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Staphylococcus Aureus Detection in Milk Using a Thickness Shear Mode Acoustic Aptasensor with an Antifouling Probe Linker †

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Abstract: Pathogen contamination of milk can pose a serious risk to health. For safe food consumption, monitoring for the presence of pathogens is critical to identify and regulate microbiological contamination. In this work, an aptasensor based on a thickness shear mode acoustic method (TSM) with dissipation monitoring was developed to detect and quantify *Staphylococcus aureus* directly in whole UHT cow's milk. The aptasensor demonstrated high sensitivity and was able to detect *S. aureus* in milk with a 33 CFU/mL limit of detection. Analysis was successful in milk due to the sensor's antifouling properties, which is based on 3-dithiothreitol propanoic acid (DTT_{COOH}), a novel antifouling thiol linker. The excellent sensitivity to detect and quantify *S. aureus* in whole UHT cow's milk demonstrates that the system is applicable for rapid and efficient analysis of milk safety.

Keywords: *S. aureus*; antifouling linker; DNA aptamer; thickness shear mode; biosensor; milk

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1. Introduction

Nutrition is a fundamental process in human life, as it allows the body to obtain nutrients necessary for growth and survival. However, food can pose a risk to human health such as through its potential contamination with pathogens [1]. The control of microbiological contamination becomes an important strategy to safeguard the quality of food and therefore human health. Biosensors can provide rapid qualitative or quantitative pathogen detection [2]. There are numerous bacteria that can be found in milk depending on different handling processes of food. There are many dangerous pathogenic bacteria capable of growing in milk and dairy products. *Staphylococcus aureus* (*S. aureus*) bacteria and their toxins can cause serious infections such as sepsis [3]. As milk has low acidity and high protein content, it provides an ideal environment for rapid growth of *S. aureus*.

A very important aspect of biosensor analysis is the ability to carry out the detection of analytes directly on raw samples, which are not previously treated. However, raw samples can limit detection due to the heterogeneity of biological liquids which can cause significant fouling of the sensing surface. As a result, antifouling chemistry is an important field for targeting non-specific adsorption (NSA) of biological fluids.

Antifouling thiol- or sulfide-based molecules are used for gold electrode sensors, as a self-assembled monolayer (SAM) can be immobilised through gold-sulfur surface chemistry [4]. In our recent work, we described the synthesis of a molecule, 3-dithiothreitol propanoic acid (DTT_{COOH},), with both antifouling and linker properties, with the ability to

prevent NSA and to anchor a probe on the sensor surface for specific recognition of the analyte. However, these properties have only been demonstrated in human serum [5]. In this work, we investigated the linking properties of DTTCOOH in whole milk. The linker was covalently bound to a DNA aptamer using to develop a thickness shear mode (TSM) aptasensor for selectively identifying *S. aureus* (a mass-sensitive sensor based on a piezoelectric quartz crystal, which resonates at particular frequencies). The resonance varies according to changes in mass on the crystal's surface [6].

2. Materials and Methods

2.1. Materials

The synthesis of 3-dithiothreitol propanoic acid (DTT_{COOH}) followed previously published methods [6]. Milli-Q water (specific resistance of 18.20 MΩ.cm) was used for preparing aqueous solutions. Ethanol was purchased from Caledon Laboratory Chemicals (Georgetown, ON, Canada). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, Mo, USA), without purifying. The lyophilised aptamer targeting *Staphylococcus aureus* was purchased from Generi Biotech (Hradec Králové, Czech Republic): 5′ NH_2-TCC CTA CGG CGC TAA CCT CCC AAC CGC TCC ACC CTG CCT CCG CCT CGC CAC CGT GCT ACA AC-3′. The aptamer's sequence follows the work by Chang et. al. [7]. The aptamer was resuspended in DNase-free TE buffer (10 mM Tris-Cl and 1 mM EDTA at pH 8.0).

The preparation of phosphate-buffered saline (PBS) involved 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at pH 7.4. *Staphylococcus aureus* KR3 was purchased from the University of Toronto Medstore (Toronto, ON, Canada). Whole UHT cow milk (3.5% fat) was bought from Walmart (Toronto, ON, Canada). The AT-cut quartz crystals (0.2 cm^2 sensing area, 8 MHz fundamental frequency) were purchased from Total Frequency Control Ltd., Storrington, UK. The crystals had gold electrodes deposited on both sides. Basic piranha solution (7 mL of 1:1:5 v/v 28–30% NH_4OH, 30% H_2O_2, Milli-Q water at 70 °C) was used to clean each crystal.

2.2. Cleaning and Surface Modification of Piezocrystals

After cleaning, the quartz crystals were functionalized in a solution of 50 μM DTT_{COOH} in absolute ethanol overnight. The surfaces were further modified in 2 mM HS-MEG-OH in absolute ethanol (25 min), 20 mM NHS and 50 mM EDC in Milli-Q water (35 min), 5 μM aptamer in Milli-Q water (90 min), and 0.1 M ethanolamine in Milli-Q water (40 min). The crystals were rinsed with Milli-Q water and dried before measurements.

2.3. Bacteria Preparation

Lysogeny broth was used to grow *S. aureus* bacteria at 37 °C overnight. The grown solution was serially diluted from 1/10 to $1/10^9$ times in PBS. Each solution was spotted onto agar plates (10 μL x 3), as well as measured using a UV-1600PC spectrometer (VWR International, Mississauga, Canada) to measure the optical density at 600 nm (OD600). The plates were incubated overnight at 37 °C and spot counted to calculate CFU per OD600. For TSM measurements *S. aureus* was grown overnight at 37 °C. The next day, 1 mL of the bacteria solution was centrifuged at 14 500 RPM. 1 mL of PBS was used to resuspend the bacteria pellet. The OD600 of the solution was used to calculate the base CFU, and then the solution was diluted to the desired CFU. The diluted PBS bacteria solution was centrifuged to pellet the bacteria, and then the bacteria was resuspended in milk.

2.4. TSM Measurements

Cleaned or modified crystals were inserted in an acryl flow-through cell (JKU, Linz, Austria) which was clamped by a holder, ensuring the internal conductors were in contact with the crystal's electrodes. Liquid flowed through the internal chamber using a GeniePlus syringe pump (Kent Scientific, Torrington, CT, USA) and a pulling syringe. The

liquid flowing through the internal chamber was in contact with one face of the crystal. A SARK-110 vector analyzer (Seeed, Shenzhen, China) was used to collect data by a Python software [8]. Experiments were measured at 8 MHz, under ambient conditions, and under a 50 µL/min constant flow. DTT_{COOH}-modified crystals were exposed to HS-MEG-OH (25 min), rinsed with PBS (5 min), activated with NHS/EDC (35 min), rinsed with PBS (5 min), incubated with aptamer solution (90 min), rinsed with PBS (5 min), incubated with ethanolamine solution (40 min), then washed with PBS (at least 15 min). Once the final PBS wash reached a stable baseline following the aptasensor functionalization, 250 µL of milk sample containing a known concentration of bacteria was flowed over the crystal surface. After milk, PBS buffer was flowed over the crystals to wash them, remove any remaining sample on the surface, and reach a final stable baseline. Each experiment was repeated three times.

2.5. TSM Data Analysis

A Python code based on Yoon et al.'s equation [9] was used to analyze the data. Excel (Microsoft®) and OriginPro 8 (OriginLab Corporation, Northampton, MA, USA) were then used to plot and statistically process the data. The aptasensor was incubated with various concentrations of *S. aureus* in triplicate. The aptasensor's antifouling ability and frequency variation according to bacteria concentration was evaluated with the Python code and Excel.

The limit of detection (LOD) has been calculated according to [10] as follows: LOD = LOB + 1.645 SD_{lc}, where SD_{lc} is the standard deviation at low concentration of the sample, and the limit of blank (LOB) is the highest apparent analyte concentration: LOB = mean blank + 1.645(SD_{blank}). The limit of quantification (LOQ) has been determined by equation: LOQ = 10 × LOD / 3.3.

3. Results and Discussion

3.1. Sensing of Staphylococcus Aureus in Milk

The aptasensor showed high sensitivity to *S. aureus* in milk. Different bacteria concentrations caused proportional decreases in the resonant frequency. The dissipation changes confirmed the sensitivity as they increased with increasing bacteria concentrations, indicating that *S. aureus* adsorbed to the crystal's surface by binding with the aptamer. As Figure 1 shows, the crystal experiences minimal frequency and dissipation shifts when exposed to milk without bacteria (8.9 ± 3.4 Hz and 2.1 ± 0.8 × 10^{-6} frequency and dissipation shifts, respectively). Bacteria concentrations in milk were proportional to changes in the frequency and dissipation; increasing cell concentration caused decreasing frequency and increasing dissipation shifts.

The Figure 1 shows the proportionality of the changes in frequency and dissipation due to increasing *S. aureus* concentrations in milk. The frequency and dissipation variations were calculated from the differences of the stable baselines (before and after sample exposure).

For all concentrations of bacteria in milk tested, a change of approximately 8.9 Hz occurred, which is the mean blank. The standard deviation of the blank (SD_{blank}) is 3.4 Hz, therefore the LOB = 14.493. The SD_{lc} of the low concentration sample is 11.49 Hz, therefore the LOD was found to be 33.4 CFU/mL. Limit of quantification has been determined as LOQ = 101.2 CFU/mL The sensor also showed a dynamic range of 10^2 to 10^6 CFU/mL, allowing it to quantify *S. aureus* in a wide range of concentrations.

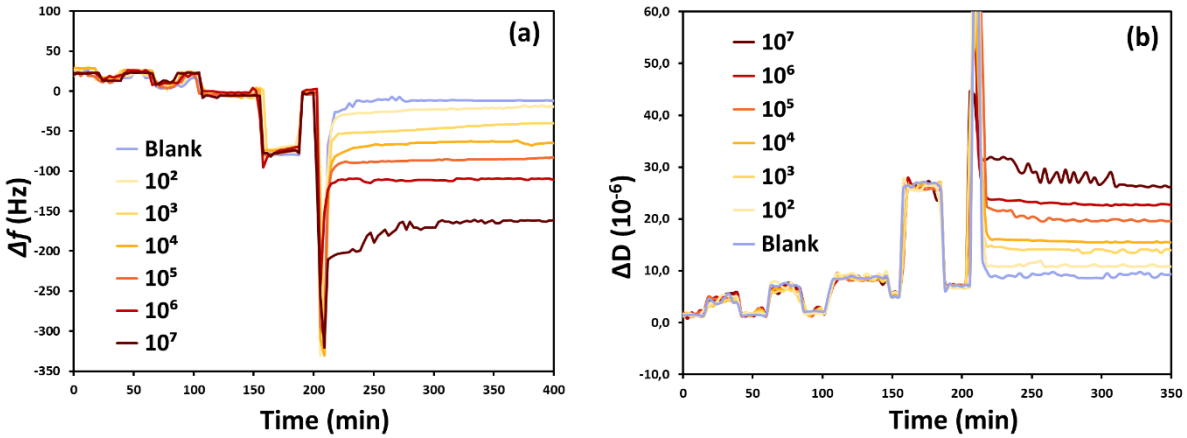

Figure 1. The (a) frequency, Δf, and (b) dissipation, ΔD, shifts as a result of exposing aptasensors to UHT cow's milk spiked with various concentrations of *S. aureus* in CFU/mL (see the legend).

Bacteria-aptamer binding was analysed by fitting the data to the Langmuir isotherm [11] (Figure 2):

$$\Delta f = (\Delta f)_{max}\,[c/(K_d + c)]$$

where the maximal frequency change is $(\Delta f)_{max}$, the dissociation constant is K_d, and bacteria concentration is c. As the K_d decreases, the binding strength of bacteria-aptamer increases. The calculated K_d and $(\Delta f)_{max}$ values for bacteria-aptamer binding in whole milk were found to be 270.9 ± 42.9 CFU/mL and 9.8 ± 3.5 kHz respectively.

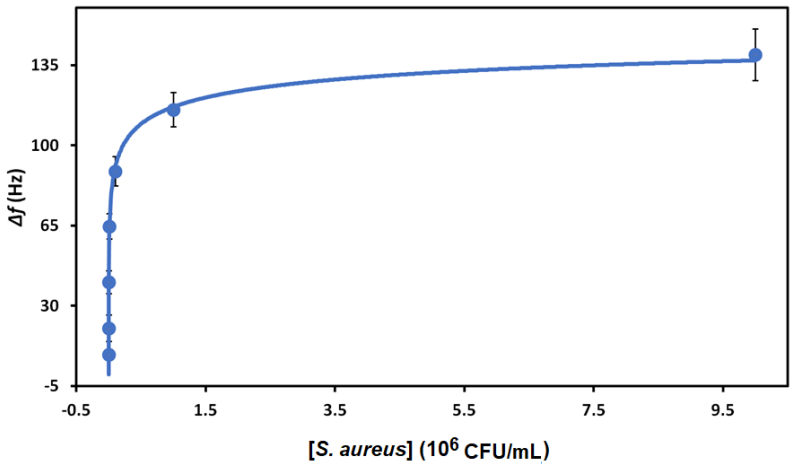

Figure 2. The Langmuir isotherm fit for the frequency shifts vs. *S. aureus* in milk. Results represent mean±S.D. obtained from 3 independent experiments.

4. Conclusions

A thickness shear mode (TSM) acoustic aptasensor was developed for detecting *Staphylococcus aureus* in whole UHT cow's milk. Detection was achieved without treating the milk due to the antifouling layer of the aptasensor, which employs the thiol linker 3-dithiothreitol propanoic acid (DTT_{COOH}). Once DTT_{COOH} was linked to the aptamer, the antifouling character of the sensor significantly improved. The developed aptasensor achieved excellent sensitivity and specificity and was able to successfully detect and quantify *S. aureus* in milk. As the 33 CFU/mL limit of detection and 101 CFU/mL limit of quantification is significantly below the EU's safe limit for bacteria in milk products, the aptasensor is practical for rapid and sensitive detection in the milk industry.

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References

1. Oliver, M.A.; Gregory, P.J. Soil, food security and human health: a review. *Eur. J. Soil Sci.* **2015**, *66*, 257–276. https://doi.org/10.1111.ejss.12216.
2. Velázquez-Ordoñez, V.; Valladares-Carranza, B.; Tenorio-Borroto, E.; Talavera-Rojas, M.; Varela-Guerrero, J.A.; Acosta-Dibarrat, J.; Puigvert, F.; Grille, L.; Revello, Á.G.; Pareja, L. Microbial contamination in milk quality and health risk of the consumers of raw milk and dairy products. In *Nutrition in Health and Disease;* Mozsik, G., Figler, M., Eds.; IntechOpen, London, UK, 2019, pp. 1–25. https://doi.org/10.5772/intechopen.86182.
3. Tremonte, P.; Tipaldi, L.; Succi, M.; Pannella, G.; Falasca, L.; Capilongo, V.; Coppola, R.; Sorrentino, E. Raw milk from vending machines: Effects of boiling, microwave treatment, and refrigeration on microbiological quality. *J. Dairy Sci.* **2014**, *97*, 3314–3320. https://doi.org/10.3168/jds.2013-7744.
4. Pawlowska, N.M.; Fritzsche, H.; Blaszykowski, C.; Sheikh, S.; Vezvaie, M.; Thompson, M. Probing the hydration of ultrathin antifouling organosilane adlayers using neutron reflectometry. *Langmuir* **2014**, *30*, 1199–1203. https://doi.org/10.1021/la4038233.
5. Blaszykowski, C.; Sheikh, S.; Thompson, M. Surface chemistry to minimize fouling from blood-based fluids. *Chem. Soc. Rev.* **2012**, *41*, 5599–5612. https://doi.org/10.1039/C2CS35170F.
6. Spagnolo, S.; Davoudian, K.; Ahmadi, S.; Chan, E.; Hianik, T.; Thompson, M. Thiol-based probe linker with antifouling properties for aptasensor development. *Chemosensors* **2022**, *10*, 435. https://doi.org/10.3390/chemosensors10100435.
7. Chang, Y.C.; Yang, C.Y.; Sun, R.L.; Cheng, Y.F.; Kao, W.C.; Yang, P.C. Rapid single cell detection of *Staphylococcus aureus* by aptamer-conjugated gold nanoparticles. *Sci Rep.* **2013**, *3*, 1863. https://doi.org/10.1038/srep01863.
8. Muckley, E.S.; Collins, L.; Srijanto, B.R.; Ivanov, I.N. Machine learning-enabled correlation and modeling of multimodal response of thin film to environment on macro and nanoscale using "lab-on-a-crystal". *Adv. Funct. Mat.* **2020**, *30*, 1908010. https://doi.org/10.1002/adfm.201908010.
9. Yoon, S.M.; Cho, N.J.; Kanazawa, K. Analyzing spur-distorted impedance spectra for the QCM. *J. Sensors* **2009**, 259746. https://doi.org/10.1155/2009/259746.
10. Armbruster, D.A.; Pry, T. Limit of blank, limit of detection and limit of quantitation. *Clin. Biochem. Rev.* **2008**, *29*, S49–S52.
11. Wilson, B.D.; Soh, H.T. Re-evaluation the conventional wisdom about binding assays. *Trends Biochem. Sci.* **2020**, *45*, 639–649. https://doi.org/10.1016/j.tibs.2020.04.005.