Capillary Flow Characterization and Application in Saliva Sampling

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Abstract

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The use of microfluidic channels is becoming increasingly prevalent in the biomedical field. As such, the characterization of capillary flow is vital to facilitate functional adaptations. The impact of progressive converging and diverging channels on open-channeled capillary flow is an area that lacks precise characterization. In this work, a theoretical model has been developed that predicts that convergent channels cause an increase and divergent channels cause a decrease in capillary flow velocity. Experimental validation of the model is presented in this work using an open-fluidic poly(methyl methacrylate) device with several liquids of varying viscosities and properties. Future work on this characterization will look at the behavior of immiscible plugs in dynamic open-microfluidic channels. Such manipulations in velocity have great potential in the application of capillary systems in biology and medicine. The implementation of open-microfluidic channels has been applied in our novel lollipop-based saliva collection tool, the CandyCollect. The

CandyCollect is a device designed to function as an alternative method of sampling for streptococcal pharyngitis diagnosis. Streptococcal pharyngitis, commonly known as strep throat, is a disease prevalent in children that is caused by *Streptococcus pyogenes*. Left untreated, strep throat can spread throughout the body and cause permanent damage to vital organs (e.g., rheumatic heart disease and post-streptococcal glomerulonephritis). Current methods of diagnosis—rapid test, bacterial culture, and polymerase chain reaction (PCR)—require a pharyngeal swab, which can be an invasive and uncomfortable procedure that can discourage the pursuit of a diagnosis. The CandyCollect, modeled to function like a lollipop for the user, can overcome the deterrents in diagnosis. Featuring an open-microfluidic channel as the bacterial capture region, the device can concentrate the bacteria during the collection period and prevent bacteria loss from the device during sampling. The device also uses isomalt candy, which functions as a built-in timer to ensure the sampling timeframe is sufficient for sample collection. Following sample collection, the device is sent to a laboratory for quantitative PCR (qPCR) analysis, wherein the detection of *S. pyogenes* is indicative of strep throat. In this work, we demonstrate functionality of the device through *in vitro* experimentation. We show that bacteria accumulate on the plasma-treated device over time, isomalt and pooled saliva do not impact the capture of bacteria on the device, and the device has a shelf-life of at least two months. A protocol for the elution of the bacteria from the CandyCollect was optimized, using 5% ethanol in eSwab buffer. Thus far, the CandyCollect has been used in two human subject research studies. The first study (IRB-exempt protocol STUDY00012318) demonstrated that the candy-based timer mechanism can be used to set a minimum time frame for sampling based on the mass of isomalt applied to the device. The user feedback was favorable, with the majority of participants ranking the handling, appearance, taste and consumption positively. In second human research study (IRB-approved protocol STUDY00013842), the

CandyCollect was consistently ranked higher than current saliva sampling methods—swab-based collection and spitting-based collection—in categories including best sampling, least invasive and most sanitary method for saliva collection. Samples from this study will be analyzed using qPCR to detect the commensal bacteria (bacteria that exists naturally in the microbiome), *Streptococcus mutans* and *Staphylococcus aureus*, to compare the efficacy of the CandyCollect to spitting-based collection and swab-based collection in healthy participants.

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Chapter 1: INTRODUCTION

Parts of this chapter are excerpted and paraphrased from my works in chapters 2-4.

This chapter serves as an introduction to some of the key topics addressed in this dissertation, including capillary flow, saliva sampling and point-of-care/at-home testing.

1.1 CAPILLARY FLOW

Capillary flow is a phenomenon in which liquid travels through narrow capillary-like structures without external intervention.[1] The theoretical model for capillary flow has undergone significant reconstruction beyond its initial fundamental representation in the Lucas-Washburn equation which characterized the phenomenon based solely upon surface tension, liquid viscosity, and contact angle and in doing so, the model can be applied to more complex scenarios beyond liquid flow in a capillary tube.[1] Capillary flow, particularly in closed systems, has been investigated since the early 1900s as investigators characterized the phenomenon of spontaneous flow of liquids into capillary structures.[2,3] Since then, capillary flow has been integrated into the development of microfluidic devices with applications including sample preparation and analyte detection.[4,5] Study of the dynamics of capillary flow in open channels—where at least one of the walls of a capillary system is removed to facilitate an air-liquid interface—is a relatively new area of study being undertaken by the field.[6] Recently, flow in rectangular and triangular uniform cross-section open channels has been investigated.[7-10] The effect of bi- and trifurcation (i.e., splitting the channel into two and three channels, respectively) on flow in open-channeled networks has also been reported within the past few years.[11]

1.2 SALIVA SAMPLING

Saliva, as a collection sample, functions as an ideal method for non-invasive sampling for disease diagnosis and progression as it does not entail the puncturing of the skin (e.g., blood, cerebral spinal fluid) for collection.[12] Saliva is primarily composed of water, but is also comprised of numerous proteins and electrocytes secreted from the salivary glands.[13] Additionally, saliva contains bacteria shed from the oral microbiome.[14] Passive drool, dried saliva spots, swab-based collection, and spit based collection are common methods for collecting whole saliva samples.[15] Currently, research is investigating the potential for saliva as a diagnostic tool for oral diseases (e.g., caries, periodontal diseases, oral cancer, Sjögren's syndrome) in addition to more generalized diseases such as diabetes mellitus, cardiovascular disease, viral infections, and numerous cancers.[16] Saliva-based tests for the detection viruses including SARS-CoV-2 and human immunodeficiency virus (HIV) have been developed and are available for diagnostic use.[17]

1.3 POINT-OF-CARE AND AT-HOME TESTING

With the impact of the SARS-CoV-2 virus and the COVID-19 pandemic, terms like "point-of-care testing" and "at-home testing" have become common terms for the general public as rapid testing and the necessity for telemedicine became a societal norm. Point-of-care (POC) testing is testing that can be performed at the site where "care"—referring to medical care—is administered (e.g., hospital, clinic, etc.). In many cases, diagnostic and biomarker testing requires sending samples to an off-site laboratory for analysis. POC devices seek to minimize the technology and analysis required to process samples to those available at clinics and hospitals.[18] This ultimately serves to decrease the time between sample collection and test readout, and to reduce the cost of performing such tests. While POC devices are typically administered by a health professional, "at-home"

testing seeks to develop methods for testing that can be administered and, in most cases, interpreted by the individual. Devices for at-home testing should include a facile method for sample collection that requires little to no training and, if the interpretation is intended to be done by the user, an easily interpretable readout. Pregnancy tests are a prime example of an at-home testing platform with these features.[19] At-home tests have become a key player in telemedicine and ultimately minimize the numerous burdens on the individual that coincide with going to a clinic (e.g., time, cost, etc.). [20]

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Chapter 2: CAPILLARY FLOW IN CONVERGING OPEN-FLUIDIC CHANNELS

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2.1 ABSTRACT

In-depth study of microflow dynamics in open channels is a relatively new area of research. Recently, flow in rectangular and triangular uniform cross-section channels has been investigated and the effect of bifurcation networks on flow has been reported.[1-6] Regarding non-uniform cross section channels, investigation of flow velocity dynamics in abrupt constrictions and enlargements has been published.[7,8] In this work, we present a study on the dynamics of open microflows in progressively converging and diverging channels. An experimental approach has been employed and a theoretical model developed to explain the change of flow velocity observed in these geometrical features.

2.2 INTRODUCTION

In open microfluidics (and in capillary flow in general), the surface tensions of the flowing liquid and solid wall, and the channel geometry govern the dynamics of the flow. In particular, experiments have shown progressively converging and diverging channels can be used to modify

the flow velocity. More specifically, we had observed that the velocity of the flow increases in a convergent and decreases in a divergent channel in previous experiments conducted by the group. In this work, we present a theoretical model for the flow in progressive convergent and divergent open channels based on the expression of the capillary force derived from free surface minimization and the expression of wall friction derived from the integration of the friction length. The model is favorably compared with experiments performed using open channels milled in poly(methyl methacrylate) (PMMA) and liquids such as aqueous isopropyl alcohol (IPA) and nonanol. This approach can be extended to confined capillary flows.

2.3 THEORY

We consider an open channel comprising three sections: first, a uniform open channel of rectangular shape, then a converging (or diverging) open channel leading again to a uniform channel. The flow regime is the viscous regime described by Lucas and Washburn where the dynamics is governed by the capillary force and wall friction. In the model, an expression for the capillary force in non-uniform channel is derived from minimal energy consideration.[9] For a channel of height h, local width w, and geometrical converging/diverging angle α, the capillary pressure P_c is

$$P_c = \gamma \frac{2h\,(cos\theta \pm sin\alpha) - w\,cos\alpha\,(1 - cos\theta)}{h\,w} \quad (1)$$

where θ is the Young contact angle. Note that this approach is based on a low capillary number assumption (small velocity). Wall friction is calculated from integration along the channel axis of the local friction length.[9] If z_0 and z_{tip} are the axial coordinates of the convergent/divergent entrance

and tip of the flow, μ the liquid viscosity, V_c the local velocity, p_c the total channel perimeter in a cross section, S_c the cross sectional area, and $\overline{\lambda_c}$, the local friction length, the expression of the pressure drop due to friction is

$$\Delta P_c = \int_{z_0}^{z_{tip}} \frac{p_c}{\overline{\lambda_c} S_c} \mu \, V_c \, dz. \tag{2}$$

2.4 RESULTS AND DISCUSSION

In the case of converging channels, our model predicts that the capillary flow will experience an increase in velocity corresponding to the angle of convergence. An increase in the angle of convergence will produce a greater increase in velocity. We conducted three IPA and three nonanol experiments in which the length of the converging region was 10 mm, 20 mm, and 30 mm for an angle of convergence of 2.86°, 1.43°, and 0.95°, respectively. Nonanol has a greater viscosity than IPA and saw a less drastic increase in capillary flow compared to the aqueous IPA. In contrast, our model predicted diverging channels cause a decrease in the capillary flow. Experimentation was consistent with this prediction. A comparison between our experimental results and model predictions is shown in Figure 2.2 for the flow of IPA solution (50% v/v) in a converging channel of length 20 mm. The experimental travel distances are closely fitted to the current model (Figure 2.2A). Some fluctuations appear in the velocity due to the derivative operator, however, in general the experimental front velocity is consistent with the current model, especially in comparison to the uniform model (Figure 2.2B). An acceleration of the flow is clearly seen in the converging section of the device (Figure 2.2, red boxes).

2.5 MATERIALS AND METHODS

Channel design was made in Autodesk Fusion 360 with a round-edged rectangular cross section designed to minimize filament formation (Figure 2.1 and Figure A-1). Devices were milled from 3.175 mm PMMA (Goodfellows) on a precision DATRON neo mill. Solvents nonanol and aqueous IPA were colored with 0.25 mg/mL of Solvent Green 3 (Sigma-Aldrich) and 1.2% blue food coloring dye (McCormick), respectively. Aqueous IPA was mixed at a 50% v/v concentration for experimentation. Solvents were added to the inlet and the capillary flow recorded on camera.

2.6 CONCLUSION

Here we show that a progressively converging open channel accelerates the velocity of the capillary flow in the convergent region while a diverging channel decelerates (and even stops) the flow. The theoretical model developed here is validated by experimental results, assessing the expression of the capillary pressure in a non-uniform channel and the use of the longitudinal integration of the friction length for the expression of the wall friction. The results are applicable to closed channel by adapting the Cassie angle and friction length. This model provides the framework for the inclusion of converging and diverging channels into more complex microfluidic devices to modify capillary flow and has significant implications for applications of open microfluidics—ranging from bioanalytical applications to chemical synthesis—where maintaining and accelerating flow is important.

2.7 FIGURES

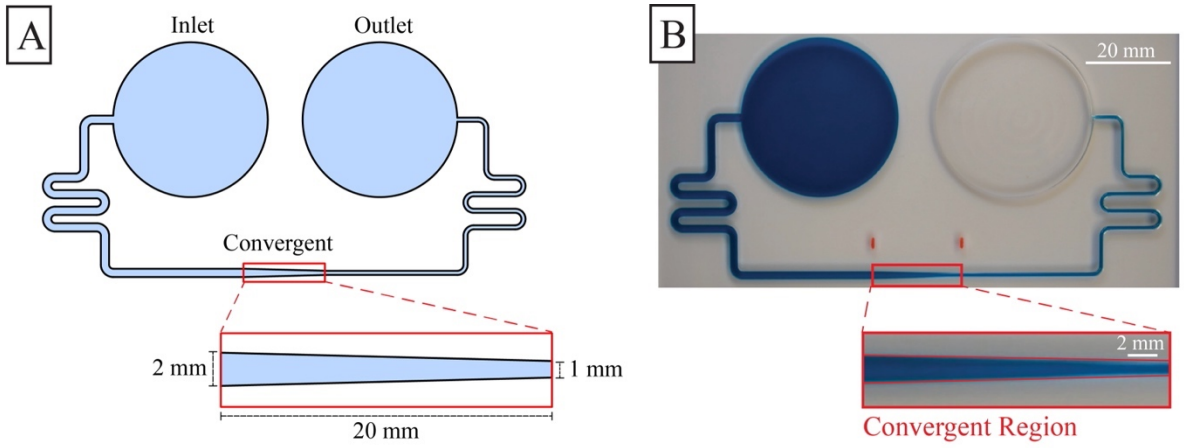

Figure 2.1: Device for convergent- and divergent-channeled experiments. (A) Pictural representation of the convergent device used for experimental conformation of theoretical model with dimensions of convergent region. More detailed dimensions of the device can be seen in Figure A-1. (B) Image of convergent device during experiment with nonanol. Liquid front is located at the interface of the outlet.

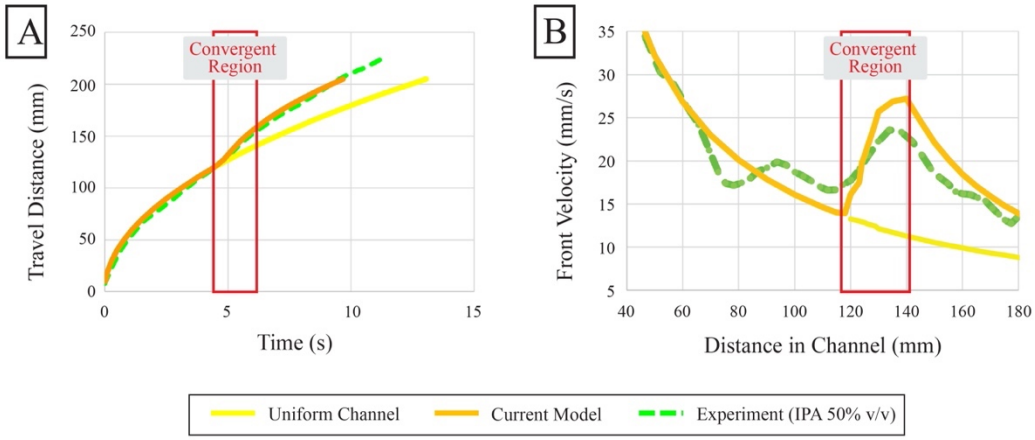

Figure 2.2: Aqueous IPA 50% v/v in convergent channel. Graphs depict a comparison between the current model for liquid behavior in a converging channel (solid orange), the model for liquid behavior in a uniform channel (solid yellow), and the experimental results from deposition of 50% aqueous IPA into the inlet of the convergent device (dashed green) for (A) the travel distance of the liquid front and (B) the velocity of the liquid front. For both the travel distance and velocity, the experimental results are more consistent with the current model in comparison to the behavior predicted for the uniform channel. The increase of velocity in the red box is indicative of acceleration of the liquid front in the convergent region.

2.8 AUTHOR CONTRIBUTIONS

JB, EB, ABT and ST conceptualized the research. JB developed theoretical model. AMM designed and fabricated experimental devices. AMM and JB planned experiments. AMM and JCT conducted experiments and data collection. ABT, ST, EB, JB, AMM, and JCT interpreted the data. AMM and JB wrote sections of the manuscript. AMM and JB made figures for the manuscript. AMM, JB, JCT, EB, ST and ABT edited and revised the manuscript. ABT, ST and EB supervised the research.

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Chapter 3: CANDYCOLLECT: AT-HOME SALIVA SAMPLING FOR CAPTURE OF RESPIRATORY PATHOGENS

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3.1 ABSTRACT

Streptococcus pyogenes is a major human-specific bacterial pathogen and a common cause of a wide range of symptoms from mild infection such as pharyngitis (commonly called strep throat) to life-threatening invasive infection and post-infectious sequelae. Traditional methods for diagnosis include collecting a sample using a pharyngeal swab, which can cause discomfort and even discourage adults and children from seeking proper testing and treatment in the clinic. Saliva samples are an alternative to pharyngeal swabs. To improve the testing experience for strep throat, we developed a novel lollipop-inspired sampling platform (called CandyCollect) to capture bacteria in saliva. The device can be used in clinics or in the home and shipped back to a lab for analysis, integrating with telemedicine. CandyCollect is designed to capture bacteria on an oxygen plasma treated polystyrene surface embedded with flavoring substances to enhance the experience

for children and inform the required time to complete the sampling process. In addition, the open channel structure prevents the tongue from scraping and removing the captured bacteria. Flavoring substances did not affect bacterial capture and the device has a shelf life of at least 2 months (with experiments ongoing to extend the shelf life). We performed a usability study with 17 participants who provided feedback on the device design and the dissolving time of the candy. This technology and advanced processing techniques, including polymerase chain reaction (PCR), will enable user-friendly and effective diagnosis of streptococcal pharyngitis.

3.2 INTRODUCTION

Acute respiratory infections are common with children (average 3-5 upper respiratory infections per year) and may lead to additional complications if left untreated.[1] In developing countries, these illnesses can be more severe, with gaps in healthcare increasing the risk of morbidity and mortality.[2] Additionally, even in developed countries, people who have limited access to clinics, such as in rural areas, or those without insurance may also be unable to receive appropriate diagnosis and treatment. Here, we develop a new sample collection platform for a common bacterial infection, group A streptococcal (GAS) pharyngitis, also known as strep throat, caused by *Streptococcus pyogenes.* Pharyngitis is one of the most common reasons for which children and young adults seek medical care and the Group A streptococcus (GAS) is the most frequent cause of bacterial pharyngitis in these age groups.[3] GAS pharyngitis is typically treated with antibiotics; it can lead to suppurative complications (acute otitis media, sinusitis, retro- and peritonsillar abscess, cervical adenitis), rheumatic fever, and organ damage if left undiagnosed and untreated.[3]

The gold standard method for diagnosis of GAS pharyngitis is swabbing the posterior pharynx and testing with a rapid antigen detection test (RADT); standard culture is performed if the RADT is negative.[4,5] However, culture is time-consuming and may delay the diagnosis.[6] Currently, there are numerous advanced techniques for the detection of *S. pyogenes*, such as polymerase chain reaction (PCR),[7,8] multiplex PCR,[9] impedimetric biosensors,[10,11] touch spray-mass spectrometry,[12] and commercial test kits (e.g., BinaxNOW® Strep A Test; OSOM® Ultra Strep A Test; BD Chek™ Group A Strep; QuickVue In-line Strep A Test). Some commercially available at-home GAS test kits, such as ezlevel Strep A test, © ezleveltests, rely on the parent swabbing the child's throat; this is not the standard of care and it does not produce reliable results.[13] Even in a clinical setting it can be challenging to obtain a high-quality swab sample of the posterior pharynx including the tonsils due to child resistance and discomfort.[14] Children may even refuse to use or be noncompliant with oral sample collection, which can lead to skewed results.[14] This problem is exacerbated in very young children and those with developmental disabilities. Recently, saliva samples have been shown to be an alternative to pharyngeal swabs for respiratory disease diagnosis.[15,16] A recent study of GAS in the saliva of children used swabs to absorb saliva and PCR for detection; 19 of 20 patients with previously confirmed GAS pharyngitis tested positive.[16] In the present work, we aim to further improve the patient experience and establish a salivary sampling open-fluidic device for *S. pyogenes* capture, which can be used comfortably in a clinic or home setting for adults and children.

Our open-fluidic sampling device, CandyCollect, is inspired by a lollipop; it includes a small polystyrene stick with one end coated in isomalt and fruit flavoring candy. We aimed to engineer a device that is both efficient at capturing bacteria in saliva samples and decreases the discomfort

experienced in traditional swabbing methods. We demonstrate that the mass of the candy can be used to inform the sampling time, effectively using the disappearance of the flavoring as a built-in timer. Additionally, we conducted *in vitro* experiments involving the target pathogen, *S. pyogenes*, to investigate its adherence to the device, the effect of sample evaporation on elution and the ability to detect bacteria on the devices using quantitative polymerase chain reaction (qPCR). We have successfully demonstrated that the open-fluidic channel prevents the tongue from scraping the surface and retains the bacteria within the channel, demonstrating that our device can capture the pathogen and accumulate more bacteria over time without disturbance from external factors. To assess the usability and feasibility of this device, we sent CandyCollects to 17 participants across the country to record the person-to-person differences in candy dissolving time and user feedback. With the growing need for decentralized medicine and telehealth solutions, our device provides an accessible sampling method for more versatility in at-home and clinical settings.

3.3 RESULTS AND DISCUSSION

3.3.1 Design of the CandyCollect device

Noncompliance with diagnostic testing in children affects accuracy and validity of diagnosis for upper respiratory infections, and the inability to access a medical professional also prevents children from receiving appropriate diagnosis and treatment. When left untreated, infections such as strep throat can have severe consequences such as rheumatic fever, organ damage, secondary infections, and death.[1,2,21] To optimize the diagnosis of streptococcal pharyngeal infections we have developed a saliva sampling device that takes design inspiration from a classic childhood treat, a lollipop. CandyCollect was designed to comfortably fit in a child's mouth, mitigate any

potential choking hazard by use of a thin candy coating rather than a large piece of candy, be compatible for use with common elution kits, such as ESwab 480C (Copan), and have optimal saliva exchange with the capture surface. The CandyCollect sampling device consists of a computer numerical control (CNC) milled polystyrene stick that is coated on the back and edges with isomalt candy. The face of the stick is not coated in candy and contains a plasma treated open-fluidic spiral channel for saliva and bacteria capture (Figure 3.1). Plasma treatment is known to facilitate bacterial adhesion on polystyrene surfaces.[22,23] Oxygen plasma treatment increases the substrate's hydrophilicity and aqueous wettability resulting in greater bacterial adhesion.[23] The aspect ratio of the channel was designed such that a tongue cannot scrape the bottom of the channel and remove captured bacteria. Isomalt, a sugar alcohol, was used for the candy because it has a low hygroscopicity (ERH of 88), meaning it will not absorb water until equilibrium relative humidity reaches 88% at 20°C,[24] which enables it to remain stable if packaged and stored for extended periods of time. Additionally, in the presence of oral bacteria, such as *Streptococcus mutans*, sucrose ferments and becomes turbid while isomalt and other polyols do not.[24] Finally, isomalt has a glycemic index of two and is safe for diabetic patients.[25] The candy also acts as a built-in timer for sampling time; it ensures the patient keeps the device in their mouth for the optimal duration to achieve capture of bacteria. When the candy flavoring disappears, the sampling is complete, and the patient can mail the CandyCollect back or schedule a pickup for analysis. By varying the amount of isomalt on the CandyCollect we can adjust for different sampling times required by other respiratory pathogens. The ability to enhance or accumulate the pathogens of interest over time shows the future potential of CandyCollect to capture a range of pathogens that are present at different concentrations. Determining how much *S. pyogenes* the device can capture

is important in determining if the device will be compatible with current quantification or rapid PCR methods such as qPCR or real-time PCR.

3.3.2 CandyCollect device enables bacteria accumulation over time and prevents loss due to mechanical scraping

Additionally, determining if bacteria collection increases over time relies on tailoring the mass and surface area ratio of the candy to control the sampling time. *In vitro,* our device can accumulate *S. pyogenes* over time and capture it in as little as 30 seconds; longer incubation time increases the number of bacteria accumulated (Figure 3.2A). Furthermore, it was important to show that the channels prevented the tongue from scraping off any captured *S. pyogenes* (Figure 3.2B). A shallow circle was milled for the flat surface of the CandyCollect device. Fluorescence microscopy images indicate the channel protects the *S. pyogenes* from being scraped off as opposed to the flat surface which shows a marked decrease in *S. pyogenes* after scraping (Figure 3.2B).

3.3.3 Shelf life tests: CandyCollect effectively captures S. pyogenes after 2 months of storage

Our device was designed to enable a high-quality, comfortable collection of pathogens from saliva in a home or clinical setting. We anticipate the devices to be stored in homes or clinics, thus it is highly likely the devices would be stored for extended periods of time before usage. It is known that over time plasma treated surfaces become more hydrophobic (water contact angle increases within 10 days and stabilizes with long storage time).[26] To test whether the time-induced changes in the plasma treated surface of CandyCollect affect its ability to capture bacteria, we conducted shelf life experiments at room temperature. Fluorescence microscopy images show that there is no significant difference observed in the images between 0 day (control group), 3 days, 1 week, and

2 weeks (Figure 3.3A). An additional experiment was conducted to test two months of storage, which also showed the device was still able to capture *S. pyogenes* efficiently compared to the control (Figure 3.3B). In the future, we plan to conduct shelf life experiments for lengths such as six months to one year.

3.3.4 Elution of S. pyogenes from CandyCollect devices and qPCR detection

The saliva on the devices will evaporate in the time between sampling and analysis if they are shipped back to the laboratory. It is important to note that we will not be culturing the bacteria from the CandyCollect device but rather eluting the bacteria from the device for qPCR analysis to detect the presence or absence of *S. pyogenes*. To evaluate whether drying affects the ability to elute *S. pyogenes,* we left the devices at room temperature overnight to evaporate. Fluorescence microscopy images showed that the *S. pyogenes* captured on devices could still be eluted from the device for further experiments (Figure 3.4A).

We also developed an elution method to efficiently remove bacteria from the devices for downstream analysis. Furthermore, we successfully established a qPCR assay to detect *S. pyogenes* eluted from CandyCollect devices (Figure 3.4B and C). The assay can determine the number of bacteria by detection of genomic DNA content. DNA content measured in the bacterial samples eluted from the devices by qPCR was highly correlated with bacterial concentrations that were incubated on the device (R^2=0.991-1, Figure 3.4C and Figure B-3). Importantly, a single DNA product was amplified when using DNA templates from *S. pyogenes*, but no products were detected when using templates from either *Streptococcus mutans* or *Staphylococcus aureus* (Figure B-4), demonstrating high selectivity in detecting *S. pyogenes*. In addition, our assay standard curve

showed linearity down to 50 fg (Figure 3.4B), representing approximately 25 CFU of *S. pyogenes*, demonstrating the ability to detect low levels of *S. pyogenes* (Figure 3.4B). Our results showed DNA was detectable at bacterial concentrations of $1x10^5$, $1x10^4$, and $1x10^3$ CFU/mL in both "dry samples" and "wet samples" (Figure 3.4C and Figure B-3). (Refer to the methods section for definitions of dry and wet samples.) *S. pyogenes* concentrations *in vivo* range from 500 to $1.5x10^4$ CFU/mL from pharyngeal samples in patients with streptococcal pharyngitis.[27] These results suggest that drying does not hinder bacterial elution from CandyCollect device nor affect the ability to detect DNA at a bacterial concentration of 10^3 CFU/mL. We will further develop the extraction protocol and qPCR assay in future work to optimize the limits of detection of our device.

The pathogens found in human saliva will have different typical concentrations, therefore, flexibility in dissolving time of the candy is desirable. To demonstrate flexibility in dissolving time, six CandyCollects with different masses of candy were created to determine how the mass and dimensions of the candy affect the dissolving time. One individual consumed three CandyCollects of each version and recorded the dissolving time (Figure 3.5A). Based on the results, the CandyCollects dissolved between less than one minute and nearly fifteen minutes, accommodating a range of sampling times that may be needed depending on the target bacteria. The full set of data including the mass and dimensions of the CandyCollects is available in Table B-1. However, the CandyCollects are not limited to this range and can be made larger to accommodate sampling times longer than fifteen minutes.

To investigate the variability in dissolving time across different people, we enrolled participants in a pilot feasibility and usability study. The participants were instructed to consume three

CandyCollects that were 0.90-1.10 g, with a diameter of 16 mm, and a thickness of 4 mm and record the dissolving time (Figure 3.5B). The average dissolving time across participants is 3.51 mins, with a minimum of 1.25 mins; the majority of participants completely consumed the candy within 2-4 mins. The full set of data is available in Table B-2. In practice, the required sampling time, which depends on the abundance of a pathogen of interest and the threshold of detection by qPCR, will be evaluated for each pathogen of interest, and the mass of the candy will be adjusted accordingly. We understand that there is personal variability in the amount of time required for the candy to dissolve, and we will adjust the mass and dimensions of the candy so that the minimum dissolving time matches the required collection time for a given pathogen.

During the collection period, the device channel will encounter saliva, bacteria (the analyte of interest) and candy. Isomalt, the primary component of the candy fixed to the device, has been demonstrated to have little impact on bacterial proliferation,[24] however the candy, in addition to saliva, both have the potential to interfere with the adhesion of *S. pyogenes* to the channels of the device, either through competitive binding or chemical action. To replicate the environment of the device during sample capture in a participant, *S. pyogenes* was incubated *in vitro* on the device in mixtures containing pooled saliva, isomalt and/or candy (Figure 3.6). Stock solutions of isomalt and the device candy were made in phosphate buffered saline (PBS). These solutions were mixed with pooled saliva in a one-to-one ratio for a final concentration of isomalt and candy of 0.5 g/mL in their respective solutions in order to maximize the concentration of the isomalt and candy in the saliva. Solutions were used to resuspend *S. pyogenes* and subsequently applied to the devices and incubated. Fluorescence images demonstrate that saliva, isomalt, and the device candy do not impact the adhesion of *S. pyogenes* to the channel of the device (Figure 3.6).

3.4 METHODS AND MATERIALS

3.4.1 CandyCollect fabrication

CandyCollect sticks were fabricated out of 2 mm thick polystyrene sheets (Goodfellow, Cat# 235-756-86), and an acrylic mold for making the silicone mold was fabricated from a 25.4 mm thick acrylic (Source One) by CNC milling (Datron Neo). Dimensions of the CandyCollect stick are provided in Figure B-1. The silicone mold used to cast the CandyCollect was made by pouring food safe silicone (smooth-sil 940, Smooth-On) into the acrylic mold, and cured for 24 hrs at room temperature. The candy was prepared following hygiene guidance from the Washington State Cottage Food Operations Law (RCW 69.22.040(2b-f(ii-iv)). The study team member who prepared the CandyCollect trained in food safety, has a Food Worker Card, and wore a mask during food preparation. In brief, the candy was prepared by gradually adding 1000 g of isomalt to 80 g of boiling water until the solution reached ~170°C. Then 3.5 mL of Chefmaster Super red gel color was added after the solution reached 165°C. Then 10 mL of LorAnn Super Strength Strawberry Candy Oil (LorAnn, Cat# 0320-0500) was added after the solution reached 170°C, and it was removed from the heat. The candy was cooled to room temperature and remelted in small portions for distribution among molds. The silicone mold was placed onto a marble slab to ensure rapid cooling, then the melted candy was poured into each individual CandyCollect shape until the CandyCollect was filled. A CandyCollect stick was placed into the candy, channels facing up, with the circular portion of the polystyrene centered in the candy. The stick was held in place for 15 s, then the next stick was placed. The sticks were pushed back into the candy as needed (using sterilized metal tools) as they set for approximately 5-10 min. Once set, they were carefully removed from the silicone mold and placed on a silicone mat on the marble to finish cooling. After

cooling, the CandyCollect were placed into oriented polypropylene bags (3x4 inch BakeBaking) and heat sealed using an impulse sealer (Metronic-Model: FS-200) on setting 3.

3.4.2 CandyCollect dissolving time in human subjects

Participant characteristics. This study was approved by the University of Washington Institutional Review Board (IRB) under IRB-exempt protocol STUDY00012318. All study procedures were performed after informed consent was obtained. A total of 17 healthy volunteers over the age of 18 years were recruited via the University of Washington Institute of Translational Health Sciences (ITHS) "participate in research" website and the study team website.

Inclusion and exclusion criteria for participant enrollment. Inclusion criterion: over the age of 18 years. Exclusion criteria: individuals who are allergic to sugar alcohols or are in vulnerable groups including pregnant women or individuals residing in a correctional facility.

Enrollment of participants. Once potential participants responded with an email sharing their interest in participating, a study team member sent them a link to a pre-screening survey which asked questions about the inclusion and exclusion criteria. Eligible participants were then asked to enter their demographic information, their first and last name, shipping address, and contact information. After signing an informed consent form on REDCap (Research Electronic Data Capture, hosted at the ITHS),[17] participants were enrolled into the study. All identifiable data were securely stored in REDCap and only de-identifiable data were obtained for further analysis.

Human subjects study procedures. Study participants were enrolled in order to test the general feasibility of the CandyCollects as a collection method. A kit containing six CandyCollect devices and an instruction card was sent to each participant. As directed on the instruction card, each participant consumed one CandyCollect at a time and recorded the total time it took to dissolve the candy on each device. Study data were collected and managed using REDCap.

3.4.3 *S. pyogenes* culture and imaging

Todd-Hewitt broth (TH broth) liquid media preparation. For the THY liquid media, 30 g of Todd-Hewitt Broth (BD Bacto™ TH broth, Fisher Scientific, Cat# DF0492-17-6) and 2 g of Yeast Extract (United States Biological Corporation, Fisher Scientific, Cat# NC9796728) (THY) were added to 0.8 L distilled water and dissolved to completion. Additional distilled water was added for a total volume of 1 L. THY liquid media was autoclaved for 30 min, cooled to room temperature and stored at 4°C.

S. pyogenes maintenance in agar plate. The *S. pyogenes* was prepared from *Streptococcus pyogenes* Rosenbach (American Type Culture Collection, ATCC®, Cat# 700294™). Freeze-dried *S. pyogenes* was wetted with 1 mL liquid media, and then transferred to another conical tube containing 4.4 mL of THY liquid media. To maintain and ensure isolated colonies, *S. pyogenes* was cultured on Trypticase™ Soy Agar (TSA II™) with Sheep Blood (BD BBL™, Fisher Scientific, Cat# B21239X). A sterile disposable inoculating loop (Globe Scientific, Fisher Scientific, Cat# 22-170-201) was dipped in liquid media containing *S. pyogenes* and streaked along the edge of the agar plate. The plate was then rotated 90° and the streaking was continued. This

was repeated two times more using the same loop. The agar plate was maintained in an incubator at 37 °C with 5% carbon dioxide overnight, then stored at room temperature for up to seven days.

Incubation of S. pyogenes in liquid media. To ensure a pure culture, fresh *S. pyogenes* from agar plates were inoculated in liquid media one day before the experiment. A pipette tip was used to pick one colony from the agar plate containing viable *S. pyogenes* colonies, and then the pipette tip was stirred in 7 mL THY liquid media in a sterile 14 mL snap-cap round bottom polystyrene tube (Falcon®, Corning, Product# 352001). *S. pyogenes* was incubated at 37 °C with 5% carbon dioxide in the incubator overnight.

Capturing, fixing and staining of S. pyogenes. The concentration of *S. pyogenes* was measured through Visible 721-Vis Spectrophotometer (vinmax) at a wavelength of 600 nm. *S. pyogenes* in liquid media was centrifuged in 1 mL at 10,000 rpm for 10 minutes. 50 µL of *S. pyogenes* suspension at the concentration of $1x10^9$ CFU/mL was applied to each CandyCollect device and then incubated for 10 minutes (unless otherwise specified). The device was rinsed with PBS and fixed with 4% paraformaldehyde (PFA) for 15 minutes. To remove excess PFA, the device was rinsed with PBS. 50 µL of Alexa Fluor™ 488 Wheat Germ Agglutinin (WGA, Invitrogen™, Fisher Scientific, Cat# W11261, 1 mg/mL) at 1: 500 dilution (v/v) was added to the channel and incubated for 30 minutes at room temperature in the absence of light. Alexa Fluor™ 488 conjugated WGA was used to stain bacteria cell wall peptidoglycans. Bacteria was green fluorescently labeled after staining. The device was rinsed with PBS to remove excess WGA. Then the device was stored in centrifuge tubes containing PBS.

Imaging and quantification. Fluorescent images of *S. pyogenes* were obtained on a Zeiss Axiovert 200 with a 10× (0.30 NA) magnification coupled with Axiocam 503 mono camera (Carl Zeiss AG, Oberkochen, Germany). Multiple channel regions (four to five) were chosen randomly from each device to take images for confirming no bias on a specific region. For all images, the contrast was adjusted uniformly using Fiji (ImageJ) software. To measure the integrated density, each image was further processed using functions from ImageJ. We used the modified procedure from Theberge *et al.,* 2015.[18] Specifically, each image was converted to 8 bits. Then the background was subtracted using "Subtract Background". Next, to convert the images into black and white, a default threshold using the "Li Dark" function was used. Three regions of interest (ROI, 200 x 200 μm^2) were selected to measure the integrated density (Figure B-2).

Statistics. Statistical analysis was performed using GraphPad Prism 9 software. One-way Analysis of Variance (One-way ANOVA) was chosen to compare groups and Tukey's multiple comparison tests were further used in evaluating significance of pairwise comparisons.

3.4.4 Cotton swab scraping assay

50 µl of *S. pyogenes* suspension at the concentration of $1x10^9$ CFU/mL were applied on each CandyCollect device (Figure 3.2B, top panel) and the device without channels which contained a milled shallow circle (Figure 3.2B, bottom panel) and incubated for 10 min. The devices were rinsed once with PBS and excess liquid was drained with KimWipes. Strokes were made on the surface of each device using a cotton swab, to mimic scraping by the tongue. Control devices were not touched by cotton swabs. To evaluate the effect of cotton swab scraping on bacteria binding, the bacteria on the device were stained and imaged following the protocol stated above.

3.4.5 Plasma treatment shelf life experiments

Oxygen plasma treatment. The devices were plasma treated by Zepto LC PC Plasma Treater (Diener Electronic GmbH, Ebhausen, Germany). The chamber was pumped down to a pressure of 0.20 mbar, gas was supplied (0.25 mbar for 2 minutes), power enabled (70W for 5 minutes). After plasma treatment, the devices were stored in OmniTrays (Thermo Scientific™ Nunc™ OmniTray™, Fisher Scientific, Cat# 12-565-296) under room temperature for zero day (control group), three days, one week, two weeks, two months with n=3 replicated devices before the experiment. For time points less than one month, devices were treated in descending order (i.e., two-week devices treated first, followed by one-week devices 7 days later) allowing for the two-week, one-week, and three-day devices to be tested on the same day. The two-month devices were tested on a different day. The zero day (control group) was done for every experiment set. The bacteria on the device were incubated, stained, and imaged following protocol stated above.

3.4.6 Saliva and candy interference experiment

A stock solution of isomalt was made at a 1.0 g/mL concentration in phosphate buffered saline (PBS). This solution was mixed with pooled saliva (Innovative Research Inc., Fisher Scientific, Cat# 50-203-6057) in a one-to-one ratio (final isomalt concentration 0.5 g/mL). This process was repeated with the device candy (composition: isomalt, water, candy oil flavoring, and gel food coloring). These solutions were used to resuspend *S. pyogenes* at a concentration of 1.7×10^9 CFU/mL.

3.4.7 Isolation and purification of genomic DNA from *S. pyogenes*

DNA was isolated using DNeasy UltraClean Microbial Kit (QIAGEN, Cat# 12224) according to the protocol supplied by the manufacturer. *S. pyogenes* was cultured overnight in THY broth. Bacterial cells were pelleted and resuspended in PowerBead Solution. 300 µL of bacteria suspension containing approximately $2x10^9$ CFU of *S. pyogenes* was transferred to each PowerBead Tube followed by the addition of 50 µL of Solution SL. The tubes were placed in a MiniBeadBeater (BioSpec Products, Bartlesville, OK USA) and bead-beating was applied twice for 30 seconds as an alternative to vortexing to facilitate cell lysis. The lysates were loaded onto MB spin column. Then DNA was eluted from each column with 50 µL of 10 mM Tris-HCl buffer. DNA concentrations were measured using BioTek Cytation 5 Cell Imaging Multi-mode Reader (Agilent, 300 Industry Drive, Pittsburgh, PA).

3.4.8 Elution of *S. pyogenes* from CandyCollect devices and preparation of cell lysates containing bacterial genomic DNA

The devices were incubated with 50 µL of *S. pyogenes* suspension for 10 min at the following concentrations: $1x10^3$, $1x10^4$, $1x10^5$, $1x10^7$, $1x10^9$ CFU/mL. Devices eluted immediately, before the suspension dried, were defined as "wet samples". Devices placed in 15 mL tubes and allowed to dry for 24 hours inside a biosafety cabinet were defined as "dry samples"; for these samples the following concentrations were used: 500, $1x10^3$, $1x10^4$, $1x10^5$CFU/mL of *S. pyogenes.* All devices were eluted in 300 µL of ESwab™ buffer (Becton, Dickinson and Company, Cat# R723482) with 5% ethanol in 15 mL round bottom tubes that accommodate the CandyCollect device. To facilitate complete removal of bacteria from devices, ~ 50 µL of 0.1 mm Zirconia/Silica beads (BioSpec Products, Cat# 11079101Z) was added to each tube and the tubes were vortexed for 50 seconds to

dislodge the captured bacteria on the devices. In addition, the tubes were heated at 85 °C for 10 min. The resulting bacteria suspension containing beads was transferred to a screw cap microtube (ThermoFisher, Cat# 3490). Bead beating was carried out on the above-mentioned BeadBeater twice for 30 sec to aid the release of DNA from bacteria. Genomic DNA in bacterial cell lysates was used as template in the qPCR assay (This protocol is used for Figure B-3).

3.4.9 DNA enrichment

Genomic DNA in bacterial cell lysates was enriched using Invitrogen™ ChargeSwitch™ gDNA Mini Bacteria Kit (Fisher Scientific, Cat# CS11301). Following the protocol supplied by the manufacturer, genomic DNA was concentrated 10 times. In brief, genomic DNA adheres on the surface of the magnetic beads when the buffer pH is lower than 6.5. Then the adhered DNA is eluted off of the beads by the provided elution buffer. Concentrated genomic DNA in bacterial cell lysates was also used as template in the qPCR assay (This enrichment procedure is used for Figure 3.4C).

3.4.10 Quantitative PCR assay for detection of *S. pyogenes*

A putative *S. pyogenes* species-specific transcription regulator, *spy1258* (GenBank accession No. AE006565), was used for detecting *S. pyogenes*. The primers/probe targeting this gene have been used in identification and/or quantification of *S. pyogenes* previously.[19,20] The primers/probe sequences designed by CDC Streptococcus Laboratory[20] were adapted for qPCR detection of *S. pyogenes* in our assay: the forward primer: 5’-GCA CTC GCT ACT ATT TCT TAC CTC AA-3’; the reverse primer: 5’-GTC ACA ATG TCT TGG AAA CCA GTA AT-3’; the probe sequence: 5'-FAM-CCG CAA C"T"C ATC AAG GAT TTC TGT TAC CA-3'-SpC6, “T” = BHQ1. The

primers were obtained from IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA). The probe was ordered from MilliporeSigma (Summit Drive Burlington, MA USA). PerfeCTa® qPCR ToughMix (VWR, Cat# 97065-954) was used with 10 µL of DNA template in the qPCR assay with a total volume of 25 µL per reaction. The final concentrations of both forward and reverse primers were 300 nM; the probe concentration was 100 nM (Figure 3.4B, 3.4C, and B-4). For select experiments, qPCR analysis was processed with primer concentration 500 nM, and probe concentration 250 nM (Figure B-3A). CFX connect Real-Time PCR Detection System (Bio-rad Laboratories, Hercules, CA, USA) was used to perform PCR and collect fluorescence data during DNA amplification. The qPCR runs 40 cycles at 95 °C for 15 s and 60 °C for 30 s after initial denaturation of 95 °C for 5 min. Data analysis was carried out using CFX Maestro software version 2.2. 1:10 serial dilutions of purified genomic DNA ranging from 50 ng to 50 fg were used as standards. PCR was performed in 96-well PCR plates (Bio-Rad Laboratories, Hercules, CA, USA) in either duplicate or triplicate.

3.5 CONCLUSION

In this work, we developed a saliva sampling device that aims to enable high-quality, comfortable collection of pathogens from saliva in a home or clinical setting. This work shows (1) the CandyCollect device can effectively capture and concentrate bacteria for further analysis, (2) the CandyCollect device is functional after storage times of up to two months, (3) candy flavoring can be used as an indicator for sampling time to facilitate effective bacterial capture, and (4) qPCR can be successfully performed on samples eluted from the CandyCollect device. In the future, manufacturing of the proposed device can be scaled up using rapid injection molding and testing of the CandyCollect in a clinical setting will provide useful information as to the widespread utility

of this device. This technology has the potential to bring sampling technologies into decentralized telemedicine and minimize the need to access a clinic. Furthermore, this CandyCollect platform and advanced sample analysis has great potential for sample collection and diagnosis in other respiratory diseases. Testing of the CandyCollect in a clinical setting will provide useful information as to the widespread utility of this device.

3.6 FIGURES

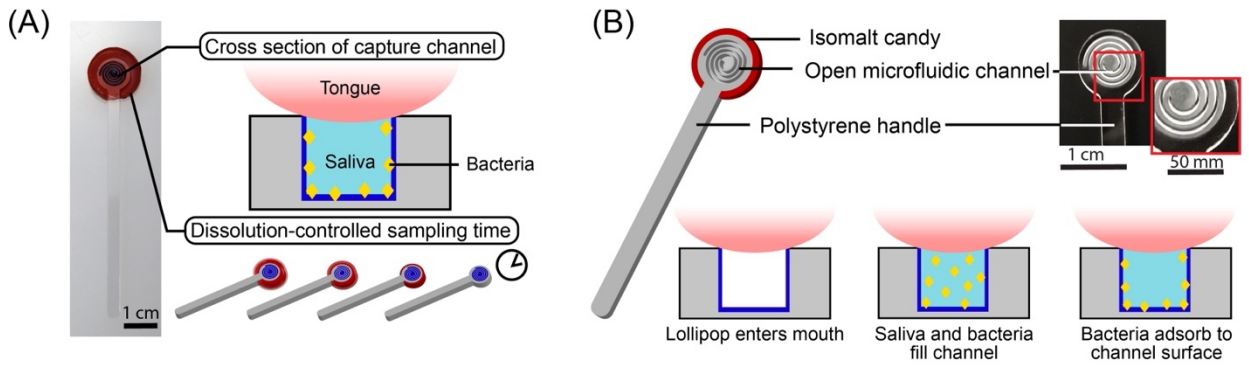

Figure 3.1: Schematic of CandyCollect device. The CandyCollect is a lollipop-inspired, at-home, saliva collection and bacteria sampling device for *S. pyogenes*, the bacteria that causes pharyngeal GAS infection (commonly called strep throat). (A) The open-fluidic channel in the CandyCollect captures the bacteria from saliva, and the candy flavoring is a built-in timer for sampling time (i.e., dissolving time of the candy). The open-fluidic channel is designed to prevent the tongue from scraping the bottom of the channel and removing the collected bacteria, allowing bacteria to accumulate during the sampling time. (B) The CandyCollect is composed of a polystyrene stick with a microfluidic channel and red isomalt candy. When the CandyCollect enters the mouth the channel fills with saliva and the bacteria adheres to the plasma treated surface of the channel.

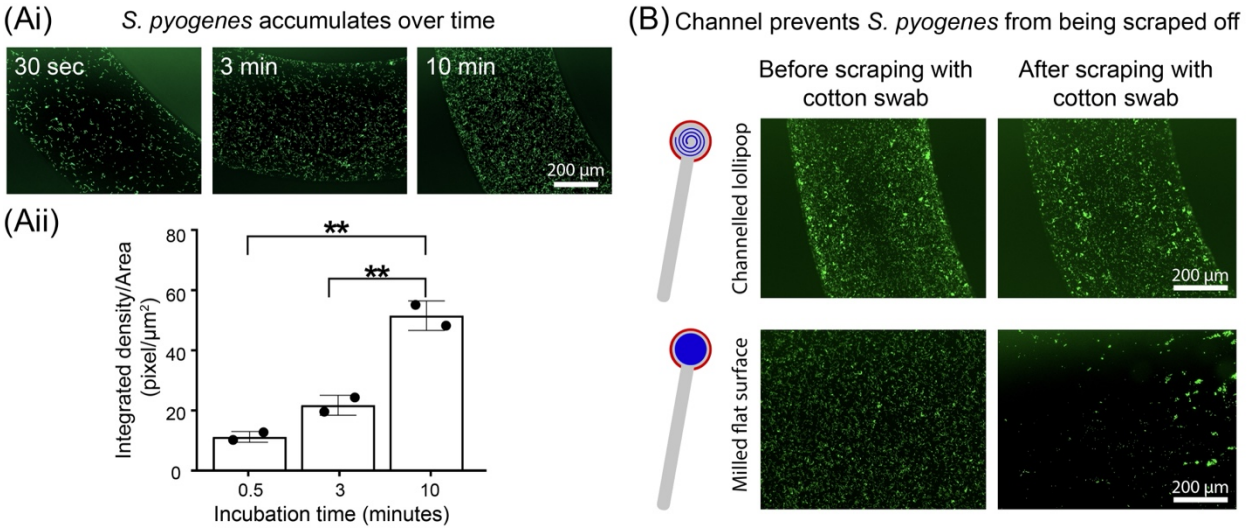

Figure 3.2: *S. pyogenes* accumulates over time, and the channel geometry prevents loss of captured *S. pyogenes*. (Ai) *S. pyogenes* captured *in vitro* on the CandyCollect accumulates over 30 sec, 3 min, and 10 min. (Aii) Quantification of the integrated density per area (pixel/µm^2). Each data point represents an individual CandyCollect; The bar graph represents the mean ± SD of n = 2 CandyCollects. Data sets were analyzed using one-way ANOVA; p-values are indicated for pairwise comparisons between different incubation time: $^{**}p \leq 0.01$ (Tukey's multiple comparison tests). (B) *S. pyogenes in vitro* remains in the channel following scraping with a cotton swab (intended to represent the tongue scraping the device) compared to markedly reduced amounts in a CandyCollect without a channel (milled flat surface) at the same concentration. Note: an incubation time of 10 min was used in B. Images are representative of 3 independent experiments, with duplicate devices and 4 images taken per replicate, and the data point plotted is the average. *S. pyogenes* was green fluorescently labeled.

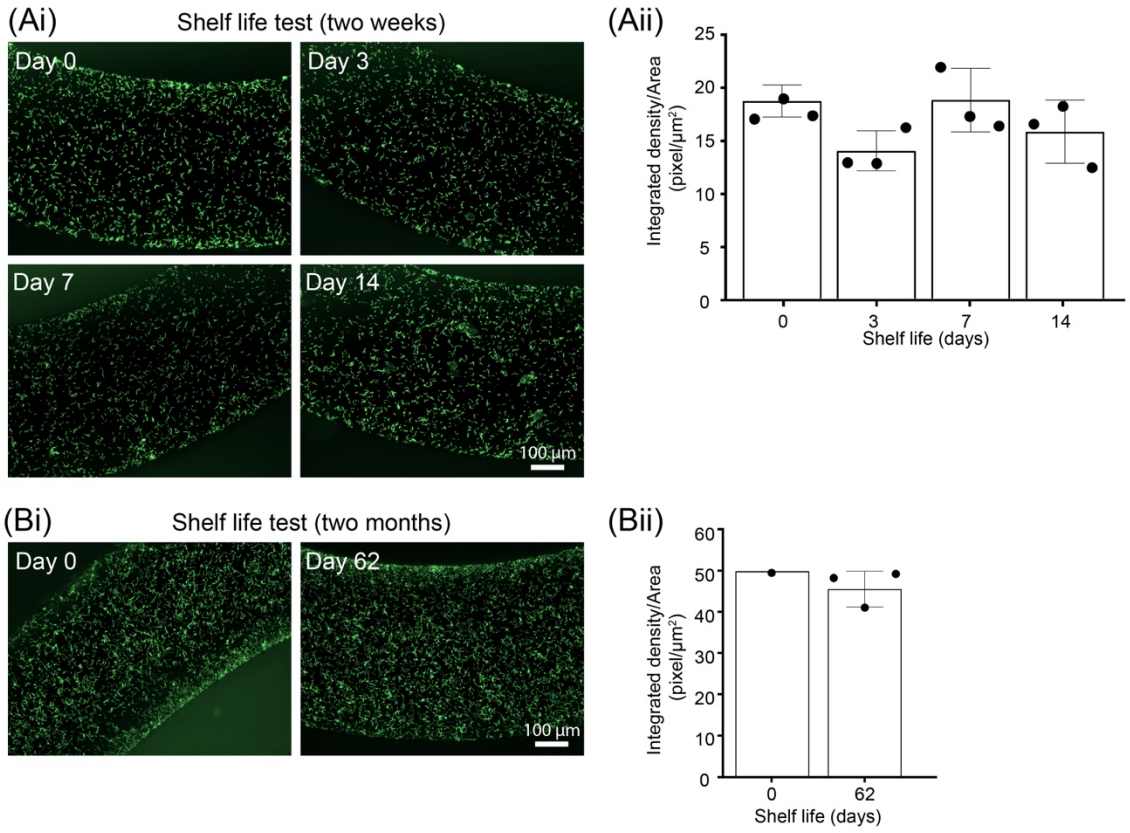

Figure 3.3: Shelf life tests demonstrate that CandyCollect effectively captures *S. pyogenes* after 2 months of storage. Devices were plasma treated and stored at room temperature for (Ai) 0 days (control group), 3 days, 1 week, 2 weeks. Fluorescence microscopy images indicate capture of *S. pyogenes* after 14 days of storage is similar to the control. (Aii) Quantification of the integrated density per area (pixel/µm^2). Data sets were analyzed using one-way ANOVA; no significant difference between shelf life periods was found. (Bi) 0 day (control group) and 2 months. Fluorescence microscopy images indicate capture of *S. pyogenes* after 62 days (~2 months) of storage is similar to the control. (Bii) Quantification of the integrated density per area (pixel/µm^2). Note: in both A and B, *S. pyogenes* at a concentration of 1x10^9 CFU/mL was incubated on the CandyCollect device for 10 minutes. Each data point represents an individual CandyCollect device (4 images were taken per device, and the data point plotted is the average); the bars represent the mean ± SD of n=3 CandyCollects. *S. pyogenes* was green fluorescently labeled.

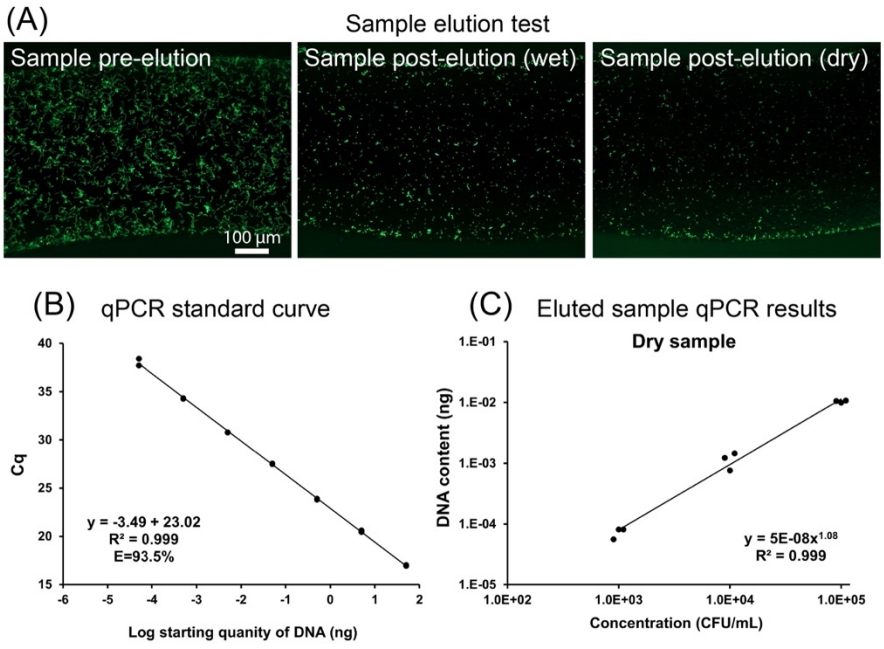

Figure 3.4: Elution tests demonstrate that *S. pyogenes* can be eluted from the CandyCollect and analyzed by qPCR. (A) Elution of *S. pyogenes* is successful in wet and dry samples. *S. pyogenes* at a concentration of $1x10^9$ CFU/mL was incubated on the CandyCollect device for 10 minutes. *S. pyogenes* was green fluorescently labeled. The image results suggest that the *S. pyogenes* can be eluted from CandyCollect device and drying does not hinder bacteria elution. (B) Standard curve for the qPCR assay. 1:10 serial dilutions of genomic DNA ranging from 50 ng to 50 fg were used as template for qPCR. Each dot represents one technical duplicate (in cases where one point is visible the duplicates were identical). The standard curve in which Cq values were plotted against starting template DNA, was linear from 50 ng to 50 fg. qPCR efficiency ranged from 93.5-100.6% across four independent experiments. (C) Device drying did not prevent elution and detection of *S. pyogenes* sampled on devices. Quantification of *S. pyogenes* by qPCR. Each data point represents an individual CandyCollect. Three concentrations of *S. pyogenes* suspension ($1x10^3$, $1x10^4$, $1x10^5$ CFU/ml) were used for the experiment. DNA content was detected in a bacterial concentration-dependent manner. Devices run through the incubation and elution protocols without bacteria loading were used as device negative controls. No-template controls (NTC) for qPCR and device negative controls had no detected signal.

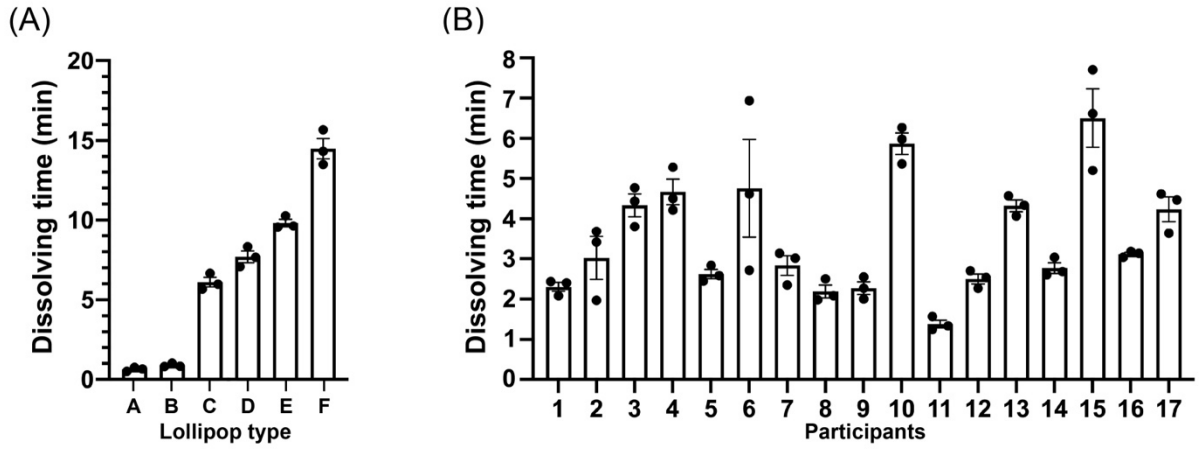

Figure 3.5: CandyCollect dissolving time can be controlled by changing the mass and size of the CandyCollect. (A) Six types of CandyCollect devices, each with a different mass and size of candy, were consumed by one individual. Bar graphs represent mean ± SEM of n = 3 CandyCollects. Please refer to Table S1 in the Supporting Information for CandyCollect mass and dimensions. (B) A single size of CandyCollect devices was sent to 17 research participants for a usability test. The mass of the CandyCollect in B is 0.90-1.10 g, with a diameter of 16 mm, and thickness of 4 mm. Bar graphs represent mean ± SEM of n = 3 CandyCollects.

Figure 3.6: Saliva, isomalt, and other candy ingredients do not interfere with *S. pyogenes* capture on the CandyCollect device. *S. pyogenes* was green fluorescently labeled. Fluorescence microscopy images of captured *S. pyogenes* in the device when incubated in solutions containing (A) pooled saliva, (B) pooled saliva and isomalt, and (C) pooled saliva and device candy (composition: isomalt, water, candy oil flavoring, and gel food coloring). Solutions were used to resuspend *S. pyogenes* at a concentration of 1.7×10^9 CFU/mL, incubated on the CandyCollect device for 2 minutes, and imaged. Images are representative of one independent experiment, with 2 replicates and 3-4 images taken per replicate.

3.7 AUTHOR CONTRIBUTIONS

EB, ABT and ST conceptualized the research. UNL designed CandyCollect devices. UNL, AMM and WCT fabricated CandyCollect devices. DLH optimized and engineered the candy to act as a

timer for collection. UNL, XS, DLH, WCT, AMM, MGT, GWH and TCC reviewed the literature and collaboratively designed experiments and the human subjects study. XS planned biological experiments. XS, WCT and AMM, conducted biological experiments and data collection. KNA, advised on work with human subjects and regulatory protocols. MGT, GWH and TCC, recruited participants, made a platform to screen participant eligibility, and packaged and shipped CandyCollects to research participants. ABT, ST, EB, UNL, XS, DLH, WCT, AMM, MGT, GWH and TCC interpreted the data. UNL, XS, DLH, WCT, AMM, MGT, GWH and TCC wrote sections of the manuscript. UNL, WCT and AMM made figures for the manuscript. ERW and GPD provided expertise on clinical relevance and sampling pathogens in saliva. UNL, XS, WCT, AMM, KNA, GPD, ERW, ST and ABT edited and revised the manuscript. ABT and ST supervised the research.

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Chapter 4: COMMENSAL BACTERIA DETECTION USING A LOLLIPOP-BASED MICROFLUIDIC DEVICE

Reproduced in part from Wan-chen Tu, Anika M. McManamen*, Xiaojing Su, Damielle L. Hieber, Meg G. Takezawa, Grant W. Hassan, Ulri N. Lee, Eden V. Anana, Molly W. Stephenson, Ingrid Jeacopello, Karen N. Adams, Erwin Berthier, Sanitta Thongpang[§] and Ashleigh B. Theberge[§]. "Commensal Bacteria Detection using a Lollipop-based Microfluidic Device." Submitted to microTAS 2022.*

**denotes co-authorship, [§]denotes co-correspondence*

4.1 ABSTRACT

In our previous work, we introduced the CandyCollect, a lollipop-inspired collection device that enables at-home collection of saliva samples for the detection of *Streptococcus pyogenes* for strep throat diagnosis. To demonstrate that CandyCollect can be used to collect the human salivary bacteria, we present that (1) CandyCollect can capture the salivary commensal bacteria *Staphylococcus aureus* and *Streptococcus mutans* that are found in healthy individuals and (2) bacteria adsorbed to a CandyCollect can be eluted from the device for downstream analysis. We further compared the feedback from healthy participants for their experiences with using CandyCollect and two other commercial saliva collection methods.

4.2 INTRODUCTION

Open microfluidic channels are becoming increasingly prevalent within the fields of biotechnology, biology, and diagnostics due to their versatile applications for achieving simple

fluid flows.[1] We previously developed the CandyCollect, a novel lollipop-inspired open-microfluidic sampling platform that simplifies the collection of saliva samples and enables at-home sampling by allowing for the device to be shipped back to a lab for microbial quantification and disease diagnosis.[2] In this work, we demonstrate versatility of the CandyCollect by evaluating its ability to capture *S. aureus* and *S. mutans*, commensal oral bacteria found healthy individuals.[3,4] Detection of commensal bacteria is useful for microbiome studies and as a positive control for diagnostics. We present a novel elution method for the removal of bacteria from CandyCollect for analysis using quantitative polymerase chain reaction (qPCR). Furthermore, we detail the structure of a clinical research study evaluating the CandyCollect in relation to commercial methods of saliva sampling.

4.3 RESULTS AND DISCUSSION

4.3.1 Bacterial adhesion and elution on CandyCollect

In vitro experiments show CandyCollect captures commensal bacteria, *S. aureus* and *S. mutans*. Our elution method, using ESwab™ buffer with Proteinase K, demonstrates efficient elution of both *S. aureus* and *S. mutans* from CandyCollect (Figure 4.2). We have also developed qPCR analysis protocol for analyzing the eluted bacteria.

4.3.2 Human Subject Research

In our human subject research study, 13 participants provided their feedback on the three sampling methods they used to collect saliva. 8 of the 13 participants rated CandyCollect as the best sampling method when asked to rank the three methods (Figure 4.3A). Additionally, CandyCollect was selected the most for questions comparing various aspects of the sampling experience (e.g., least invasive, most sanitary) (Figure 4.3B). Overall, the reception of the CandyCollect was

positive, indicating—in terms of user perspective— CandyCollect presents a viable method for saliva collection. Ongoing work will increase the sample size of this study.

4.4 METHODS AND MATERIALS

4.4.1 Device fabrication

The device was milled out of 2 mm polystyrene sheets by CNC milling (Datron Neo) (Figure 4.1). Following milling, the device is sonicated with isopropyl alcohol (IPA) for 30 minutes and then sonicated with 70% ethanol for 30 minutes. Device was then oxygen plasma treated following the protocol in our previous publication.[2]

4.4.2 *In-vitro* bacteria capture and elution

S. aureus and *S. mutans* were purchased from ATCC; *S. aureus* was cultured in Bacto™ Tryptic Soy Broth (BD211825) (TSB), and *S. mutans* was cultured in TSB supplemented with 0.2 % yeast extract (BD212750) (TSY). The bacteria on the device were incubated, stained, and imaged following protocol stated in our previous publication.[2] All devices were eluted in ESwab™ buffer with 3% Proteinase K in 15 mL round bottom tubes that accommodate the CandyCollect to dislodge the captured bacteria on the devices. Fluorescent images were obtained following the protocol established in our previous publication.[2]

4.4.3 Human subject study (UW IRB-approved protocol STUDY00013842)

Study participants were enrolled to access the preferences of CandyCollect in comparison to two commercial sampling methods. A package containing CandyCollects, ESwabs™, SpeciMAX Stabilized Saliva Collection Kits, and an instruction card was sent to each participant. A participant collected saliva samples, mailed the samples back to our lab, and recorded feedback on the different methods. Study data were collected and managed using REDCap.

4.5 FIGURES

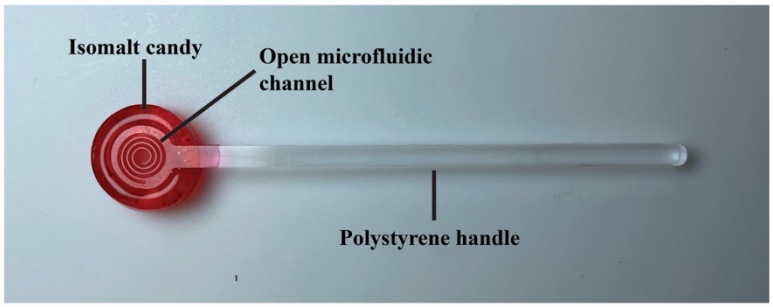

Figure 4.1: Schematic of CandyCollect device. CandyCollect device is composed of a polystyrene handle and face. Face has a spiral open-microfluidic channel for bacterial capture. Isomalt candy (composition: isomalt, water, candy oil flavoring, and gel food coloring) is inlaid on the back of the face to function as a timer for saliva collection.

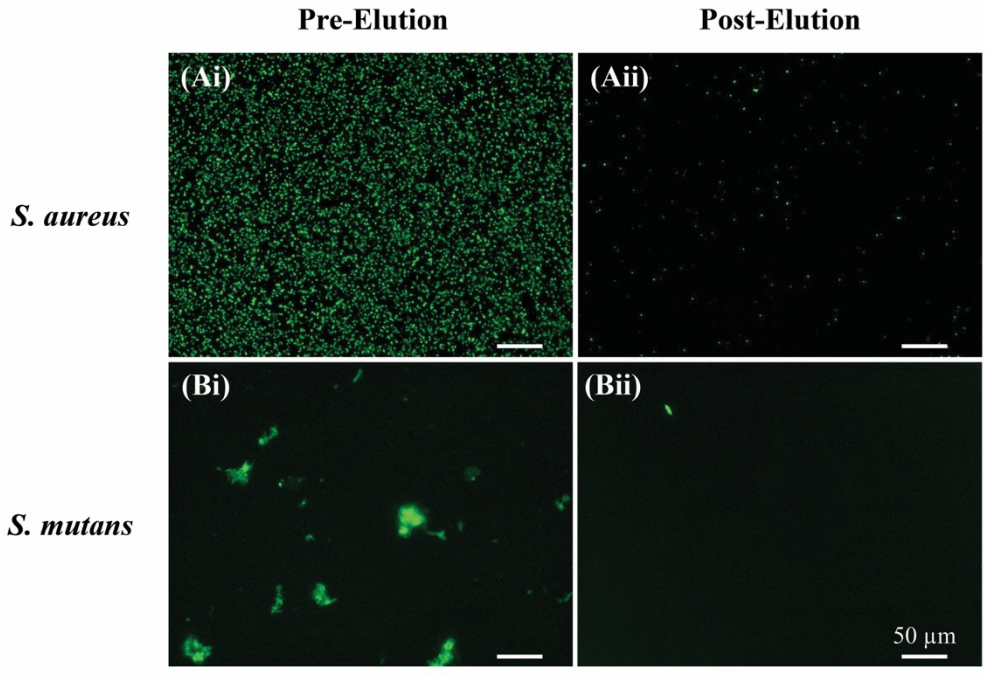

Figure 4.2: Fluorescent images of bacteria on CandyCollect device. (A) *S. aureus* and (B) *S. mutans* were captured in vitro on the CandyCollect at a concentration of $1x10^9$ CFU/mL and were eluted by ESwab™ buffer with 3% Proteinase K. *S. pyogenes* was green fluorescently labeled by Alexa Fluor™ 488 Wheat Germ Agglutinin (WGA, Invitrogen™, Fisher Scientific, Cat# W11261, 1 mg/mL).

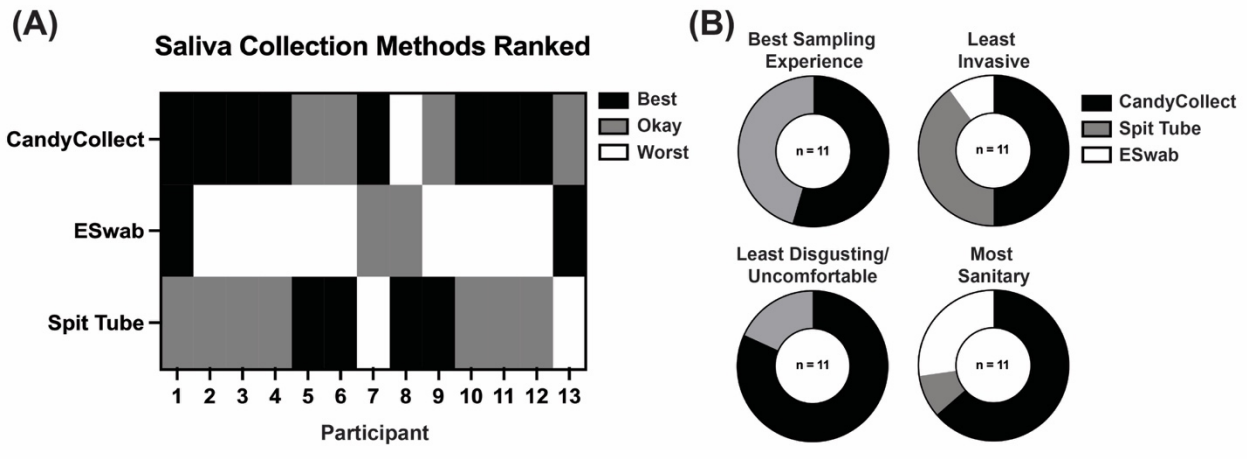

Figure 4.3: Comparison of saliva collection methods by participants in clinical research study by healthy participants. (A) Participants were asked to rank the three methods of saliva collection—CandyCollect, Eswab™ (swab-based collection) and Spit Tube—from best to worst. CandyCollect was ranked best by the majority of participants. (B) Participants were asked additional questions regarding their positive experience amongst the three sampling methods; for all questions, CandyCollect was selected most frequently indicating an overall positive experience.

4.6 CONCLUSION

We have demonstrated that the CandyCollect can capture both *S. aureus* and *S. mutans*, in addition to *S. pyogenes* (previously established) and that *S. aureus* and *S. mutans* can be eluted from the CandyCollect device. This demonstrates our device is both versatile and facilitates a clinical research study with healthy subjects where bacterial detection of *S. aureus* and *S. mutans* can be evaluated. Furthermore, we have demonstrated that the CandyCollect has an overall positive reception by the public, making it potentially competitive in the market.

4.7 AUTHOR CONTRIBUTIONS

EB, ABT and ST conceptualized the research. AMM, WCT, UNL, EVA and MWS fabricated CandyCollect devices. DLH, EVA and MWS manufactured isomalt candy. XS, WCT, AMM reviewed the literature and collaboratively designed experiments and the human subject study.

AMM, WCT, and XS planned biological experiments AMM, WCT, XS and IJ conducted biological experiments and data collection. KNA, advised on work with human subjects and regulatory protocols. MGT, GWH, recruited participants, made a platform to screen participant eligibility, and packaged and shipped CandyCollects to research participants. ABT, ST, EB, XS, WCT, AMM, DLH, MGT, GWH, EVA, MWS, and IJ interpreted the data. WCT, and AMM wrote sections of the manuscript. WCT and AMM made figures for the manuscript. ERW and GPD provided expertise on clinical relevance and sampling pathogens in saliva. WCT, AMM, XS, ST and ABT edited and revised the manuscript. ABT and ST supervised the research.

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Chapter 5: CONCLUSIONS AND FUTURE WORK

Parts of this chapter are excerpted and paraphrased from my works in chapters 2-4.

In this dissertation, three projects have been presented exploring capillary flow in its fundamental behavior and use in the CandyCollect device.

Chapter 2 presented a fundamental microfluidics project characterizing the capillary flow behavior of a liquid in a converging (and diverging) open-fluidic channel. This work shows that a progressively converging open channel accelerates the velocity of the capillary flow in the convergent region while a diverging channel decelerates (and even stops) the flow. This model provides the framework for the inclusion of converging and diverging channels into more complex microfluidic devices to modify capillary flow and has significant implications for applications of open microfluidics—ranging from bioanalytical applications to chemical synthesis—where maintaining and accelerating flow is important. In the future, this model will be further developed by incorporating aqueous droplets in an immiscible carrier fluid to increase the model's complexity and to characterize the droplet behavior in converging and diverging channels.

Chapters 3 and 4 presented the development and assessment of the CandyCollect through the approach of *in vitro* experimentation and human subject research. Through *in vitro* experimentation, we have demonstrated that the CandyCollect, with the microfluidic channel facilitating capillary action, can successfully capture *Streptococcus pyogenes*, *Streptococcus mutans*, and *Staphylococcus aureus*, with an efficacy of up to 2 months post-plasma treatment. Future experiments will assess longer time points to determine the maximum time post-plasma

treatment for efficient bacterial collection on the CandyCollect device. The device's ability to capture multiple bacteria demonstrates the versatility of the CandyCollect. This is indicative of the device's potential to function as a general platform for bacterial collection across various diseases wherein oral bacterial concentration would be characteristic for a diagnosis. Two methods for bacterial elution from the CandyCollect—one for *S. pyogenes* and one for *S. mutans* and *S. aureus*—was also presented with highly efficient bacterial removal. The *S. pyogenes* method has also been demonstrated to be non-inhibitory to analysis using quantitative polymerase chain reaction (qPCR), thus facilitating downstream analysis of samples collected using the CandyCollect. Future work will evaluate the novel elution method for *S. mutans* and *S. aureus* with respect to qPCR inhibition to ensure the methods are complimentary. Through human subject research, we have demonstrated the efficacy of the isomalt-based timer system and presented an overwhelmingly positive reception towards the CandyCollect device from the public. In the future, the samples from the latest study (UW IRB-approved protocol STUDY00013842) will evaluated in comparison to the commercial methods for bacterial concentrations of *S. mutans* and *S. aureus*. Subsequent studies will investigate CandyCollect reception by children and assessment of *S. pyogenes* concentration in patients with strep throat.

Characterization of capillary flow, namely in open-microfluidic systems is still a novel field of study with many facets still to be explored. This exploration is valuable in its application to numerous technologies including the development of at-home testing devices that have the potential to decentralize telemedicine and minimize the need to access a clinic.

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APPENDIX A

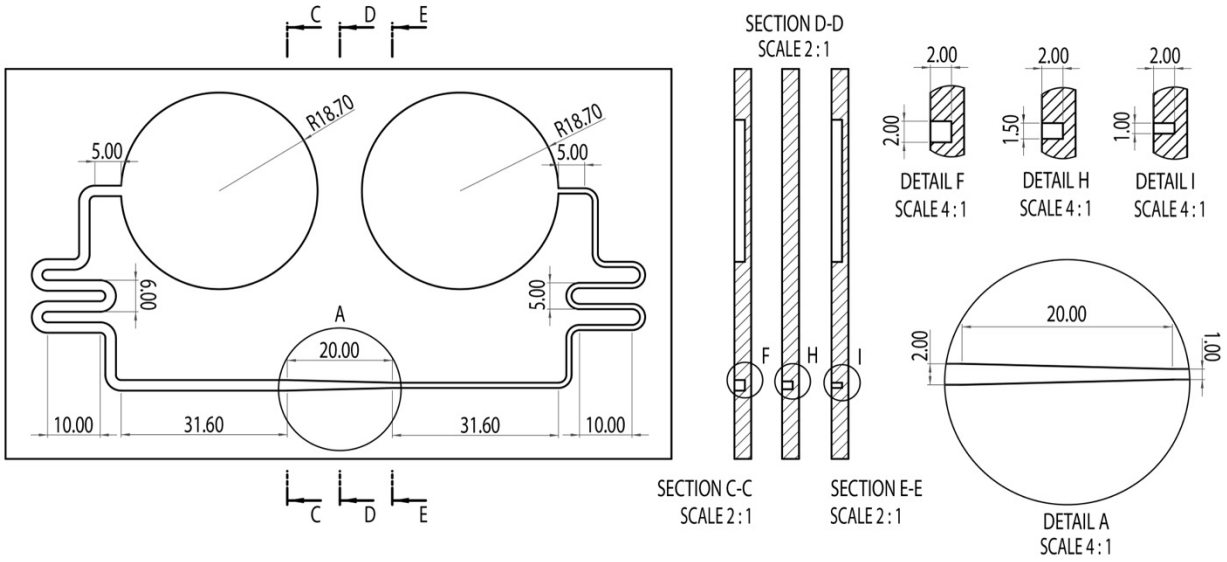

Figure A-1: Schematic diagrams illustrating the dimensions, in mm, of the experimental convergent/divergent open-fluidic device. Round channel edges are achieved through the use of a round endmill tool (corner radius = 0.1905 mm).

APPENDIX B

*Reproduced in part from Ulri N. Lee *, Xiaojing Su*, Damielle L. Hieber, Wan-chen Tu, Anika M. McManamen, Meg G. Takezawa, Grant W. Hassan, Tung Ching Chan, Karen N. Adams, Ellen R. Wald, Gregory P. DeMuri, Erwin Berthier, Ashleigh B. Theberge[§], and Sanitta Thongpang[§]. "CandyCollect: At-home saliva sampling for capture of respiratory pathogens". Biorxiv, 2021.10.19.464911; doi: https://doi.org/10.1101/2021.10.19.464911*

**denotes co-authorship, [§]denotes co-correspondence*

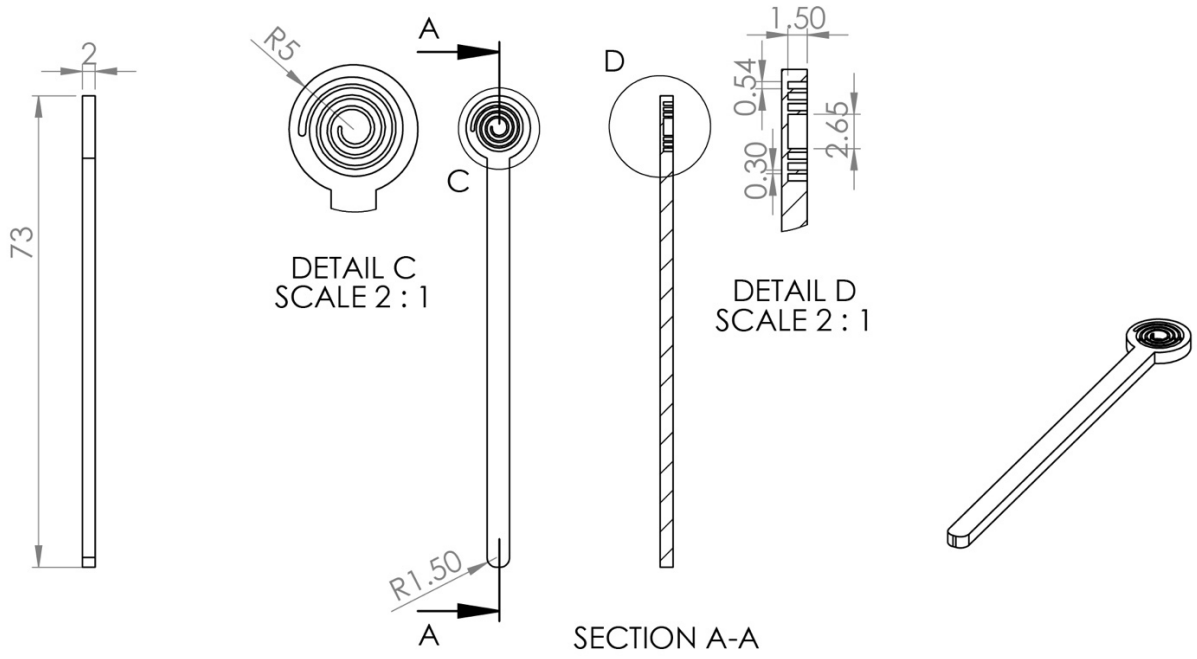

Figure B-1: Schematic diagrams illustrating the dimensions, in mm, of the CandyCollect milled stick.

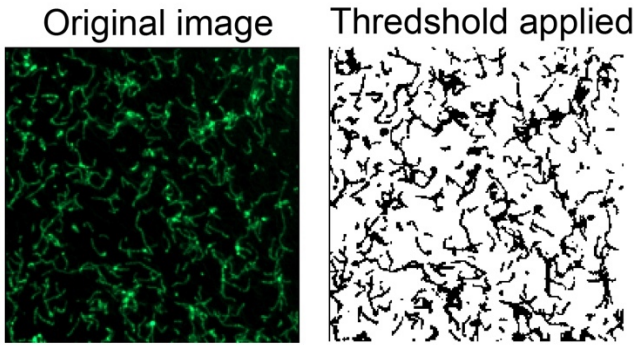

Figure B-2: Qualification of integrated density. Image process before and after threshold applied in one given region of interest (ROI).

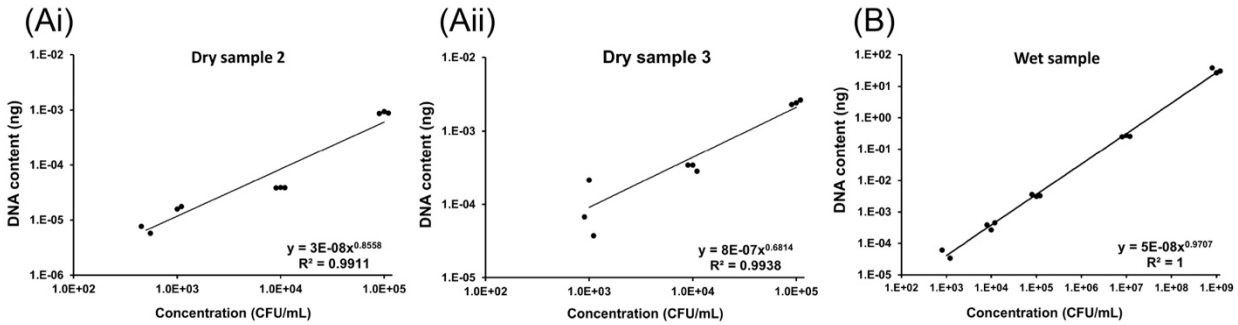

Figure B-3: Analysis of *S. pyogenes* eluted from CandyCollect devices. (Ai-ii) Quantification of *S. pyogenes* from dry CandyCollect samples by qPCR. Shown here are two additional independent experiments performed in addition to the experiment shown in Figure 4C (note: the experiments in this figure were performed without enrichment of DNA, whereas DNA enrichment was performed in the experiment shown in Figure 4C). Each data point represents an individual CandyCollect. Three concentrations of *S. pyogenes* suspension $1x10^3$, $1x10^4$, $1x10^5$ CFU/mL were used. (B) Quantification of *S. pyogenes* from wet CandyCollect samples by qPCR. Each data point represents an individual CandyCollect. Five concentrations of *S. pyogenes* suspension $1x10^3$, $1x10^4$, $1x10^5$, $1x10^7$, $1x10^9$ CFU/mL were used. (Note: the procedures performed for the experiments shown in Figure S3A differed slightly from the procedure for Figure S3B; refer to the methods section for details.)

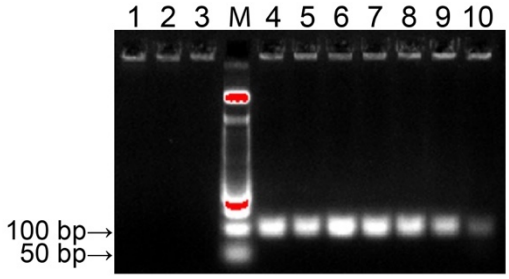

Figure B-4: Agarose gel electrophoresis demonstrated high selectivity of the qPCR assay. qPCR was performed using the primers/probe targeting *spy1258*. The templates were 1:10 serial dilution of DNA (50 ng – 50 fg) from *S. pyogenes* and 50 ng of DNA from *S. mutans* and *S. mutans*. 3% agarose gel was used to separate DNA products from the qPCR reactions. A single PCR product with a size of about 100 bp, an expected amplicon size, was only shown in each of the DNA samples from *S. pyogenes* (lane 4-10). No PCR products were shown in DNA samples from *S. mutans* (lane 1), *S. aureus* (lane 2), and no template control (lane 3). Note: lane M is 50 bp DNA ladder.

Table B-1: Mass, diameter, thickness, and dissolving time of CandyCollects in Figure 4A.

Lollipop	Mass (g)	Diameter (mm)	Thickness (mm)	Time (min)
A1	0.04	n/a*	n/a	0.75
A2	0.04	n/a	n/a	0.50
A3	0.04	n/a	n/a	0.65
B1	0.08	n/a	n/a	1.03
B2	0.08	n/a	n/a	0.83
B3	0.08	n/a	n/a	0.82
C1	4.70	27.12	4.52	6.67
C2	4.16	27.12	4.52	5.98
C3	4.09	27.12	4.52	5.67
D1	4.06	23.00	6.46	8.33
D2	4.12	23.00	6.46	7.67
D3	4.27	23.00	6.46	7.08
E1	7.44	30.00	7.60	10.28
E2	7.62	30.00	7.60	9.55
E3	6.32	30.00	7.60	9.63
F1	6.92	23.00	12.92	14.32
F2	7.45	23.00	12.92	13.50
F3	7.53	23.00	12.92	15.67

*Note: CandyCollects A1-B3 did not have measurable diameter and thickness because a mold was not used to apply the candy. Instead a small drop of isomalt was applied and allowed to form a thin layer on the stick. The diameter of the round area on the stick is 1 cm and the thickness is 2 mm.

Table B-2: Mass, diameter, thickness, and dissolving time of CandyCollects in Figure 4B.

Participant	Mass (g)	Diameter (mm)	Thickness (mm)	Time (min)
1	0.96	16	4	2.08
	1.14	16	4	2.43
	1.07	16	4	2.40
2	0.93	16	4	3.42
	1.12	16	4	3.68
	0.97	16	4	1.97
3	1.02	16	4	4.77
	0.88	16	4	3.80
	1.07	16	4	4.43
4	1.07	16	4	5.28
	0.88	16	4	4.50
	1	16	4	4.22
5	1.06	16	4	2.83
	1.11	16	4	2.58
	1.09	16	4	2.45
6	0.98	16	4	2.72
	1.15	16	4	6.93
	0.97	16	4	4.62
7	1.07	16	4	2.35
	1	16	4	3.02
	1.09	16	4	3.13
8	1.12	16	4	2.50
	1	16	4	1.98

	0.96	16	4	2.08
9	1.13	16	4	2.55
	1.03	16	4	2.27
	0.95	16	4	2.00
10	1.01	16	4	6.27
	0.83	16	4	5.98
	0.96	16	4	5.37
11	0.92	16	4	1.25
	1.04	16	4	1.33
	1.05	16	4	1.57
12	0.99	16	4	2.70
	1.02	16	4	2.53
	0.98	16	4	2.27
13	1.08	16	4	4.33
	0.95	16	4	4.07
	0.96	16	4	4.57
14	0.92	16	4	2.68
	1.01	16	4	2.60
	0.97	16	4	3.03
15	0.97	16	4	6.62
	1.02	16	4	7.70
	0.94	16	4	5.20
16	0.93	16	4	3.18
	0.95	16	4	3.13
	0.95	16	4	3.03
17	1.01	16	4	4.62

	1.04	16	4	4.47
	0.95	16	4	3.63