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Research Article

Molecularly Imprinted Chitosan Modified Quartz Tuning Fork Sensors for Real Time Biosensing in Liquid Environment

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ABSTRACT

Several new sensing technologies have emerged to meet the escalating demand for accurate and rapid diagnosis. We present an overview of the development of highly sensitive and selective Quartz Tuning Fork (QTF)-based sensors in a liquid environment, which will be critically important for contemporary diagnostic methods reliant on sensing technologies. The purpose of this study is to modify QTF prongs using molecularly imprinted chitosan, in combination with the operation of a quartz tuning fork as a piezoelectric crystal for biomedical applications. Through real-time data acquisition, we evaluate QTF resonance frequency shifts in dry and liquid environments using a model protein, BSA. As a result, the QTF-based sensor fails to detect BSA in dry conditions. It is however possible to measure frequency shifts ranging from 5 to 25 µg /mL within a liquid matrix. There is a rapid equilibration response time of 2 to 10 minutes depending on the concentration of BSA in the sensor. With the developed QTF-based sensor, a sensitivity of 1.1069 Hz/ µg has been achieved within the liquid matrix. As a result of the excellent properties of molecularly imprinted chitosan, it has been possible to develop a QTF-based biosensor capable of acquiring real-time data even when it is in liquid solutions.

Keywords: *Quartz tuning fork, Mass sensitive sensor, Molecularly imprinted polymer, chitosan, bovine serum albumin*

Sıvı Ortamda Gerçek Zamanlı Biyoalgılama için Moleküler Baskılı Kitosan Modifiye Kuvars Ayar Çatalı Sensör Geliştirilmesi

ÖZET

Yüksek hassasiyet ve seçicilikle çalışan, doğru ve hızlı teşhis talebini karşılamak üzere bir dizi yeni duyusal teknoloji ortaya çıkmıştır. Bu çalışma kapsamında, sensör teknolojilerine dayalı çağdaş teşhis yöntemleri için kritik önem taşıyan sıvı ortamda çalışan Kuvars Ayar Çatal (QTF) tabanlı sensörler tasarımı üzerine detaylı bilgi sunmaktadır. Bu çalışma, biyomedikal uygulamalar için bir çevirici olarak kuvars ayar çatalın piezoelektrik kristal olarak kullanılması ile, QTF çatallarını moleküler baskılı kitosan ile modifiye etmeyi amaçlamaktadır Gerçek zamanlı veri toplama ile, model bir protein olan BSA kullanarak QTF rezonans frekansındaki değişimleri hem kuru hem de sıvı ortamlarda takip edilmiştir. Elde edilen sonuçlara göre, QTF tabanlı sensör, kuru koşullarda BSA tespiti yapamamaktadır. Ancak sıvı matris içinde 5 ila 25 µg/mL aralığında frekans değişimleri ölçülebildiği gösterilmiştir. Sensördeki

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BSA konsantrasyonuna bağlı olarak, tepki süresi BSA konsantrasyonuna bağlı olarak 2 ila 10 dakika arasında değişmektedir. Geliştirilen QTF tabanlı sensörle, sıvı matris içinde 1.1069 Hz/ µg hassasiyette ölçüm yapabilmektedir. Moleküler baskılı kitosanın üstün özellikleri sayesinde, sıvı çözeltilerde bile gerçek zamanlı veri toplama yeteneğine sahip bir QTF tabanlı biyosensör geliştirilmiştir.

Anahtar Kelimeler: *Kuvars ayar çatalı, Kütle hassas sensör, Moleküler baskılı polimer, Kitosan, Sığır serum albumin*

I. INTRODUCTION

Modern healthcare systems are increasingly dependent on diagnosis technologies, which have revolutionized medical practice by providing accurate and timely disease identification. An accurate diagnosis is not only essential to enhancing patient outcomes, but also to improving the efficiency of healthcare management and reducing healthcare expenses. The advancement of diagnostic methodologies across various medical disciplines has been driven by sensing technologies, which have emerged as indispensable tools. The use of sensing technologies provides real-time and non-invasive methods for the detection of biomarkers, the monitoring of physiological changes, and the assessment of disease progression in real time. These tools have the potential to enhance the early detection and monitoring of disease, as well as the development of personalized treatment plans [1]. However, materials are a key component of an effective sensor, since they dictate the specificity of recognition, the limits of detection, and the overall quality and reliability of the results. Since polymer-based receptors are scalable, simple, offer an extended shelf-life and are cost-effective, they have gained significant attention. Furthermore, they promise to reduce performance variations between batches [2,3]. Polymer-based biosensors also offer automated manufacturing with predefined attributes for both single and multiple analyte detection, enabling enhanced personalized healthcare applications [4]. The sensor industry has access to a wide range of polymerization techniques. A particularly promising method for crafting high-quality functional materials on sensor substrates has emerged with the use of molecular imprinting.

Molecular imprinting is a technique used to create specific cavities within three-dimensional polymeric networks [5]. Upon eliminating the template molecules from the polymer structure, these cavities are revealed. These cavities possess complementary characteristics in terms of size and configuration. In addition, they possess complementary characteristics in relation to the interaction points and coordination spheres of template molecules. A broad range of research fields, including chromatography, biotechnology, environmental science, food safety, biomedical sciences, and notably, biosensors, have benefited from MIPs, which offer promising substitutes for their biological counterparts [4,6].

A crucial initial step in the synthesis of molecularly imprinted polymers (MIPs) is the selection of the appropriate functional monomer, as it determines how complementary interactions can be established with matrix molecules. An amino polysaccharide derived from chitin by deacetylation, chitosan contains three distinct reactive functional groups – one amine group, two primary and secondary hydroxyl groups – and is the most abundant nontoxic, biodegradable, and biocompatible amino polysaccharide in nature [7]. A wide range of chemical modifications can be carried out on this structure, including aldehyde-ketone interactions, grafting, hydrogen bonding with polar entities, crosslinking, etc. As a result of reactive functional groups present along the polysaccharide chain of chitosan, the material is flexible, has excellent film-forming capabilities, is highly adherent, is biocompatible, has a noteworthy mechanical resilience, and may be structurally modified. This material has phenomenal properties that make it an ideal material for the creation of MIPs and the construction of several types of transducers including electrochemical, optic, and physical transducers for sensor design [8,9].

There has been significant interest in mass-sensitive biosensors derived from Quartz Tuning Forks (QTFs) for biomedical applications in recent years, primarily due to the high-quality factor ($10^3 – 10^5$), the cost-effective fabrication, the low energy consumption, and the inherent stability of these devices. The use of QTFs has proven useful for a variety of applications, including time-frequency standard systems, atomic force microscopy, gas and humidity sensing, quartz-enhanced photoacoustic spectroscopy (QEPAS), and, more recently, biosensing investigations [10-25]. The technique relies on the detection of biomolecules within gaseous or liquid media, whereby the mass of the target analytes is converted into a measurable signal, known as the resonant frequency (*f*). With the assistance of an oscillator circuit and a frequency counter, the frequency of this signal can be quantified. The effectiveness of this sensing mechanism is determined by the resonator's performance, which is closely linked to the quality factor and the dynamics of interactions between target biomolecules and the resonator surface [16]. Since the transducer's surface is chemically inert, the establishment of a recognition layer is critical for modifying the transducer. Not only is this layer useful for enhancing sensitivity and selectivity, but it also addresses the requirement that the transducer interact with the biomolecule being detected. Despite this, it is important to strike a balance between thickness and uniformity because excessively thick recognition layers can result in a precipitous decline in the transducer's quality factor and consequently its sensitivity [16].

Based on these above-mentioned advantages, the present study explored the potential integration of QTFs and molecularly imprinted technology, to establish an innovative paradigm for biosensor development. Throughout this research, molecularly imprinted chitosan was used as an interface layer between the prongs of the QTF and the model protein. By choosing molecular imprinting, it was possible to create a binding interface capable of recognizing a specific protein model, bovine serum albumin (BSA). Through dry state and real-time measurement of shifts in the QTF resonance frequency in response to varying BSA concentrations, the effectiveness of the molecularly imprinted chitosan thin film was assessed by taking advantage of QTFs' inherent responsiveness to mass changes on their surface. Based on the correlation between these frequency shifts and BSA concentrations, the molecularly imprinted chitosan-QTF system has proven to be highly specific and sensitive for the detection of proteins. This achievement highlights the potential for the combination of molecularly imprinted polymeric materials and QTF-based sensor technologies to create innovative diagnostic tools with many applications, ranging from medical diagnostics to environmental monitoring.

II. EXPERIMENTAL

A. MOLECULAR IMPRINTING OF BSA

Molecular imprinting of chitosan/glutaraldehyde resin is accomplished in the following manner described by Monier and El-Sokkary [26]. Hydrophilic glycol chitosan solution was prepared by mixing 15 grams of chitosan (Mw: 190,00-310,00, Sigma-Aldrich, St. Louis, MO, USA) with 1000 mL of deionized water and 0.5% acetic acid solution (Sigma-Aldrich, St. Louis, MO, USA) was added to the mixture. To ensure mixing, it was vigorously stirred using a magnetic stirrer for 72 hours. As a next step, 10 mL of 0.1 M BSA (Sigma-Aldrich, St. Louis, MO, USA) was added to the chitosan solution, and it was stirred for two hours. In the following hour, 5 mL of glutaraldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) was added and stirred. The gelatinous resin was dried in a 60°C oven for 24 hours after this period. A mortar and sieve were used to grind the dried resin into particles with a diameter of approximately 150 µm. To prepare the desired adsorbent to separate BSA from samples, 3 g of particles were stirred for 24 hours in 1000 mL of 0.1 M HCl solution to wash out the BSA molecules. To ensure maximum removal of the imprinted BSA molecules, this step was repeated three times to ensure that they were removed as much as possible from the chitosan network. The imprinted BSA molecules could cross-link with the chitosan network during the reaction, interfering with subsequent adsorption and extraction procedures. Non-imprinted chitosan was synthesized in a similar manner, without the addition of the BSA to the solution.

B. SURFACE MODIFICATION OF THE QTF'S PRONGS WITH MOLECULARLY IMPRINTED CHITOSAN

Before modification, the hermetic case of bare QTFs (32.768 kHz, Shoulder Crystal, China) was removed and crystals were sonicated in ethanol for 30 minutes. Then, the prongs were washed with deionized (DI) water several times and dried with a gentle flow of nitrogen [20,22,24-25]. The prongs of QTFs were modified with BSA imprinted chitosan solution by simple dipping method for 60 minutes. The repeatability of prongs' modified area was controlled with Asensis QTF F-master device. Real time monitoring of the frequency was performed during all surface modification periods with the Asensis QTF F-master device and the frequency of dried QTFs were also measured. The change in frequency ($\Delta f=f_1-f_2$) was calculated for each measurement [25].

C. CHARACTERIZATION OF QTF BIOSENSOR

The chemical groups were determined by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) (PerkinElmer Spectrum TwoTM FT-IR Spectrometer, USA) analysis. ATR-FTIR spectra measured with a range of 4000-1000 cm^{-1}.

D. BSA DETECTION VIA MOLECULARLY IMPRINTED CHITOSAN MODIFIED QTF BIOSENSOR

Unmodified, non-imprinted and imprinted chitosan modified QTF-based biosensors were used in both dry and liquid state BSA monitoring. The interaction of prongs' modified area with BSA was controlled using the Asensis QTF F-master device. The specified immersion level of the device corresponded to a full revolution of the stepper motor which is 40 µm. When the "immerse" command was activated, it was moved 40 µm in one step, and the process continues until the fork was interacted with different BSA concentrations. Consequently, the interaction length of forks with liquid could be either 40 µm or less than that value with a single immerse command.

D. 1. Biosensor Testing in Dry State

Firstly, the biosensors were immersed in 200 µL of PBS solution for 10 min. Before interacting with BSA, prongs were washed with DI water, dried with nitrogen gas, and frequency measurements performed to monitor baseline frequencies. Then, the QTF-based biosensors mentioned above were immersed into different BSA concentrations (0-41 µg/mL BSA in 0.1 % v/v in PBS, pH 7.4) for a period of 10 min, washed three times with PBS (pH 7.4, 4 ºC) to remove unbounded BSA and nitrogen dried. Frequency shifts before and after interaction were recorded in a dry state with the QTF controller [20,22, 24].

D. 2. Real Time Monitoring in Liquid State

Real time monitoring of BSA with QTF-based biosensor was performed by recording all changes occurred after immersion of prongs in solution. Unmodified, non-imprinted and imprinted chitosan modified QTF-based biosensors were interacted with different BSA concentrations (0-41 mM BSA in 0.1 % v/v in PBS, pH 7.4). The base frequency was recorded after frequency reached to equilibrium in BSA solution right after immersion. The frequency shifts after interaction with BSA were recorded real time with the QTF controller and the highest shift was accepted as a response of QTF-based biosensor.

III. RESULTS AND DISCUSSION

The chemical change after molecular imprinting process was evaluated with ATR-FTIR analysis (Figure 1). In the spectrum of hydrophilic glycol chitosan before molecularly imprinted (non-imprinted

chitosan), a broad peak was observed at 3310 cm^{-1} and 1642 cm^{-1} indicating –OH/NH_2 stretching and C– O stretching of the acetyl group (amide-I) [27-29]. In the molecularly imprinted chitosan's ATR-FTIR spectrum, the bands at 1520 cm^{-1} and 1245 cm^{-1} correspond to vibrations of the N-H bending and the -NO_2 stretching vibration, respectively. Other characteristic peaks for chitosan did not shift from their original position.

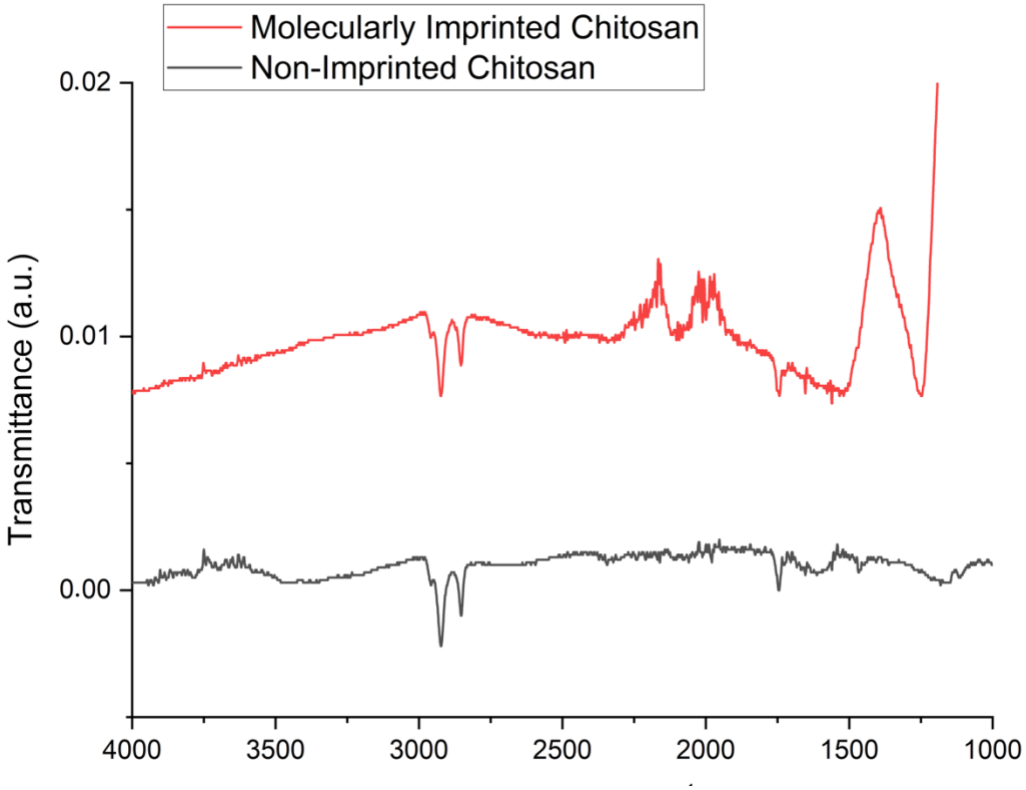

Figure 1. *ATR-FTIR spectra of the non-imprinted and molecularly imprinted chitosan.*

A novel biosensor designed as a model for BSA detection operates by monitoring changes in the resonance frequency of the QTFs. A change occurs when mass accumulates on the surface due to the selective interaction between molecularly imprinted chitosan and BSA biomolecules. Inert prongs on the QTFs are effectively activated by molecularly imprinted chitosan films using dip-coating technique, allowing selective interaction with BSA to be achieved. BSA detection is reflected in the observed decrease in the resonance frequencies of QTFs (Figure 2). Thus, in the initial phase of the experiments, the resonance frequencies of unmodified QTFs in air, and BSA were measured to determine how air damping and matrix effects affect frequency.

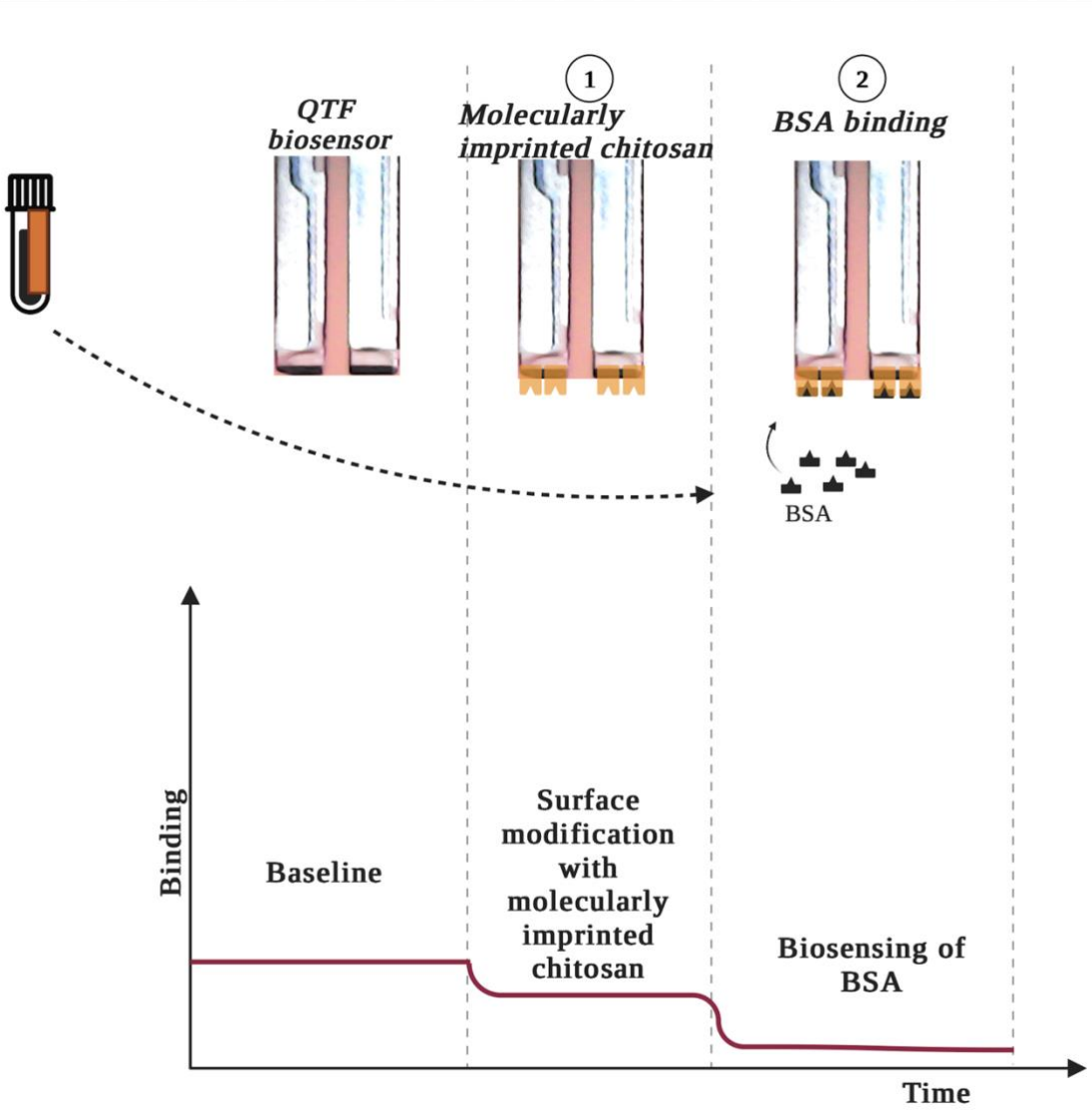

Figure 2. *Schematic representation of biosensing of BSA with molecularly imprinted chitosan modified QTF-based sensor.*

Due to their user-friendly design, QTFs with dimensions of 3.10x8.20 mm and a resonance frequency of 32,768 Hz in vacuum were selected and utilized for this purpose rather than QTFs with dimensions of 2.10x6.20 mm. Upon removal of the hermetic case and cleaning, the average frequency was calculated as 32,757.3 ± 2.3 Hz (n=30) because of the prongs having been cleaned and dampened by the air. Control experiments were conducted using a non-imprinted chitosan coating on the prong of the QTF, and frequency changes approximately 9.8 Hz (n=10) were caused by chitosan thin film formation. In the next step, BSA detection was conducted, and a frequency change of approximately 0.2 Hz (n=10) was detected. Since the frequency change is less than the error value of 2.3 Hz, the difference is not considered a change. This result indicates that the non-imprinted chitosan thin film does not detect BSA selectively from the matrix.

Then, first dry-state detection was performed with molecularly imprinted chitosan modified QTF-based biosensor with varied BSA concentration between 0-41 µg/mL. But no significant frequency change was observed in the given concentration range. However, during real time measurement, after the frequency reached to equilibrium, even in lowest concentration, a linear change in resonance frequency shift was observed until 25 µg/mL of BSA. Due to the steric effect, remaining BSA cannot interact with the active side of thin film since the concentration-consequent active sites of molecularly imprinted

chitosan reduce the concentration of BSA in the prongs. As a result, when a concentration of 25 µg/mL of BSA is exceeded, the surface of the QTF-based sensor also loses its sensitivity (Figure 3).

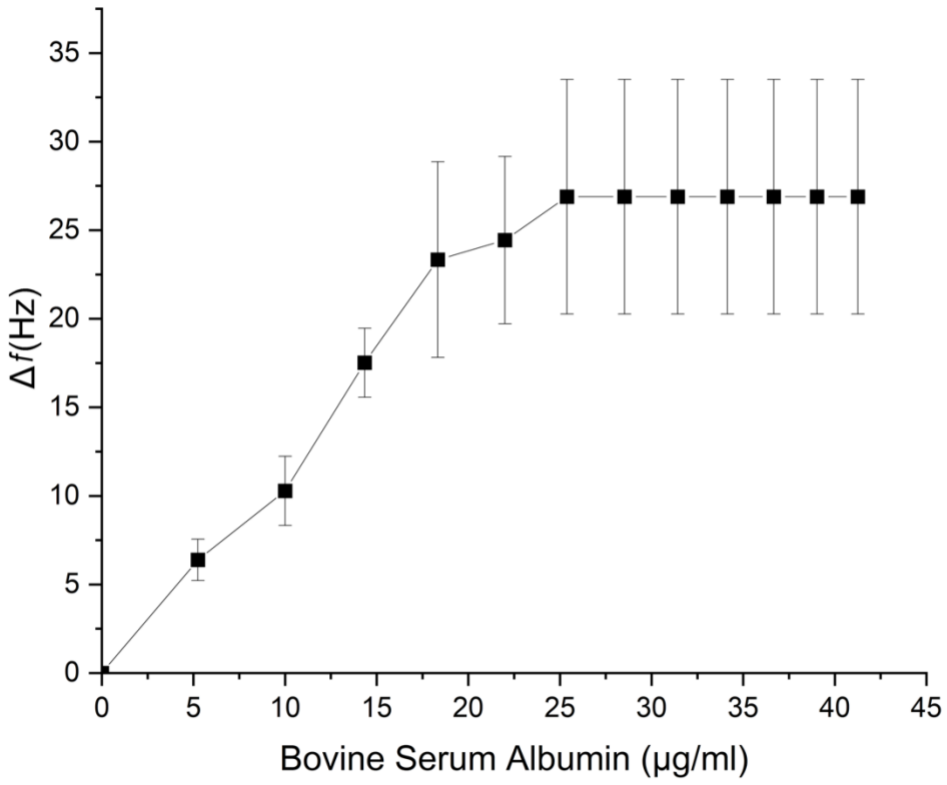

Figure 3. *QTF response modified with molecularly imprinted chitosan, which is interacted with a solution containing BSA in certain amounts with real time monitoring (n=3)*

This newly developed biosensor in liquid displayed a linear range of 5 to 25 µg/mL with a correlation coefficient of $R^2 = 0.9796$ (Δf (Hz) = 1.1069C + 0.4789). Due to the capillary effect formed between QTF prongs, the biosensor responded within 2-10 minutes. In this study, it was determined that the biosensor had a sensitivity of 1.1069 ± 0.1812 Hz/ µg (0.09 µg/0.1 Hz) (Figure 4).

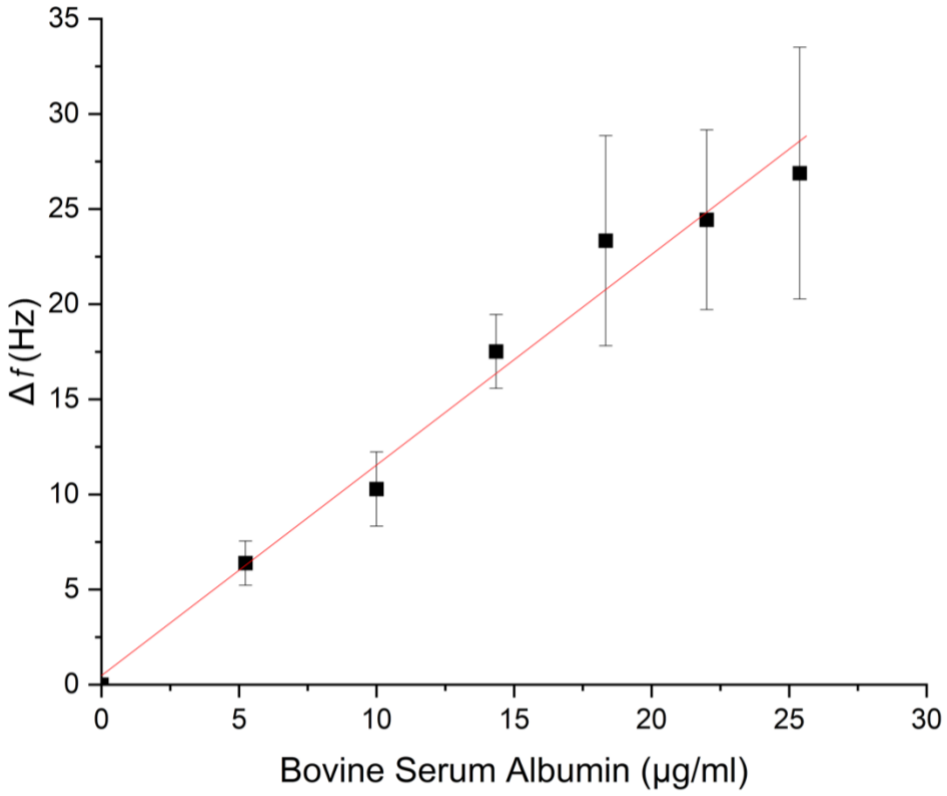

Figure 4. *The calibration curve obtained for the QTF-based sensor for the detection of BSA with real-time monitoring (n=3)*

We have recently conducted extensive studies in our research group investigating the adaptation of QTFs for biosensing applications by utilizing plasma polymerization processes and a variety of precursor materials [20,22,24-25]. Three different amino-containing precursors-amylamine, n-heptylamine, and diaminocyclohexane- were initially used to modify the QTF prongs. Amylamine-modified surfaces yielded the most favorable results following extensive optimization efforts. Testing of these amylamine-functionalized QTFs with 50 µg/mL of BSA resulted in a frequency shift response of approximately 5 Hz [20]. As a follow-up to our previous studies, we examined a two-step strategy that included the application of a hydrocarbon-based plasma film (heptane) as a pre-coating layer, and the application of an amino-group-containing precursor (ethylenediamine) as the capping layer. As a result of this configuration, there was a frequency shift of 20 ± 3.6 Hz for the same BSA concentration [22]. According to a recent study involving plasma polymerization, hexane pre-coating significantly enhanced QTF response, exhibiting a 32-fold increase in QTF response compared to amylamine-modified QTFs, and a fourfold increase in QTF response compared to those modified with heptane and ethylenediamine [24]. Despite this, due to the high cost of the plasma device and the challenges associated with reproducibility associated with thin film production by plasma polymerization, alternative methods to modify the surface of the film were explored.

The effective utilization of QTF-based biosensing strategies remains a crucial component despite progress in QTF-based biosensing strategies. Our current research is focused on developing a more accessible and reproducible method of surface enhancement like molecularly imprinted polymer modified QTF-based biosensor. The present study identifies a novel approach to addressing the limitations of plasma polymerization and presents a viable alternative for improving the performance and feasibility of QTF-based biosensors for improved diagnostic applications. As a result of this study, our lowest concentration that could be measured was 5 mM, which is 100 times higher than our previous studies, and the response was 100 times and 400 times lower with a 6.38 Hz frequency shift compared to our previous studies.

According to literature, the sensitivity of unmodified QTF sensors is calculated to be approximately 1.3 ng/0.1 Hz [28]. Interestingly, the surface modification of the QTF prongs resulted in a significant sensitivity reduction by approximately 6.9 times compared to bare crystals and 20 times compared to plasma modified QTFs. This decrease in sensitivity may be due to the thickness of thin films produced by dip coating. Based on these findings, even though dip-coating have been used for different applications due to their simplicity and easiness to operate, it is apparent that surface modification of QTF's prongs can be complex and impact the performance characteristics of resultant QTF-based biosensors by decreasing quality factor of crystals [30]. Therefore, more investigation could be performed to increase the sensitivity by changing dip coating parameter with a more controllable device. Apart from all these limitations, this study presents the first real-time monitoring of biomolecules using a QTF-based sensor.

IV. CONCLUSION

Medical practice has been transformed by diagnostic technologies, which facilitate the accurate and timely identification of diseases in healthcare systems. In addition to improving patient outcomes, accurate diagnosis improves the efficiency of healthcare management and reduces costs. Sensor technology has become an indispensable tool for sensitive and selective diagnosis across diverse medical domains. It is possible to identify biomarkers in real time, monitor physiological activity, and assess disease progression using these technologies. These technologies are highly promising in the areas of early disease detection, monitoring, and personalized treatment planning. This context makes the role of materials within sensing platforms paramount, since they influence the specificity of recognition, the limits of detection, and the quality of the results. Various polymer-based receptors have attracted substantial attention due to their scalability, simplicity, extended shelf-life, and cost-effectiveness, in addition to promising to reduce batch-to-batch performance variability. The availability of a variety of polymerization techniques has allowed innovative materials to be designed on sensor substrates, with molecular imprinting being particularly promising. Through the process of molecular imprinting, specific cavities may be created within three-dimensional polymeric matrixes to reveal complementary characteristics with respect to size, configuration, interaction points, and coordination spheres. Choosing the right monomer for complementary interactions with matrix molecules is one of the most critical steps in the synthesis of MIPs. The flexibility, film-forming capability, biocompatibility, mechanical resilience, and structural adaptability of chitosan make them an ideal substrate for MIPs, allowing sensor design.

A growing interest in mass-sensitive biosensors using QTFs has been observed in the biomedical field, given features such as high-quality factor, low cost, low energy consumption, and inherent stability. Although chemically inert prongs require a recognition layer for enhanced sensitivity and selectivity. In line with these advantages, our study examines the fusion of QTFs with molecularly imprinted technology to create an innovative biosensor paradigm for QTF-based sensor technologies. Molecular imprinting facilitates specific recognition of BSA by using molecularly imprinted chitosan as an interface between QTF prongs and model proteins. We have confirmed the efficacy of the molecularly imprinted chitosan film by using dry and real-time measurements of QTF resonance frequency shifts.

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