CandyCollect: An Open-Microfluidic Device for the Direct Capture and Enumeration of Salivary-Extracellular Vesicles

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Abstract

Extracellular Vesicles (EVs) are membrane-derived vesicles shed by cells into the extracellular space that play key roles in intercellular communication and other biological processes. As membrane-bound cargos of nucleic acids and other proteins that are abundantly found in virtually every biofluid including blood, urine, and saliva, EVs are widely regarded as promising biomarkers for disease detection. While it is an increasingly promising biofluid from which to isolate EVs, saliva poses challenges due its complexity and heterogeneity—cells, debris, and other proteins can inhibit the isolation of EVs by traditional platforms. Here, we employ the CandyCollect, a lollipop-inspired sampling device with open microfluidic channels, as a non-invasive and patient-friendly alternative for the capture of salivary EVs. The CandyCollect simplifies sample preparation by effectively pre-concentrating EVs on the device surface before EVs are eluted off of the CandyCollect, labeled with cholesterol-tagged oligonucleotides, and subsequently detected by qPCR with primers specific for the tagged oligos to enumerate the relative number of EVs. We demonstrate that downstream EV cargo analysis can be performed using Simoa. Overall, the CandyCollect ushers a new method to capture, enumerate, and analyze salivary EVs.

MAIN TEXT

Introduction

Extracellular Vesicles (EVs) are membrane-derived vesicles continuously shed by cells (*1-3*) into the extracellular space. Containing DNA, RNA, mRNA, miRNA, and other proteins in their cargo (*4, 5*) that are virtually identical to those of their cell-of-origin (*6*), EVs facilitate intercellular communication (*7*) and a variety of biological processes, ranging from development and homeostasis (*8*) to cancer evolution and metastasis (*7*). While much research has focused on understanding the genesis and function of EVs (*9*), recent attention has pivoted to harnessing EVs' clinical potential as biomarkers for a range of diseases (*10-13*), including cancer and neurodegenerative disorders (*14-16*).

A key challenge to employing EVs clinically, however, is their isolation from biofluids such as blood, urine, saliva, and cerebrospinal fluid (CSF) where they are abundantly present at concentrations ranging from 10^6 EVs/mL in CSF to 10^7-10^9 EVs/mL in saliva or urine to 10^{10} EVs/mL in blood (*6, 17-21*), and their subsequent analysis. Traditional methods to isolate and/or analyze EVs include ultracentrifugation (*22, 23*), transmission electron microscopy (*24*), nanoparticle tracking analysis (*25-27*), dynamic light scattering (*28*), and flow cytometry (*29, 30*). While they can provide information on EV concentration and size distribution (*31*), these methods suffer from multiple limitations such as low EV yields and integrity, expensive instrumentation, and intensive labor (*32-35*). Moreover, they cannot easily identify or quantify subpopulations nor readily enable successful downstream applications such as cargo analysis. More recent methods of EV isolation such as precipitation (*36-38*), size exclusion chromatography (*39, 40*), immuno-affinity capture (*41, 42*), and the use of microfluidic platforms (*43-49*) have helped to alleviate some of these shortcomings. Sample loss, non-EV contamination, and incompatibility with subsequent analysis still pose ongoing issues in even the more contemporary methods (*33-42, 50, 51*). Overall, less expensive and time-effective platforms that allow for the high recovery and purity rates of isolated EVs that do not impede subsequent analysis are crucial if EVs are to achieve their truest potential as biomarkers.

Just as there is a challenge in developing an EV technology for efficient capture and analysis, so too is there a challenge in choosing the appropriate biofluid from which to isolate EVs. Among the aforementioned biofluids, saliva holds significant promise. Compared to blood and CSF, saliva can be easily collected non-invasively in person or remotely. Yet, saliva is a complex, heterogenous biofluid consisting of cells, debris, proteins, enzymes, and mucins (*52, 53*) that can make EV isolation challenging. Here, we employ the CandyCollect (*54-56*), a novel lollipop-inspired, saliva sampling device consisting of open microfluidic channels, to directly capture salivary EVs for enumeration and cargo analysis. Previously used to capture respiratory pathogens in saliva (*54-56*), we demonstrate that the CandyCollect effectively concentrates salivary EVs onto its surface. We further demonstrate that we can release the EVs from the

CandyCollect by eluting them off of the device for subsequent enumeration and cargo analysis. For enumeration, we draw on a method we previously used to enumerate virus and liposomes (*57*): specifically, we tag the EV lipid membranes with oligonucleotides (oligos) and subsequently perform qPCR to amplify and quantify the oligo tag. For cargo analysis, we employ the ultrasensitive immunoassay, Simoa (*58*).

Results

CandyCollect design and working principle of EV capture and enumeration

The CandyCollect device is a lollipop-shaped polystyrene stick that has open microfluidic channels milled into the "lollipop" end (**Figure 1** and **Materials and Methods**). The lollipop end is treated with oxygen plasma, which has been shown to facilitate adhesion to pathogens in the mouth (*59, 60*). The back of the lollipop is coated with isomalt candy and the face of the device is left bare, leaving the plasma-treated microfluidic spiral open for saliva to flood the channel and capture EVs (**Figure 1A**). The thickness of the isomalt candy on the CandyCollect device serves as a built-in timer for sampling (*54*), encouraging participants to incubate the device in their mouth for a set time.

Following incubation in the mouth, the CandyCollect device is immediately submerged in Qiagen exoEasy Maxi Kit elution buffer (Cat# 76064) for 10 min to release the EVs bound to the open microfluidic channels of the CandyCollect device (**Figure 1B**). After performing a buffer exchange into phosphate buffered saline (PBS) solution (**Materials and Methods**), isolated EVs are labeled with oligos using our previously published method (*57*). Specifically, a 3' cholesterol-tagged single-stranded DNA oligo (universal anchor) that had been pre-hybridized to a qPCR detection oligo self-embeds into the EV lipid bilayer membrane. A 5' cholesterol-tagged universal co-anchor oligo is then introduced which subsequently self-embeds into the EV lipid bilayer membrane and hybridizes with the universal anchor, thereby stabilizing the complex (**Figure 1C** and **Materials and Methods**). Labeled EVs are next isolated with paramagnetic beads (Dynabeads, Cat# 65305) and functionalized with anti-CD63 antibody (Biolegend Cat #353017). CD63 was targeted, as it is a pan-EV marker (*50*). qPCR with primers specific for the detection sequence oligo was performed on prepared EV samples, thereby measuring the relative number of EVs (**Figure 1D**). See **Materials and Methods** for details.

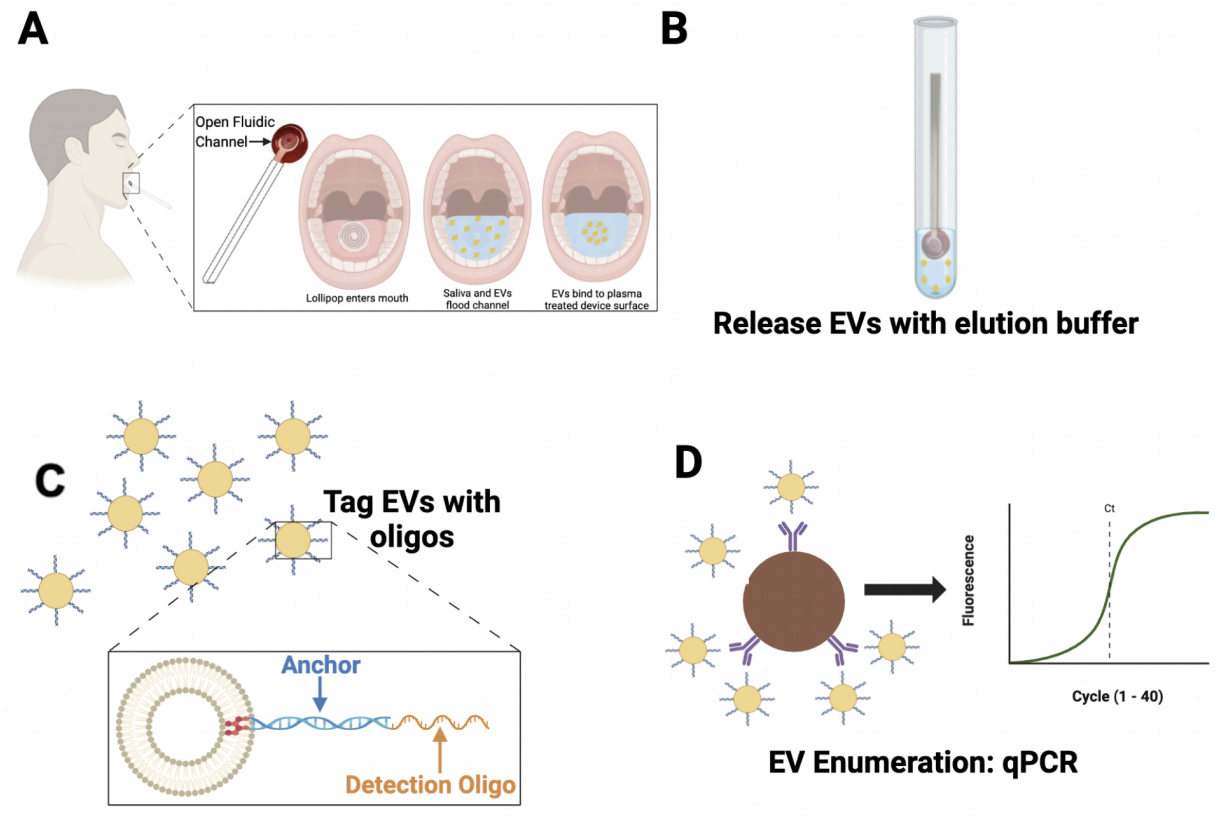

Figure 1. Workflow: Salivary EV isolation and analysis. A) CandyCollect device with a spiral open fluidic channel for EV capture and time-release isomalt/strawberry flavor (red) (*54-56*). B) EVs are released from CandyCollect using Qiagen exoEasy Maxi Kit elution buffer. C) EV lipid bilayer membrane is labeled with cholesterol-tagged oligos (*57*). D) Capture EVs with antibody-coated paramagnetic beads and wash away excess oligos. Subsequently, perform qPCR with primers for (detection) oligos, thereby measuring relative number of EVs (*57*).

CandyCollect device effectively captures EVs after 3-5 min incubation in the mouth

To determine the minimum incubation time needed to achieve saturation of EVs on the CandyCollect device, three healthy human participants were instructed to suck on a total of twelve candy-free CandyCollect devices each: three at 1 min, three at 3 min, three at 5 min, and three at 10 min (**Figure 2** and **Materials and Methods**). To mitigate the introduction of any extra variables, sample collection for each experiment was conducted in the morning, between the hours of 9am and 11am, and at least 30 min after eating or drinking. No breaks were taken between CandyCollects used. To minimize bias, each participant was asked to incubate the CandyCollect devices in their mouth in different orders of sampling time: Participant C completed sample collection in descending order of incubation time (starting with 10 min and finishing with 1 min), Participant B completed sample collection in ascending order of incubation time (starting with 1 min and finishing with 10 min), and Participant A completed sample collection in a random order (i.e., starting with 5 min, then 3 min, then 10 min, and

finally 1 min). EVs were immediately eluted off of the CandyCollect and analyzed using the qPCR-based method described above and in **Figure 1C** and **D**. To ensure we quantified only the oligonucleotide labels associated with EVs, we considered dC_T between each sample and a non-EV, PBS-based negative control processed in parallel.

Across all three participants, a one min CandyCollect incubation led to significantly less capture of EVs as compared to longer incubation times. Notably, CandyCollects that were incubated in the mouth for 3 min, 5 min, and 10 min all captured the same relative number of EVs, suggesting that a 3 to 5 min incubation is likely sufficient for EV saturation.

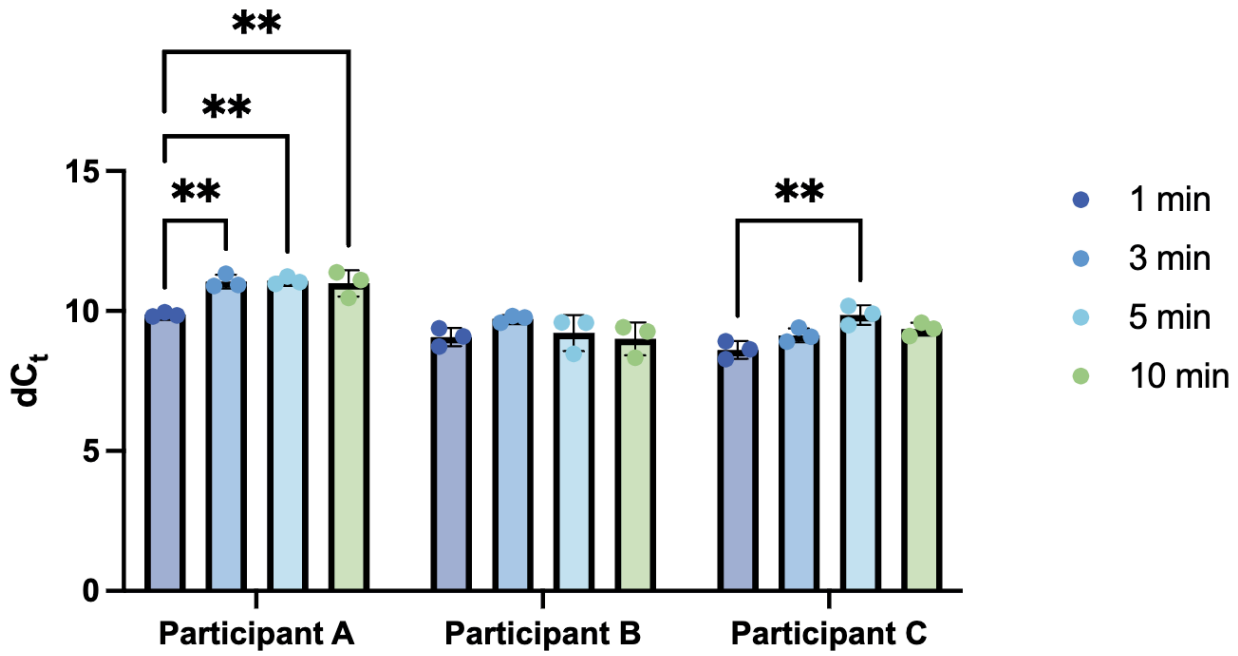

Figure 2. Incubation Time Optimization. The relative number of salivary EVs isolated from three healthy human participants using the CandyCollect platform. Each participant was asked to suck on a total of twelve CandyCollects without isomalt candy: three for 1 minute (dark blue), three for 3 minutes (medium blue), three for 5 minutes (light blue), and three for 10 minutes (green). EVs were then eluted off of each CandyCollect and enumerated using the oligo labeling and subsequent qPCR-based method described in **Fig. 1**. There was no significant difference between 3 and 5 min CandyCollect incubation in a given individual. The qPCR signal is presented as dCt, the difference in threshold cycle between each sample and a non-EV, PBS-based control. Error bars = standard deviation. **:$p<0.01$, Two-Way ANOVA followed by Tukey multiple comparisons test.

EV capture is robust among individuals

To investigate how robust EV capture with the CandyCollect is across individuals, seven healthy human participants were instructed to suck on three candy-free CandyCollect devices for 5 min each. EVs were immediately eluted off of each device and enumerated as described in **Figure 1C** and **D**. Each data point (colored dot) at the top of each bar in **Figure 3** is representative of one individual CandyCollect device from a given participant. While a significant difference in EV capture can be observed across participants as expected, there is less variation in EV capture between devices within a given participant.

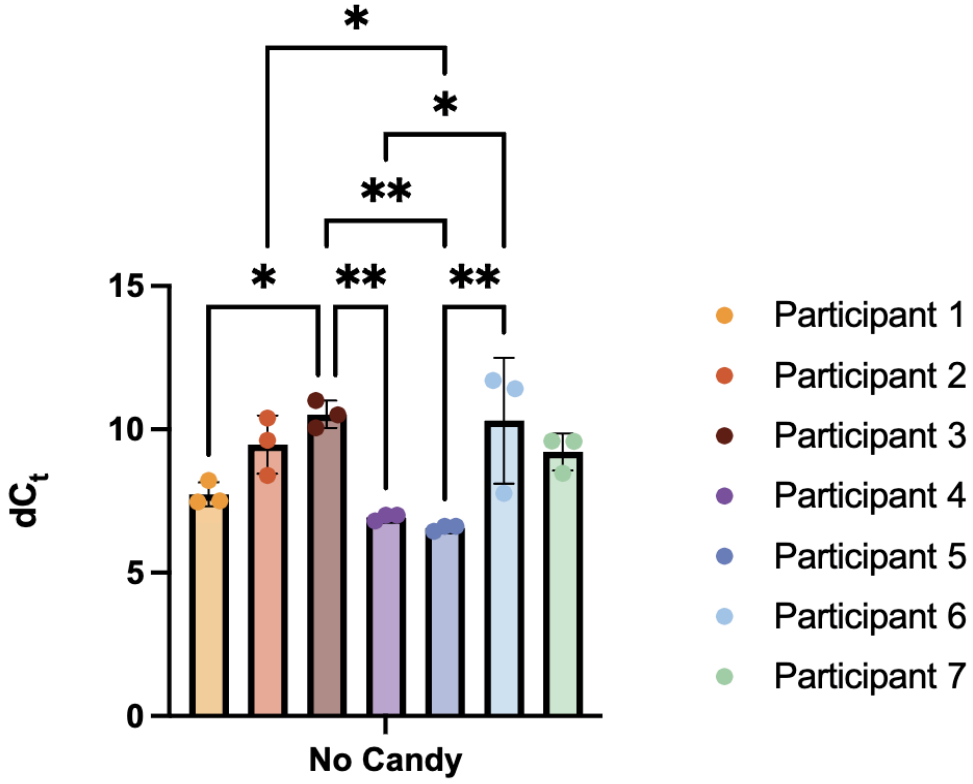

Figure 3. EVs captured from 7 healthy human participants. After a 5 min incubation in the mouth with 3 CandyCollects without candy, EVs were eluted off of each CandyCollect device and enumerated using the oligo labeling and subsequent qPCR-based method. The qPCR signal is presented as dCt, the difference in threshold cycle between each sample and a non-EV, PBS-based control. Error bars = standard deviation. *: $p<0.05$; **:$p<0.01$, Two-Way ANOVA followed by Tukey multiple comparisons test.

Isomalt candy does not interfere with EV capture or enumeration

To determine whether isomalt candy interferes with EV capture and subsequent enumeration, three participants were instructed to suck on six CandyCollects each, three that were candy-free and three that were coated with 0.5 g of isomalt candy (**Materials and Methods**). For the candy-coated CandyCollects, participants were instructed to remove the device from the mouth

after exactly 5 min, regardless of if all of the candy had completely dissolved or not. In most participants, 5 min was enough time to dissolve 0.5 g of candy. In some participants, 5 min was not sufficient to dissolve all of the candy. All devices including those with trace amounts of isomalt candy were processed identically according to our method as described in **Figure 1C** and **D.**

The data suggest that there is no significant difference in EV capture by devices with or without isomalt candy ($p = 0.7297$) and therefore the presence of candy should not inhibit or influence experimental findings when using the CandyCollect (**Figure 4**).

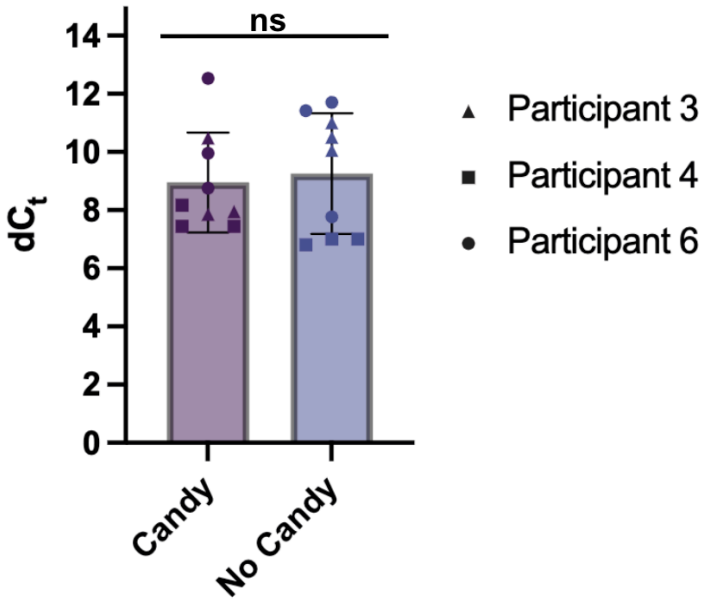

Figure 4. Comparison between candy-coated and bare CandyCollect devices. Three healthy human participants were asked to suck on three isomalt candy-coated and three candy-free CandyCollect devices, each for 5 min. EVs were eluted off of each CandyCollect device and enumerated using our oligo labeling and subsequent qPCR-based method. No significant differences in the number of EVs were observed in the face of the isomalt candy. The qPCR signal is presented as dCt, the difference in threshold cycle between each sample and a non-EV, PBS-based control. Error bars = standard deviation. ns = no significance ($p=0.7297$), two-tailed paired t-test.

Single molecule array (Simoa)

As a proof-of-concept for any future EV cargo analysis for disease detection, CandyCollect samples were analyzed by Simoa assays to investigate the presence of total Tau protein. Total Tau Simoa assays were first validated in CandyCollect samples pooled from two healthy individuals. The total Tau Simoa assays show a LOD of 0.043 ± 0.004 pg/mL ($N = 3$) and dilution linearity over 2 to 64 dilution factors ($R^2 = 0.99$). Spike-and-recovery values of 58% and

56% were achieved at an 8x dilution factor. 24.40 pg/mL and 53.63 pg/mL total Tau was detected in the pooled and individual sample, respectively. The Simoa analysis of total Tau in CandyCollect samples shows feasibility in detecting protein biomarkers in saliva samples collected by the CandyCollect devices. Future studies include determining the presence of protein biomarkers from CandyCollect samples as free protein, membrane-bound protein, or protein from EV cargo.

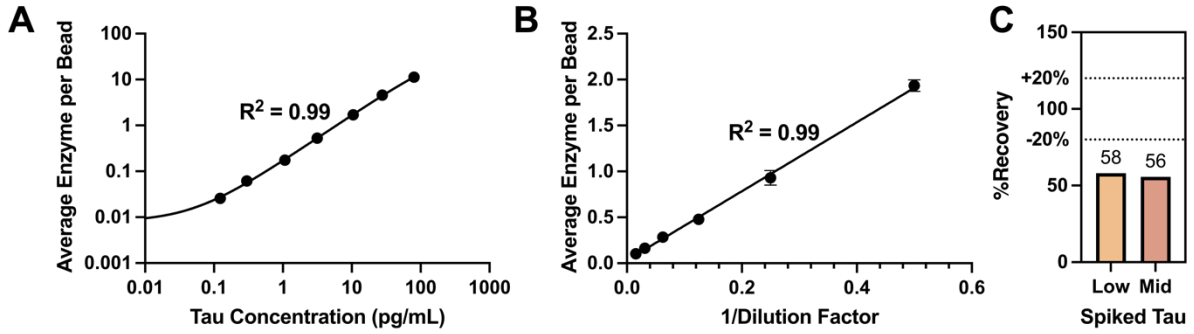

Figure 5. Single molecule array (Simoa) assay for total Tau in Candy Collect samples. *Data are the average of two replicates and the error bars represent standard deviation across duplicate measurements. A) Calibration curve of Tau Simoa assay (N = 3) with 4-PL fit. B) Dilution linearity of Candy Collect sample. C) Spike-and-recovery of total Tau Simoa assay in Candy Collect samples using 2 pg/mL and 21 pg/mL spike concentrations.*

Discussion

We have demonstrated a novel saliva sampling device—the CandyCollect—that can capture EVs for enumeration and cargo analysis. Specifically, we show that the CandyCollect can effectively capture and concentrate EVs in minutes. Furthermore, EVs can be eluted off of the device and labeled with cholesterol-tagged oligos whose detection sequence can be amplified by qPCR for EV enumeration. Finally, as proof-of-principle, we show that EV cargo analysis can be performed using Simoa. Overall, the CandyCollect has the potential to advance EV biomedical and clinical research.

Materials and Methods

Ethics Statement: Human subjects studies were conducted under an IRB-approved protocol (UC Berkeley Committee for Protection of Human Subjects, Protocol ID: 2024-07-17631), and all subjects provided written informed consent prior to taking part in the study.

Fabrication and Preparation of CandyCollect Devices

CandyCollect device milling

The DATRON neo, a computer numerical control (CNC) milling machine, was used to mill the CandyCollect devices from 2 mm (GoodFellow, Cat# 235-756-86) and 4 mm (Goodfellow,

CAT# 725-602-13) thick polystyrene sheets. After milling, the CandyCollect devices were sonicated with isopropanol (IPA) (FisherScientific, A451-4) and 70% v/v ethanol (FisherScientific, Decon™ Labs, 07-678-004)(*1, 2*). An engineering drawing of the CandyCollect design is provided in the Supplementary Materials and was previously published in Lee *et al*. (*54*).

CandyCollect device surface activation

CandyCollect devices were surface activated by plasma treating the devices with oxygen using a Zepto LC PC Plasma Treater (Diener Electronic GmbH Plasma Treater, EbHausen, Germany). The following protocol is consistent with previous publications) (*54, 55*). Briefly, atmospheric gas in the chamber was removed until 0.20 mbar of pressure was reached. The chamber was then filled with oxygen gas until a pressure of 0.25 mbar was achieved and subsequently exposed to 70 W of voltage for 5 min (*54, 55*).

CandyCollect devices with candy

Isomalt candy was added only to 4 mm-thick polystyrene CandyCollect devices. Lab members were trained in food safety and wore gloves and a mask to prepare and apply isomalt candy to CandyCollect devices. The isomalt candy was prepared as described in previous published papers (*54-56*). Briefly, heated isomalt was gradually added to water. The final addition of isomalt included food color. As the heated isomalt cooled, strawberry candy flavoring was folded into the mixture. The strawberry isomalt was poured onto a marble slab to set. During this time, the plasma-treated CandyCollect devices were cleaned using hot water and dish soap. Portions of the strawberry flavored isomalt candy were remelted. A silicone mold was used to apply 0.5 g of candy to CandyCollect sticks. After setting, CandyCollect devices with candy were moved to individual mylar bags (Belle KR, Cat# B0BZDCC8F8). Devices were stored in food-safe containers with a desiccant (Dry and Dry, Cat# B00DYKTS9C).

CandyCollect devices without candy

CandyCollect devices without candy were cleaned by washing with hot water and soap. After drying, they were stored in food-safe containers. Participants used both 2 mm- and 4 mm-thick polystyrene devices based on availability of materials, but this difference in device thickness does not affect the channel geometry or EV capture.

EV Capture with, and Elution from, CandyCollect Device

CandyCollect devices, with and without isomalt candy, were incubated in a healthy participant's mouth with the open microfluidic channel facing down on the tongue for 1, 3, 5, or 10 min. Following incubation, each CandyCollect device was immediately submerged in 400 uL of Qiagen exoEasy Maxi Kit (Cat# 76064) elution buffer for 10 min to release EVs bound to the open microfluidic channels of the CandyCollect device. The CandyCollect device was then

removed from the elution buffer, and the buffer itself was transferred to a 0.5mL 10 kDa Amicon Ultra Centrifugal Filter (Millipore, Cat# UFC501024) and centrifuged for 15 min at 14,000 rcf at 4°C. After disposing the flow through, 400 µL of sterile phosphate buffered saline (PBS) was added to the filtration unit in order to perform a buffer exchange, as the Qiagen elution buffer is not compatible with the downstream oligonucleotide labeling step (described below). Centrifugation was performed again at 14,000 rcf at 4°C for 15 min. The Amicon filter was next transferred to a new collection tube and inverted before centrifuging at 1,000 rcf for 2 min at 4°C. Approximately 62 µL of EVs in PBS was recovered from the filter.

Preparation and Functionalization of Colloids

1 µL of Dynabeads M-270 Streptavidin beads (Cat# 65305) per sample was pipetted into a nuclease-free microcentrifuge tube and washed with 200 µL of 0.2% bovine serum albumin (BSA) before being placed on a magnetic rack. After a pellet formed on the wall of the tube (due to the presence of a magnetic field), the buffer was carefully pipetted out before repeating two more 0.2% BSA washes. After the third and final wash, the beads were resuspended at a 1:10 ratio (10x the volume originally pipetted from the colloid stock bottle) in 0.2% BSA and biotinylated antibody (anti-CD63 antibody, Biolegend Cat #353017) was added at 10 µg per mL of diluted colloids and incubated on a hula rocker for 30 min. Following incubation, the colloids were placed back on the magnetic rack and the remaining solution was removed. Three more 0.2% BSA washes were performed before the colloids were again resuspended, this time with 3% BSA, and incubated on a hula rocker for > 30 min.

Cholesterol-Oligonucleotide Labeling of EVs and Capture of EVs on Colloids

Universal and co-anchor strands (*57, 61*) (Table S1) consisting of 48 and 20 nucleotides long, respectively, 3' and 5' cholesterol tags were purchased from IDT. The detection strand, also purchased from IDT, is based on the Arabidopsis thaliana FLG22-inducted receptor-like kinase 1 (FRK1) gene because of its lack of sequence homology with human, viral, and bacterial genes, preventing unintended amplification with human samples (*57*). The length of the detection strand—130 nt long—was chosen to maximize qPCR efficiency while also maintaining labeling efficiency. See Carey *et al*. (*57*) for additional information regarding oligo sequence design.

Cholesterol-universal anchor and detection oligo were prehybridized at a 1:1 ratio (oligos were resuspended in Molecular Biology-Grade H_2O at 100 uM to a final concentration of 50 µM) and incubated at 37°C for 15 minutes. This stock solution was further diluted 1:100 in nuclease-free H_2O to obtain a 500 nM working solution. 100 µM co-anchor was diluted 1:200 to obtain a 500 nM working solution.

10 µL of EVs in buffer out of the total 62 µL that was recovered from each Amicon filter (as described above) was pipetted to fresh microcentrifuge tubes. 1 µL of 500 nM anchor + detection oligo solution was added to each EV sample and incubated at room temperature for 15 min.

Following incubation, 1 µL of 500 nm co-anchor oligo solution was added to each EV sample. 10 µL of the prepared diluted functionalized colloid solution described above were added to each EV sample (each sample had a final volume of 22 µL) and incubated on hula rocker for 60 min. Following incubation, each sample was placed on a magnetic rack and the remaining solution was removed. Each pellet was resuspended in 200 µL of 0.2% BSA and transferred to a fresh microcentrifuge tube. 0.2% BSA wash was repeated four more times with each sample being transferred to a new microcentrifuge tube with every wash before the pellets were resuspended in 26 µL of nuclease-free water.

qPCR

500 nM primers were prepared by diluting each forward and reverse FRK1 primers 1:100 in nuclease-free water. Mastermix consisted of 10 µL of PowerUp SYBR Green Master Mix (Applied Biosystems Cat# A25776) and 2 µL of 500 nM forward/reverse primer mixture per reaction. To each tube containing 26 µL of sample, 39.4 µL of master mix was added and mixed thoroughly before pipetting 20 µL to each well on the qPCR plate. qPCR machine is set according to 'standard' (i.e. not 'fast') parameters in the PowerUp SYBR Green Master Mix handbook.

Single molecule array (Simoa)

Sample Collection for Simoa Analysis

CandyCollects were incubated in a healthy participant's mouth with the open microfluidic channel facing down on the tongue for 10 minutes. Following incubation, each CandyCollect device was immediately submerged in 400 uL of Qiagen exoEasy Maxi Kit (Cat# 76064) elution buffer for 10 min to release EVs bound to the open microfluidic channels of the CandyCollect device. The CandyCollect device was then removed from the elution buffer, and the buffer, itself, was transferred to a 0.5mL 10 kDa Amicon Ultra Centrifugal Filter (Millipore, Cat# UFC501024) and centrifuged for 15 min at 14,000 rcf at 4°C. After disposing the flow through, 400 µL of sterile phosphate buffered saline (PBS) was added to the filtration unit in order to perform a buffer exchange, as the Qiagen elution buffer is not compatible with the downstream oligonucleotide labeling step (described below). Centrifugation was performed again at 14,000 rcf at 4°C for 15 min. The Amicon filter was next transferred to a new collection tube and inverted before centrifuging at 1,000 rcf for 2 min at 4°C. Approximately 62 µL of EVs in PBS was recovered from the filter.

Simoa Assays for total Tau

Frozen Candy Collect samples (-80˚C) were thawed on ice. For lysis, Triton X-100 was added to the samples to make a final concentration of 1%, followed by incubation for 45 minutes on ice. Subsequently, the suspension was centrifuged at 3,000 g, 4˚C for 15 minutes to collect the supernatant. Validation of the total Tau Simoa assay in the samples was performed using the Neurology 3-Plex A Advantage Kit (Quanterix, USA) with an HD-X Analyzer (Quanterix, USA)

according to manufacturer protocols. All calibrators and samples were run in duplicates. The limit of detection (LOD) was calculated as the concentration corresponding to three standard deviations of the first calibrator added to the background signal. For dilution linearity, recombinant total Tau protein was spiked into the sample, followed by total Tau signal measurements in serially diluted samples.

Signal percent recoveries were examined in the samples with spiked Tau protein. Two concentrations were chosen at low and mid-range concentrations (2 pg/mL and 21 pg/mL). The percent recoveries were calculated by subtracting the measured signal of the spiked samples with the measured signal of the samples without the spiked protein, then multiplying by 100%.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 10.3.1 software. Data were presented as mean +/- standard deviation. Statistical tests used in this paper included a One-Way ANOVA with Tukey's multiple comparison (5-Minute Incubation between 7 participants), Two-Way ANOVA with Tukey's Multiple Comparison, and a two-tailed paired t test comparing CandyCollects with candy vs. CandyCollects without candy. Specific statistical tests and sample sizes can be found in the appropriate figure captions. $p < 0.05$ was considered statistically significant.

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Competing Interests: Ashleigh B. Theberge, Xiaojing Su, Erwin Berthier, and Sanitta Thongpang filed patent 63/152,103 (International Publication Number: WO 2022/178291 Al) through the University of Washington on the CandyCollect oral sampling device. Erwin Berthier and Ashleigh B. Theberge filed patent 63/683,571 through the University of Washington on a related platform. Ashleigh B. Theberge reports filing multiple patents through the University of Washington and receiving a gift to support research outside the submitted work from Ionis Pharmaceuticals. Erwin Berthier is an inventor on multiple patents filed by Tasso, Inc., the University of Washington, and the University of Wisconsin. Sanitta Thongpang has ownership in Salus Discovery, LLC, and Tasso, Inc. Erwin Berthier has ownership in Salus Discovery, LLC, and Tasso, Inc. and is employed by Tasso, Inc. However, this research is not related to these companies. Sanitta Thongpang, Erwin Berthier, and Ashleigh B. Theberge have ownership in Seabright, LLC, which will advance new tools for diagnostics and clinical research, potentially including the CandyCollect device. The terms of this arrangement have been reviewed and approved by the University of Washington in accordance with its policies governing outside work and financial conflicts of interest in research. Lydia L. Sohn has filed a patent 62/942,216

through the University of California, Berkeley on the DNA labeling of extracellular vesicles for enumeration. The other authors have no conflicts of interest to disclose.

Data and materials availability: Additional materials/information will be provided upon request.

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