obtaining data in the field. The ultrasonic method is an attractive candidate for portable systems that can be deployed in non-laboratory environments.

Ultrasound is prevalent in industrial and medical applications, however, its utility as a spectroscopic characterization method for chemical systems is in the early stages of research and development. Time of flight and attenuation studies have spun off numerous applications for the characterization of simple systems, yet the potential for frequency domain analysis of ultrasound in non-linear, complex systems have not been fully explored. Continuing investigations are necessary before systems can be developed that will provide quantitative information with minimal or no operator expertise.

This thesis demonstrates the viability of using ultrasonic spectroscopy as a method for quantification of biologically relevant molecules. Specifically, the work focuses on the development two ultrasonically active biosensors that respond to the presence of vitamin D$_3$. First, the molecular imprinting technique based on acrylamide hydrogel polymers is used to demonstrate the applicability of the hydrogel method on the quantification of vitamin D. Next, a novel approach to sensor design is demonstrated in which native binding proteins are used as recognition elements embedded within a cellulose hydrogel network. Frequency analysis of the ultrasound signal as it propagates though the solutions that contain hydrogel sensors is demonstrated. The methods are described demonstrated in principle in samples of phosphate buffered saline solution spiked with 25-OHD$_3$. Furthermore, an effort is made to quantify samples of human serum containing endogenous 25-OHD$_3$. The project described in this thesis serves as one of many prerequisite proof-of-concept procedures for extending the application of ultrasound to routine practice in chemical analysis.
CHAPTER 2

Ultrasonically Active Hydrogel Sensors for the Quantification of Vitamin D$_3$

2.1 Foreword

The goal of this chapter is to outline and discuss the process for creating ultrasonic sensors that are capable of responding to the presence of vitamin D$_3$. The techniques for sensor design discussed in Section 1.10 provide the basis for the work in this thesis. In the following manuscript, two sensor synthesis protocols are discussed with different merits and potential applications.

The first sensor implements chemistry similar to that used to conjugate antibodies with hydrogels. Proteins are attached to cellulose networks in order to create sensors with molecular recognition. The use of vitamin D binding protein as a molecular recognition element is investigated as a novel technique for imparting target recognition to acoustic sensors. Vitamin D binding protein (VDBP, or Gc-globulin) is a readily available carrier protein that is endogenous to humans. This protein’s primary function is to provide transportation for vitamin D metabolites. VDBP demonstrates a high binding efficiency towards different circulating metabolites of vitamin D with greatest affinity towards 25-hydroxycholecalciferol, the metabolite of diagnostic interest. VDBP can be used as a target recognition entity with multiple analyte detection capabilities. Conjugated with
cellulose, the incorporation of VDBP within a hydrogel sensor is demonstrated for multiple analytes in serum and buffer solutions.

The second sensor is an extension of the work done by Dion and Troïani [137] on molecular imprinting of acrylamide-based polymer hydrogels. By tailoring the recognition pockets to specifically target 25-hydroxycholecalciferol, highly selective recognition can be achieved among the presence of other vitamin D metabolites and interfering species in phosphate buffer and human serum.

A novel method for the detection and quantitation of 25-hydroxycholecalciferol is presented in this work. Transmission ultrasonic spectroscopy was used to monitor the changes in the frequency profile of each sensor after analyte introduction. Ultrasound spectroscopy was demonstrated on complex samples without preprocessing or chemical separation steps. Quantitative 25-hydroxycholecalciferol measurements in unfiltered human serum is also demonstrated in this chapter. Removing the need for sample pretreatment is advantageous, since it reduces sample preparation time and greatly reduces the overall analysis time. The ultrasound method coupled with acoustically-responsive hydrogel sensors can improve the current clinical model by allowing measurements to be made on site with minimal expertise.
2.2 Manuscript

Ultrasonically Active Hydrogel Sensors for the Quantification of Vitamin D₃

Authors:
Andrew G. Dafoe, Jonathan R. Dion, and David H. Burns

2.3 Abstract

Research invested in point-of-care techniques for the analysis of biological samples is growing rapidly. To meet the needs of modern clinical healthcare, high-throughput analytical techniques are in development to achieve shorter turnaround times for routine analyses. Ideally, testing should be done "on-site" to provide immediate results for rapid diagnosis. Recent clinical research has identified vitamin D as an important factor in human health and well-being. Vitamin D status is currently evaluated using HPLC, GC-MS, RIA, CL, CPB, or ELISA protocols, all of which require skilled personnel, chemical reagents, and expensive equipment. We propose that ultrasound can be used as an inexpensive alternative for point-of-care clinical measurements. The recent development of ultrasonic spectroscopy as a method for characterizing liquid systems shows promise for low-level biological compound detection. The work presented in this manuscript involves the synthesis of hydrogel entities with embedded recognition sites to facilitate interaction with vitamin D metabolites. To evaluate the feasibility of using ultrasound to determine vitamin D status in patients, two sensors were designed to target 25-hydroxycholecalciferol, the primary circulating vitamin D metabolite in the human body. The first sensor involved native vitamin D binding protein groups embedded within a 3D cellulose network (R²=0.92, SEE=1.4 nM). The other sensor used molecular imprinting to create molecular recognition elements within an acrylamide based hydrogel copolymer (R²=0.88, SEE=11.4 nM).
Experimental results indicate that frequency domain analysis of ultrasonically active hydrogel sensors is a viable method to quantify vitamin D content in serum.

2.4 Introduction

2.4.1 Background

Vitamin D status in patients is becoming a greater global concern as new studies reveal issues related to deficiency. The active form of vitamin D plays a major role in immune system regulation [17], and serves to maintain calcium and phosphate levels in the blood [22]. Insufficient vitamin D has been linked to psychological problems including depression, irritability, tiredness, mood swings, sleep difficulties, weakness, inattentiveness, and pain [12]. Asthenic patients often exhibit metabolic vitamin D deficiencies as compared to non-asthenic patients [138]. Other more serious complications may arise given prolonged periods of extreme deficiencies [12, 13, 16, 18, 138]. In general, a patient is considered vitamin D deficient when their blood vitamin D content is below 30 ng/mL. Rickets is one of many examples in which vitamin D deprivation has been linked to a serious medical complication. In 1822, Jędrzej Śniadecki discovered a connection between the softening/weakening of bones with low vitamin D level. Historically, the remedy for rickets was to expose the patient to sunlight, which Śniadecki suggests "as one of the most efficient methods for the prevention and cure of this disease" [18, 139]. Today, three main treatment options are available to combat vitamin D insufficiency: sunlight exposure, artificial UVB irradiation, and dietary supplementation. As excess exposure to high energy UV radiation poses potential health risks [140], the standard clinical treatment for vitamin D deficiency is through oral administration.

Vitamin D can be ingested from a fortified diet, or synthesized naturally in the skin through ultraviolet irradiation [141]. Cholecalciferol (vitamin D₃) is the stable, naturally occurring form of vitamin D that is produced in the skin through UV photolysis of 7-dehydrocholesterol. It is much more effective at treating deficiency in humans than plant-based ergocalciferol (vitamin D₂) [30]. Vitamin D₃ becomes hydroxylated
in the liver to 25-hydroxycholecalciferol (25-OHD₃), the primary circulating metabolite. Further hydroxylation occurs via enzyme mechanisms in the kidney to form the active 1α,25-hydroxycholecalciferol (1α,25-OHD₃) metabolite [22, 142]. Developing an assay for measuring 25-OHD₃ levels in patients is an issue of accuracy as well as selectivity, since not all vitamin D metabolites are helpful in determining patient vitamin D status. The serum half-life of cholecalciferol is approximately 24 hours, so any values recorded are indicative of recent UV exposure or treatment, and not baseline levels. As well, the synthesis of 1α,25-OHD₃ is highly regulated by parathyroid hormone (PTH) and has a low serum half-life (approximately 4-6 hours). Instead, the best candidate for the assessment of patient vitamin D status is the 25-OHD₃ metabolite, whose serum half-life is approximately 3 weeks.

Currently, high-performance liquid chromatography and gas chromatography coupled with tandem mass spectrometry detection systems are standard reference tools for quantifying 25-OHD₃ [20, 22]. Although mass spectrometry devices can selectively quantify different metabolites with high accuracy, they lacks ease-of-use and multi-sample analysis capabilities of other routine clinical techniques. An alternative to column separation techniques is the use of immunoassays, which offer low detection limits and high sample throughput. Immunoassay methods are routinely used in clinical applications, however there is often a high amount of inter- and intra-assay variability [16, 18, 20–22, 143]. It is worth investigating different methods for quantifying 25-OHD₃ with a focus on improved accuracy, reproducibility, and sample throughput.

2.4.2 Ultrasound spectroscopy

Acoustics have a wide range of applications in many diverse fields from process and quality control in industry, to imaging and soft tissue therapy in medicine. Ultrasound refers to sound waves whose frequency exceeds 20 kHz. Waves in this frequency region have many uses including flaw detection of industrial building components [73, 144] and non-invasive medical imaging [145]. Recently, ultrasound spectroscopy has been
Table 2–1: Analysis range and sample requisite for modern 25-OHD$_3$ assays [23].

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sample (µL)</th>
<th>Range (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioimmunoassay</td>
<td>50</td>
<td>4-400</td>
</tr>
<tr>
<td>Enzyme-Linked Immunosorbent Assay</td>
<td>50</td>
<td>6.3-250</td>
</tr>
<tr>
<td>Chemiluminescence</td>
<td>20</td>
<td>17.5-300</td>
</tr>
<tr>
<td>Liquid Chromatography</td>
<td>500</td>
<td>15-150</td>
</tr>
<tr>
<td>Competitive Protein Binding</td>
<td>50</td>
<td>8-312</td>
</tr>
</tbody>
</table>

used in place of optical spectroscopy. Although highly accurate and robust, optical spectroscopy can be difficult to employ on solids and in highly scattering media. Light is subject to absorption and scattering effects, which can lower the intensity of the analytical signal, resulting in diminished signal-to-noise ratios. Ultrasound, in contrast, is a mechanical wave, which can penetrate concentrated samples such as blood and tissue. Undiluted quantification of an otherwise optically non-transmissive sample is possible using ultrasound. Many studies report the characterization of chemical systems using ultrasonic methods [54–57, 132, 133, 136, 137]. Modeling the behavior of sound and its interaction with viscous media gained popularity following a study published by Stokes in which he discussed the correlation between sound attenuation and liquid viscosity [146]. More recently, the specific interactions that exist between acoustic waves and the media though which they propagate have been explored [34, 55, 147–149]. These interactions include a variety of linear and non-linear phenomena, which can be exploited to obtain physical and chemical information about the system.

With recent progress in hydrogel manufacturing, micron-sized hydrogel networks can be synthesized with uniform sizes and unique features. These tunable microgels have broad application potential in ultrasonic contrast agents that are used in medical imaging [150]. Sensors and contrast agents that are fabricated with these materials have
intrinsic qualities which make them suitable for ultrasonic imaging [151]. Hydrogel contrast agents readily reflect a large percentage of acoustic energy due to the large acoustic impedance disparity between the hydrogel and the surrounding media. Interaction between the hydrogels and the incident pressure waves cause oscillations to reverberate within the agent, generating secondary acoustic waves from the surface of the sensor. The behavior of these oscillations is non-linear since the compression phases of the sensor occur faster than the relaxation. Secondary resonance frequencies are generated through this non-linear phenomenon, giving rise to harmonic acoustic waves that are characteristic of the size and stiffness of the contrast agent.

A simplification of the Minnaert equation for microbubble resonance describes the dependence of the resonance frequency on the physical properties of the system under measurement:

\[
 f_0 \approx \frac{1}{2\pi a_e} \sqrt{\frac{3K_p}{\rho_L}}
\]

where \( f_0 \) is the resonance frequency of the microgel, \( a_e \) is the oscillator radius, \( K_p \) is the modulus (or stiffness) of the microgel, and \( \rho_L \) is the density of the surrounding media. Changes in the resonance frequency of the sensor are possible through the introduction of mechanisms to alter the size and stiffness of the microgel. Binding events between the microgel and its target molecule will induce changes in both the size and stiffness of the sensor. Through this binding interaction, a frequency shift of the secondary waveform emission will occur. Quantitation of the target species can be achieved by modelling the frequency shift as a function of analyte concentration. The use of NIPA-based hydrogels have been explored for this type of application [137]. The gel itself is very sensitive to environmental changes. Depending upon the stimulus, NIPA-based hydrogels will undergo changes in water content, size, stiffness, or a combination of all three [137, 152–159]. This transformation is reversible, which allows the sensor to be used multiple times. When stimulated, the swelling and expansion behavior of these hydrogels alter the frequency at which they resonate. This in turn can impart significant signal changes to the passing
ultrasound wave, leading to frequency shifts that are dependent on sensor status. We have developed a quantification method that uses ultrasound to monitor hydrogel biosensors in solution. The hydrogels were designed to selectively bind to 25-OHD$_3$ so that any measured change in biosensor size and stiffness is a direct function of analyte concentration.

2.4.3 Designing acoustic sensors

In order to develop a sensor that is capable of selectively responding to the presence of a target analyte, it is necessary to create permanent docking elements. Antibodies are assigned this task due to their high affinity and specificity for the target molecule. However, antibody activity is highly dependent on the media, which can lead to non-specific binding and other accuracy-hindering phenomena [160]. Furthermore, antibodies are expensive to obtain in their purified form. Molecular imprinted hydrogels are an attractive alternative, as they can be inexpensively synthesized with high target specificity.

One approach in hydrogel sensor design is to adapt a naturally occurring compound that already has an affinity for the analyte in question and augment it such that an acoustic response is produced. Gc-globulin (vitamin D binding protein, or VDBP) is a protein that exists naturally in blood [161]. Its primary function is to circulate vitamin D metabolites to neighboring tissues and organs throughout the body. VDBP has a high affinity for 25-OHD$_3$, which makes it an excellent choice as a sensor recognition element. A micron-scale hydrogel network with interspersed functional groups capable of chemically bonding to VDBP is required for this sensor design. In this work, carbodiimide chemistry is used to couple carboxyl moieties in carboxymethyl cellulose to amine groups present on proteins [136]. For this experiment, a three-dimensional cellulose-based hydrogel network has been functionalized with VDBP to create an acoustic sensor capable of selectively targeting 25-OHD$_3$. Upon specific targeting and binding of the protein structures to the vitamin D metabolite, the acoustic profile of the solution is measured using the ultrasonic instrumentation presented in this work.
Figure 2–1: Principles of molecular imprinting. Monomers assemble around the analyte in solution. Polymerization takes place, preserving the monomer formation around the template. The template is then removed, leaving behind analyte affinitive pockets. These pockets serve as recognition sites for the sensor.

Molecular imprinting is another way that recognition elements can be embedded in a polymer network (see Figure 2–1). N-isopropylacrylamide (NIPA) monomers are copolymerized with methacrylic acid (MAA) to form long NIPA-co-MAA chains in solution. These chains are further cross-linked with N,N’-methylenebisacrylamide (MBA) in order to create 3D networks [137, 158, 159, 162, 163]. The MAA moieties will arrange in a thermodynamically favorable configuration around the vitamin D metabolite through hydrogen bonding interactions. Once the polymerization is complete, the template molecules are removed either through successive solvent extractions or multi-stage dialysis. In the absence of the vitamin D metabolites, the hydrogel sensor retains the analyte pocket structures. These features persist indefinitely, allowing for reintroduction of the target species at a later time. The advantage of NIPA-based hydrogels is that they can be tuned to swell or shrink depending upon the stimulus, such as temperature, pH, ion content, or hydrogen bonding [137, 153–159]. The resultant deformation in hydrogel size and shape can be measured using the ultrasound instrumentation presented in this work.

2.5 Materials and Methods

Deionized water purified by a Millipore Milli-Q OM-154 water purification system (Billerica, MA) was used for all experiments. All chemicals and reagents were obtained from Sigma-Aldrich (Oakville, ON). Unfiltered human serum samples from 15 individual
donors were obtained from Bioreclamation (Liverpool, NY). Initial serum 25-OHD₃ levels were obtained externally using a Liaison® direct competitive chemiluminescence immunoassay (CLIA) carried out by the Weiler group (School of Dietetics and Human Nutrition, McGill University). This external analysis step was necessary for verification of this technique. A BI-200SM dynamic light scattering spectrometer, equipped with a TurboCorr digital correlator and 532 nm laser source (Brookhaven Instruments Corporation, Holtsville, NY), at a scattering angle of 90 degrees, was used for characterizing the size of the synthesized hydrogel sensors.

2.5.1 Synthesis of cellulose conjugated Gc-globulin sensor

A 2-(N-morpholino)ethanesulfonic acid (MES) buffer was prepared by dissolving 0.9760 g of MES (sodium salt) and 0.8768 g of NaCl in 50 mL of H₂O (0.1 M MES, 0.3 M NaCl, pH 6.5). A carboxymethyl cellulose solution was prepared by dissolving 0.1000 g of carboxymethyl cellulose (CMC, 250,000 MW) in 10 mL of MES buffer and vortexed gently for 24 hours to ensure complete dissolution. A diluted CMC solution was then prepared by adding 150 µL of the buffered CMC solution to 30 mL of MES buffer to achieve a 0.005% solution by weight. The coupling agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), was then added in the amount of 63.45 µg to the 0.005% cellulose solution, followed by 25.85 µg of N-hydroxysuccinimide (NHS). The reaction mixture was stirred for 15 minutes, followed by an addition of 3.0 mg Gc-globulin (≥ 90% (SDS-PAGE), ≈51.9 nmol). To ensure coupling, the solution was stirred for 4 hours. Over the course of the coupling reaction, 15 µL of divinyl sulfone was added every 45 minutes to initiate crosslinking of the cellulose chains. After 4 hours of reaction, the samples were dialyzed in phosphate buffered saline for 4 days, with 1 buffer change per day (0.1 M PBS, 0.15 M NaCl, pH 7.4). This was done to ensure complete removal of any unreacted species remaining in the reaction vessel. The final mixture was diluted to 300 mL with PBS. A series of standard solutions for calibration purposes were prepared with 4 mL of the final sensor mixture dispensed into 18 sample vials. An addition
of 25-OHD$_3$ dissolved in ethanol was administered to the standard sensor solutions to give 6 calibration standards ranging from 1.6-11.1 nM in triplicates. Counter additions of ethanol were added to the low concentration standards in order to ensure the same ethanol content for every solution. Dynamic light scattering measurements of the raw sensor solution showed an estimated mean particle diameter of 0.201 µm. Upon addition of excess analyte, the sensor size increased to 0.281 µm.

### 2.5.2 Synthesis of cellulose conjugated Gc-globulin sensor for serum study

Mildly acidic buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) was prepared by dissolving 4.3440 g of MES (sodium salt) and 3.5070 g of NaCl in 200 mL of H$_2$O. A CMC solution was prepared by dissolving 0.1000 g of carboxymethyl cellulose (250,000 MW) in 10 mL of MES buffer and vortexed gently for 24 hours. A 100 µL volume of this CMC solution was added to 9.9 mL of MES buffer to obtain a 0.01% solution by weight. The diluted solution was vortexed for an additional hour to ensure full dissolution of the CMC. The coupling agent, EDC, was added in the amount of 51 µg to the 0.005% cellulose solution, followed by 15 µg of NHS. The reaction mixture was vortexed for 15 minutes. To this solution, 1.0 mg of Gc-globulin ($\geq$ 90% (SDS-PAGE), $\approx$ 17.3 nmol) was added. The solution was left to react for 4 hours under a gentle vortex. Over the course of the coupling reaction, 10 µL of divinyl sulfone was added every hour (starting 30 minutes into reaction) to crosslink the cellulose chains. After 4 hours, the solution was dialyzed in phosphate buffered saline solution for 4 days, with 1 buffer change per day (10 mM PBS, 0.15 M NaCl, pH 7.5). This was done in order to remove any unreacted species present in the solution. The final mixture was diluted to 19 mL with PBS. The sensor solution was added to 5 different 200 µL serum samples in aliquots of 3.8 mL. A standard addition experiment was carried out by adding 10 µL aliquots of 25-OHD$_3$ dissolved in ethanol. Each standard addition raised the concentration by approximately 2 nM. The signal contributions from the addition of ethanol were decoupled from concentration of the analyte by adding an additional volume of ethanol between 8 and 19.5 µL following each standard addition. The volume of this
additional alliquot of ethanol was randomized to prevent correlation with increasing analyte concentration during data processing.

2.5.3 Synthesis of imprinted NIPA-co-MAA hydrogel sensor

A three-neck round bottom flask was fitted with a condenser and nitrogen line. To it, 100 mL of deionized water was added, followed by 1.3500 g of N-isopropylacrylamide (NIPA), 0.1500 g of methacrylic acid (MAA), 0.0211 g of N,N’-methylenebisacrylamide (MBA). Since dissolved oxygen present in the reaction solution can lead to premature chain termination, the reaction vessel was purged with nitrogen for 4 hours. Furthermore, the reaction vessel was covered with aluminum foil, as the 25-OHD₃ metabolite is sensitive to light. A 50 µg measure of 25-OHD₃ dissolved in 50 µL of ethanol was then added to the reaction vessel. The solution was stirred for 15 minutes, followed by an addition of 0.0650 g ammonium persulfate (APS) to initiate the free-radical polymerization reaction. As well, 120 µL of tetramethylethylenediamine (TEMED) was added as a reaction accelerator. The reaction was left for 10 hours in a closed nitrogen environment. The final solution was dialyzed in 4 L of PBS buffer (10 mM PBS, 0.15 M NaCl, pH 7.5) over the course of 2 days with 3 buffer changes. The sensor was diluted to 200 mL with the dialysis buffer solution and aliquoted into sample vials (4 mL each). A total of 18 samples were used for the molecularly imprinted sensor experiment in PBS. External standards were prepared by the addition of 25-OHD₃ dissolved in ethanol to give solutions of 25-150 nM in triplicates. Counter additions of ethanol were introduced in order to ensure the same ethanol content for every solution. Dynamic light scattering measurements were then made to determine the mean particle diameter of the hydrogel. The solution was found to contain particles with an estimated mean particle diameter of 1.585 µm. Upon addition of excess analyte, the sensor size was reduced to 0.922 µm.

2.5.4 Synthesis of imprinted NIPA-co-MAA hydrogel sensor for serum study

A stock solution of reaction reagents was prepared by dissolving 0.2500 g of NIPA, 0.0250 g of MBA, and 0.0250 g of MAA into 25 mL of deionized water. A 2.5 mL aliquot
of the stock monomer solution was dispensed into a reaction flask covered in aluminum foil. To this, a measure of 15 µg of 25-OHD$_3$ dissolved in 15 µL of ethanol was added. The solution vessel was purged with nitrogen for 30 minutes with gentle stirring. Following this, 0.6 mg of APS was added to initiate polymerization. Additionally, 3 µL of TEMED was added as an accelerator. The reaction was left under a closed nitrogen environment with gentle stirring for 10 hours. The template molecule, as well as unreacted chemical reagents, were removed by dialysis (first in a 25:75 ethanol-water mixture, followed by deionized water, followed by 50:50 ethanol-water, followed by three more deionized water solutions) over the course of 4 days. A 300 µL aliquot of the final sensor solution was added to 5 different unfiltered serum samples of 4 mL each. A standard addition experiment was carried out by adding 10 µL aliquots of 25-OHD$_3$ dissolved in ethanol. Each standard addition raised the concentration by approximately 11.2 nM. The variations associated with the additions of ethanol were decoupled from concentration of the analyte by adding an additional random volume of ethanol (between 8 and 19.5 µL).

2.5.5 Instrumentation

Ultrasonic frequency pulses were generated by a Model 500PR pulse generator (Panametrics Inc. Waltham, MA.) and subsequently digitized using a Handyscope HS3 USB oscilloscope (TiePie engineering. Sneek, FR) sampling at 50 MHz with 12-bit
resolution. Ultrasound transducers (Technisonic, Fairfield CT.) with a frequency response centered at 5.0 MHz were used to generate and receive the ultrasonic pulse. A 3.5 mL aluminum sample cell with a path length of 2.5 cm was used. In the case of the sensor experiments where the standard matrix was phosphate buffered saline, acetate windows were used to separate the sample from the transducer face. To minimize signal loss, the head of the transducer was coupled to the acetate window with petroleum jelly. For all subsequent serum experiments, the transducers were threaded and fastened on both ends of the cell with the transducer element in direct contact with the sample solution. The cell was temperature controlled using a Peltier device set to maintain a temperature of 25 degrees Celsius. The experimental configuration is illustrated in Figure 2–2. All data processing was done in MATLAB (The MathWorks Inc., Natick, MA).

2.5.6 Data processing

For each ultrasonic pulse, a 100 µs window of data was collected by the receiving transducer and measured by the oscilloscope. In order to reduce the effect of signal variation, 1500 replicate spectra were collected for each sample. To account of acquisition time drift and fluctuations in sound arrival time, the signal profiles were aligned with one another in the time-domain. This was achieved though a simple peak-alignment algorithm. The occurrence in time of the maximum signal value for each spectrum was indexed. The most common index is selected and any spectrum whose signal maximum deviates from that index is shifted appropriately. Once aligned, the spectra were averaged together to improve the signal to noise ratio. A fast Fourier transform (FFT) algorithm was applied in order to convert the averaged time-series data into the frequency domain.

The intensity of the acoustic signal has been found to drift over the experimental time frame. A number of contributing factors have been found to account for this error, inducing transducer temperature and pulser runtime. In order to correct for this, the frequency profiles were area-normalized. Random frequency fluctuations were also reduced in the data during post-processing by using a boxcar smoothing function.
Once all the experimental spectra had been collected and processed, a stage-wise multilinear regression (SMLR) algorithm was used to correlate changes in magnitude of the ultrasonic frequency profiles with analyte concentration [164]. The SMLR algorithm recursively determines multiple frequency regions that collectively serve as predictors of analyte concentration. Starting with the region of highest correlation, the algorithm calculates the residuals of regression. Next, the algorithm continues searching through the subset of frequencies for further correlations. Once the process is complete, a linear combination of the frequency subset selected can be used to predict the concentration of analyte. The analyte concentration can be predicted using the following equation:

\[ C = \alpha_0 + \alpha_1 f_1 + \alpha_2 f_2 + ... + \alpha_n f_n \]

where \( C \) is the predicted analyte concentration, \( f_1-\)\( n \) are the selected frequencies, and \( \alpha_{0-\ n} \) are the weighting coefficients.

To ensure parsimony in the model, an F-test (\( \alpha = 0.05 \)) for regression models was applied to determine the appropriate number of frequencies to describe the data. This involves calculating the residual sum of the squares (RSS) of two sequential models and determining if the difference is statistically significant. If the inclusion of an additional factor provides no significant gain in predictability, then that additional factor is omitted and the current model is used.

In order to verify the validity of the model generated by the SMLR process, an independent test set of samples was used. This set consists of concentrations of vitamin D that were not used in the original formulation of the model. The accuracy of the prediction equation is assessed by attempting to predict these independent samples. Accurate prediction of the test samples confirms whether the frequencies of the model have been appropriately selected and describe the desired trend, as opposed to undesired trends, which can arise from random errors and unforeseen experimental factors.
2.6 Results and Discussion

In order to examine sensor-analyte recognition and changes in the ultrasound signal, it was necessary to carry out the assay first in the absence of interfering species. The preliminary analyses were carried out in a phosphate buffered saline solution to stabilize the media pH. The pH of the media was adjusted to physiological levels to evaluate sensor response in human serum conditions.

2.6.1 Interferent-free buffer studies

For the NIPA-co-MAA imprinted polymer assay in PBS, the results of the interference-free test are shown in Figure 2–3.a and 2–3.b. For the molecularly imprinted sensor, the most parsimonious model was obtained with 4 factors at frequencies of 0.83, 1.51, 2.93 and 3.43 MHz. The vitamin D metabolite, 25-OHD₃, was quantified within the concentration range of 25-150 nM. This calibration model had a standard error of estimation (SEE) of 12.3 nM and a coefficient of determination (R²) of 0.96. This model provided a modest quantification of 25-OHD₃ within the healthy physiological range (25-125 nM). The variation in the signal is well correlated with the change in concentration as seen in the mean-centered spectra in Figure 2–4.a. It is clear from the figure that the introduction of the analyte species results in an intensity increase of the 4 MHz center frequency, as well as other subtle shifts in different regions of the spectrum.

The regression results for the Gc-globulin coupled hydrogel sensor are shown in Figure 2–3.c and 2–3.d. The model for the cellulose conjugated Gc-globulin hydrogel sensor achieved parsimony with 4 factors at frequencies of 0.57, 1.18, 2.70, and 4.70 MHz. With this model, 25-OHD₃ was estimated in the concentration range of 1.6-11.1 nM with a standard error of estimation (SEE) of 0.868 nM and a coefficient of determination (R²) of 0.95. This model was able to quantify 25-OHD₃ at levels 20 times less than those found in healthy adults. The mean-centered spectra in Figure 2–4.b illustrate the change in signal intensity with respect to analyte concentration. Specifically, regions of large variation are centered around 5 MHz, which overlaps with the optimal response region.
Figure 2–3: Estimation of 25-OHD₃ concentration with two different sensor types. 

a) Acoustic profile of the molecularly imprinted hydrogel sensor with bars indicating the weight and frequency of model factors. 
b) Validated estimation of 25-OHD₃ in the molecularly imprinted NIPA sensor using points not present in the original formulation of the model (X). 
c) Acoustic profile of the cellulose-conjugated protein hydrogel sensor with bars indicating the weight and frequency of model factors. 
d) Validated estimation of 25-OHD₃ in the cellulose-conjugated protein sensor using independent concentration sets not present in the original formulation of the model (X).
Figure 2–4: Comparison of ultrasonic spectra for low, intermediate, and high concentrations of 25-OHD₃ for a) the molecularly imprinted NIPA sensor, and b) the cellulose-conjugated vitamin D binding protein sensor. The spectra have been mean-centered and smoothed using a simple moving average algorithm for clarity.

of the transducers. It is interesting to note that this trend is apparently opposite to that of the shifts seen in Figure 2–4.a. This trend is consistent with the data obtained through DLS particle sizing measurements. Introduction of 25-OHD₃ to the Gc-globulin sensor resulted in a mean particle diameter increase of 42% (from 0.201µm to 0.201µm). In comparison, the NIPA-based molecularly imprinted sensor had undergone a 40% reduction in size after analyte introduction (from 1.585µm to 0.922µm). These opposing trends are evident in Figure 2–4, where a frequency intensity increase in one is roughly observed as a decrease in the other.

2.6.2 Serum studies

In order to determine if 25-OHD₃ could be measured using the molecularly imprinted NIPA hydrogel in the presence of interfering species, serum samples were used in place of PBS. Quantification of the vitamin D metabolite, 25-OHD₃, was successfully achieved as indicated by the regression results illustrated in Figure 2–5. The initial values of 25-OHD₃ in the serum samples prior to standard addition were externally verified by a Liaison® chemiluminescence immunoassay. Once serum 25-OHD₃ levels were obtained, the values
Figure 2–5: Regression model for the molecularly imprinted hydrogel sensor in human serum samples. a) Selected profiles of the hydrogel samples with cofactors from the SMLR results (grey bars). Initial 25-OHD₃ values obtained from Liaison® chemiluminescence immunoassay. b) Known concentrations of 25-OHD₃ plotted against those estimated by the SMLR. Concentration points used to validate the model shown as X.

Figure 2–6: Regression model for the cellulose-conjugated binding protein sensor in human serum samples. a) Selected profiles of the hydrogel samples with cofactors from the SMLR results (grey bars). Initial 25-OHD₃ values obtained from Liaison® chemiluminescence immunoassay. b) Known concentrations of 25-OHD₃ plotted against those estimated by the SMLR. Concentration points to validate the model shown as X.
were incorporated into the model. In order to minimally dilute the serum samples, the sensor was prepared in a much smaller volume as outlined in Section 2.5.4. The reduced volume of sensor in the presence of higher concentrations of vitamin D during synthesis allowed for the creation of higher binding capacity sensor. As a result, the addition of less sensor was necessary to achieve an appropriate dynamic range of calibration in the serum. After data acquisition, a simple moving average algorithm (SMA) was employed with a 0.33 MHz bandwidth to reduce variation in the signal. The most parsimonious model was obtained with 3 factors at frequencies of 1.77, 1.95, and 2.40 MHz. With this model, the concentration of 25-OHD$_3$ was estimated in the concentration range of 41.4-134 nM with a standard error (SEE) of 11.4 nM and a coefficient of determination ($R^2$) of 0.88.

Serum samples were used in place of PBS as the matrix in order to determine if 25-OHD$_3$ could be measured using the cellulose-conjugated Gc-globulin sensor in the presence of interfering species. Quantification of the vitamin D metabolite, 25-OHD$_3$, was successfully achieved as indicated by the regression results illustrated in Figure 2–6. The initial values of 25-OHD$_3$ in the serum samples were obtained externally before standard addition. The initial serum 25-OHD$_3$ concentration values from these external results were incorporated into the model. In order to ensure that the sensor was not saturated with 25-OHD$_3$ upon addition into the serum matrix, the serum was diluted 20-fold. A SMA algorithm with a 0.33 MHz bandwidth was used to reduce the variation in the signal. The most parsimonious model was obtained with 2 factors at frequencies of 1.41 and 2.16 MHz. With this model, the concentration of 25-OHD$_3$ was estimated in the concentration range of 1.5-13.8 nM with a standard error (SEE) of 1.4 nM and a coefficient of determination ($R^2$) of 0.92. This model provided a reasonable quantification of 25-OHD$_3$ well below the healthy physiological range, allowing for sample dilution if necessary.

2.6.3 Multi-analyte capabilities of the Gc-globulin sensor

Gc-globulin has an affinity towards all three serum metabolites of vitamin D$_3$ [161]. Therefore, it is important to determine if all three could be quantified independently using
Table 2–2: Estimating different vitamin D₃ metabolites using the cellulose-coupled 
Gc-globulin sensor. Quantities were estimated in solutions containing 2-11 nM of the 
metabolite of interest.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>R²*</th>
<th>Frequencies chosen (MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OHD₃</td>
<td>0.98</td>
<td>0.63, 5.36, 5.89</td>
</tr>
<tr>
<td>Cholecalciferol</td>
<td>0.93</td>
<td>3.39, 5.00, 6.46</td>
</tr>
<tr>
<td>1α,25-OHD₃</td>
<td>0.96</td>
<td>1.61, 4.73, 4.46, 6.28</td>
</tr>
</tbody>
</table>

*R² is the coefficient of determination

the cellulose-conjugated Gc-globulin sensor. The concentration range for this experiment 
was between 2-11 nM for all three vitamin D metabolites. The results are summarized in 
Table 2–2. The chosen frequencies for each metabolite are heavily weighted in the 4-6 MHz 
region of the spectrum. Another interesting feature is that the frequencies selected for the 
model are different depending on which vitamin D metabolite has been added. Differences 
in model frequencies suggests a different sensor response for each vitamin D metabolite. 
This allows for multi-analyte quantitation possibilities, whereby signals relevant to different 
metabolites can be differentiated from one another in the same solution. Additionally, 
this technique could be useful for actively determining selectivity coefficients and binding 
behavior of similar protein species.

2.7 Conclusion

Quantification of the major circulating metabolite of vitamin D, 
25-hydroxycholecalciferol, has been demonstrated with two different hydrogel sensors. 
Furthermore, by measuring in unfiltered serum, the feasibility of using hydrogel sensors in real-world analyses has been demonstrated. It was also shown that multiple metabolites can be quantified by the same sensor, as was shown by the Gc-globulin coupled cellulose 
hydrogel experiments. Detection ranges of 25-hydroxycholecalciferol (2-150 nM) fell 
well within the levels typical of healthy human subjects (25-250 nM). Investigations into
biocompatible hydrogel systems, such as those presented, have the potential to be useful for in-vivo applications. NIPA-based sensors could be a competitive approach to vitamin D analysis due to their low cost and ease of synthesis. As well, the ability to measure binding events of Gc-globulin antigen receptors with target analyte species demonstrates the multifaceted potential of the ultrasonic measurement system. With the availability of custom made, highly purified proteins and antibodies, adaptable sensors can be engineered to accommodate a wide variety of target compounds. Future studies in ultrasound spectroscopy include the investigation of multi-targeting sensors, such as the Gc-globulin sensor presented in this work. Multi-sensory hydrogels can have tremendous implications for modern clinical measurements, providing a vast array of diagnostic information using a single sensor entity. Future research in ultrasonically active biosensors will also include applications to other biologically relevant target molecules.

Acoustic monitoring of hydrogel sensors has been demonstrated, in principle, as an effective method for point-of-care analysis. With the ability to propagate through optically opaque and highly scattering media, ultrasound allows for unique approaches to clinical measurements. Real-time sample analysis, combined with minimal operating expertise, affordable instrumentation, and readily available, inexpensive sensors make ultrasound a promising candidate for the next generation of intelligent biosensing.

2.8 Acknowledgments

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CHAPTER 3

Conclusions and Future Work

3.1 Conclusions

The application of ultrasound in the quantification of hydrogel sensors biological systems has been shown to greatly benefit medical sciences. Moreover, its expanding use in modern medical and industrial sectors has led to major research gains, as the equipment and instruments are regularly updated. With further progress in instrumental design, ultrasound spectroscopy will likely become commonly used in clinical measurement protocols. The novel hydrogel sensor design presented in this thesis illustrates a way in which the ultrasound method can be used in a clinical setting.

The work presented in this thesis demonstrates the improvement potential for the current clinical measurement model using ultrasonically active hydrogels for analytes in complex liquid media. Furthermore, hydrogel sensors have been demonstrated as novel candidates for quantification in serum samples. The adaptability of the hydrogel sensors allows for dynamic measurements to be made for a wide range of target biocompounds. As was described, analysis of transmitted ultrasonic waves through sensor solutions can provide accurate quantitative information for the target analyte compound. In addition, measurement times for each sample were less than a few minutes. Coupled with the fact that measurements can be made without the need for extensive sample preparation, ultrasonically active biosensors may raise the current standard for clinical measurements.
The novel method for incorporating protein structures into ultrasonically active hydrogel sensors shows promise for future research. The chemistry used to conjugate Gc-globulin amine groups to cellulose hydrogels is easily adaptable for other polypeptide chains. The ability to probe proteins for resonance information is a huge gain from a research perspective. Protein-conjugated acoustically active hydrogel sensors can be custom made on a needed basis given the multitude of purified proteins available for research. The availability of these proteins is beneficial for research groups studying binding and adhesion, as the ultrasound method can be used for measuring changes in the effective mass and size of the protein during binding events.

The applicability of acoustically responsive hydrogel networks in providing accurate quantitative information about their chemical targets was clearly demonstrated. The acoustic measurements made on the hydrogel systems showed that the sensor frequency shifts in response to an increasing concentration of vitamin D. In the current study, in phosphate buffered saline solutions were set to mimic pH level found in the human body. In these experiments, coefficients of determination exceeded 0.95 for both the Gc-globulin hydrogel sensor and the molecular imprinted hydrogel sensor after 4-factor multiple linear regression models were applied. Furthermore, the sensor capabilities were tested in unfiltered human serum, where coefficients of determination were 0.88 and 0.92 for the imprinted and conjugated sensors respectively. In both media studies, the cellulose-conjugated hydrogel with vitamin D binding protein moieties quantified vitamin D better than the molecularly imprinted hydrogels. Detection limits and standard errors for the Gc-globulin sensor were more than 10 times lower, and the coefficient of determination was consistently higher than those observed using the molecularly imprinted hydrogel sensor. Although the molecularly imprinted hydrogel sensors did not quantify to the same degree as the cellulose-conjugated protein sensor, they serve as less expensive alternatives to antibody and protein-based assays. The methods for rapid and simplified quantification of vitamin D proposed in this thesis is readily adaptable for clinical settings.
From a global perspective, hydrogel biosensors can make vitamin D screening more common and readily available, providing an abundance of data for vitamin D deficiency studies. Efficient delivery of vitamin D information to both clinicians and research scientists would aid in the continuation of studies relating to the short- and long-term impact of vitamin D deficiency. In addition, the ultrasound method employed to evaluate the hydrogel sensor response can be adapted as a rapid laboratory or clinical office measurement system, removing the dependence on complex protocols for measuring vitamin D. Furthermore, and perhaps most importantly, the use of hydrogel biosensors can improve patient care, since a greater amount of diagnostic information can be made readily available for physicians.

3.2 Future Work

To further explore the capabilities of ultrasonic frequency analysis as a quantitative detection method, many possibilities for improvement should be explored. Acoustic measurement systems are extremely sensitive to environmental variability. In order to minimize the variations associated with environmental instability, steps must be taken in order to control certain experimental parameters. For example, temperature, since it is one of the most dynamic parameters in any experimental configuration, must be rigorously controlled. Acoustic wave propagation itself can generate regions of dissimilar temperature within a media. Differences in temperature lead to variations in sound speed, which in turn introduce variability in measured intensity values. Another parameter that can introduce instability is sample volume. In the current study, measurements were made using an open sample cell, where liquid was manually injected. Any fluctuation in sample volume can result in signal variations due to pressure increasing as a function of sample depth.

The development of a closed, fully automated ultrasound system would serve to alleviate the problems of temperature and sample volume variability. Automated flow injection systems can be configured to make highly reproducible injections in order to minimize sample volume variations. Isothermal conditions can be readily imposed on closed sample cells as well. Sample cells built from aluminum or stainless steel can utilize
a contact-based electrical temperature controller, such as a Peltier device. By controlling
the volume of injection and the temperature of the cell, measurement reproducibility would
greatly improve.

The frequency response of the ultrasonic transducers used in the research presented
here were centered at 5 MHz. At these frequencies, the subharmonic resonance oscillations
of the ultrasonic sensors can be measured. It is important to note, however, that the
recorded subharmonics were several generations removed from the expected fundamental
oscillating frequency of the hydrogel. The intensities of subharmonic frequencies decrease
significantly as the order of the harmonics increase as shown in Figure 1–6. In order to
bypass the issue of low-intensity harmonics, transducers with higher frequency ranges
can be used to monitor lower-order subharmonic resonance frequencies (i.e. resonance
frequencies closer to the fundamental). When switching to transducers with higher
frequency ranges, it is important to consider that ultrasonic penetration depth decreases
as frequency increases. Smaller sample cell path lengths must be used in order to
accommodate the loss of penetration depth.

In order to improve the measurement of hydrogel subharmonic frequencies, it is
crucial that the particles be synthesized with a consistent size distribution. A system of
polymer hydrogels with large variations in particle diameter will lead to a broad distribution
of resonance frequencies. Methods for controlling particle sizes and maintaining tight
distributions have been reported [154, 158]. One common technique involves the use of
a surfactant during polymerization. Polymerizing in the presence of a surfactant such as
sodium dodecyl sulfate (SDS) results in tunable, tightly distributed microgel particle sizes.
With narrow size distributions, the ensemble of particles will resonate at similar oscillation
frequencies as one another. Theoretically, narrower size distributions would improve the
signal-to-noise of the measurement, resulting in lower detection limits and better calibration
models.
The cellulose-based protein sensor described previously demonstrates an effective method for monitoring binding events in solution. It was shown that protein modification is possible in order to create unique acoustic signatures. In addition, binding events occurring between proteins and their target compounds can be monitored. The conformational changes brought on through binding events can be exploited to characterize systems of proteins. The characterization of proteins in solution is traditionally done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), an extensive procedure that involves separation of proteins based on differences in electrophoretic mobility. Procedures such as SDS-PAGE take several hours to complete, provided that all materials, reagents, and samples are prepared ahead of time. Using ultrasound, mixtures of proteins can potentially be monitored and characterized in real-time.

The groundwork for ultrasonic spectroscopy innovations and potential applications have been well developed in recent years. However, both the instrumentation and experimental protocol could benefit from the aforementioned modifications. With the proposed method for developing novel hydrogel biosensing entities, combined with the use of a fully automated and portable system, hydrogel sensors will become indispensable tools for routine chemical analysis.
References


