Conditioning saliva for use in a microfluidic biosensor†

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This report details an approach to saliva conditioning for compatibility of raw patient samples with microfluidic immunoassay components, principally biosensor surfaces susceptible to fouling. Stimulated whole human saliva spiked with a small molecule analyte (phenytoin, 252 Da) was first depleted of cells, debris and high molecular weight glycoproteins (mucins) using membrane filtration. This process significantly reduced but did not eliminate fouling of biosensor surfaces exposed to the sample. An H-filter, which separates solutes from mixed samples based on their diffusion in laminar flow, was used to extract the analyte from the remaining large molecular weight species in the filtered saliva sample. Patient samples treated in this way retained 23% of the analyte with 97% and 92% reduction in glycoproteins and proteins, respectively, and resulted in 3.6 times less surface fouling than either untreated or filtered saliva alone. These sample conditioning steps will enable the use of fouling-sensitive detection techniques in future studies using clinical saliva samples.

Introduction

Chip-based pretreatment of patient samples is critical to the development of micro total analysis systems (μTAS) for use in biomedical diagnostics. Saliva is an attractive sample fluid for home patient monitoring as it is simple and painless to collect. Due to its high viscosity and glycoprotein content, whole saliva is incompatible with some microfluidic devices. Therefore, some form of sample pretreatment is necessary, but the pretreatment should be automated to the extent possible for home use. We report here on work done as part of a project aimed at developing an automated salivary diagnostic immunoassay system that consists of a miniaturized surface plasmon resonance imaging (SPRI) instrument and a disposable microfluidic immunoassay card. A two-step method for conditioning a stimulated whole saliva sample is shown to greatly reduce its viscosity and propensity to foul the biosensor surface, while retaining a concentration of a small molecule analyte that reliably reflects its original concentration.

We are focusing on therapeutic drug monitoring of antiepileptic drugs (AEDs) in saliva as a test case. The case has been made for why patients taking these drugs should be monitored: they have a therapeutic range with a readily discernible and temporally coupled pharmacodynamic end-point (*i.e.* seizure control); they show marked inter- and intra-individual dosage requirements; and seizure control is improved when the drug and active metabolites are measured and the data used appropriately.[1,2] Many AEDs, including the drug evaluated in this study, phenytoin, have previously been studied in saliva as their concentrations in saliva strongly correlate with plasma concentrations[2–4] and they are stable with storage in saliva samples.[5,6]

Saliva samples are notoriously difficult to process due primarily to the presence of particulate matter and mucins.[7] Mucins are responsible for the viscous and often stringy consistency of samples that makes pipetting and aliquoting laborious and inaccurate.[8] The challenge is to selectively remove these interferents while maintaining an analyte concentration that quantitatively correlates to its original concentration. Current approaches to salivary conditioning involve a freeze–thaw cycle followed by centrifugation[9,10] or specialized saliva collection (*e.g.* the Salivette[11–15]). Such methods must be performed in centralized laboratories by trained technicians and are therefore not amenable to rapid point-of-care testing.

The "H-filter" is a microfluidic device that separates low molecular weight compounds from larger proteins or particles by differential diffusion in laminar flow.[16] We previously demonstrated that the high mucin concentration in saliva, responsible for its non-Newtonian viscoelastic behavior, makes it incompatible with H-filter operation.[17] However, membrane filtration removes both large particles and sufficient mucin to enable the use of an H-filter for further sample processing.

In this report we demonstrate reduction of biosensor (SPR) surface fouling by patient saliva samples using a two-step filtration and H-filter extraction method (Fig. 1). Biochemical characterization of the sample matrix at various stages of the conditioning process is presented. Experimental results correlate well with the computational simulations based on levels of interferent removal and target analyte enrichment; this demonstrates the effectiveness of this sample conditioning process for use of human saliva and other challenging samples in μTAS applications.

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† In honour of Andreas Manz on his retirement as Chair of the *Lab on a Chip* Editorial Board.

Experimental

Saliva was collected (University of Washington Human Subjects Review Committee IRB# 32079) from human subjects not undergoing AED therapy. Subjects were instructed to refrain from eating and drinking anything other than water for the hour prior to collection. A small square of Parafilm was used to aid in the masticatory stimulation of saliva and subjects drooled into a clean tube (~ 2 ml). Whole human saliva (WHS) samples were collected between 9.30 and 10 am each day and briefly stored at 4 °C. Although contamination due to blood leakage in the oral cavity[19] was not a concern here, samples were still visually inspected for blood and discarded if discolored. Samples were pooled and vortexed for one minute. The WHS pool was spiked with the antiepileptic drug, phenytoin (Sigma, St. Louis, MO, USA), to a concentration of 20 μM (therapeutic phenytoin concentrations are 4–8 μM free and 40–80 μM total).[2]

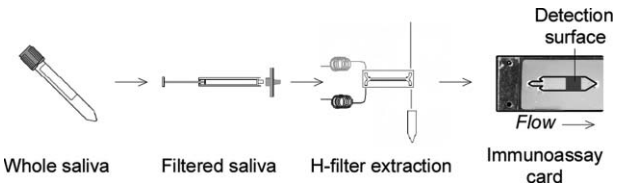

Fig. 1 Sample conditioning flow chart. The process begins with sample collection followed by off-card membrane filtration and on-card H-filter extraction. Surface fouling was measured with SPRI. These steps were performed separately such that aliquots could be taken and analyzed after each step. Device integration into a single card has been demonstrated in a separate publication.[18]

Biomolecular assays

Aliquots of the pooled whole saliva, filtrate and H-filter extraction were collected and assayed for interferent (protein and glycoprotein) and analyte (phenytoin) concentration. Total protein was measured using the bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Inc. Rockford, IL, USA) according to package guidelines. The periodic acid–Schiff (PAS) glycoprotein assay was performed as previously described[17] (reagents supplied by Sigma). Aliquots were also tested for free and total phenytoin concentrations by the University of Washington's Clinical Chemistry Reference Testing Service (RTS). The analytical measurement range is 10–160 μM for the measure of total phenytoin (turbidimetric assay) and 4–8 μM for the free measurement (enzyme multiplied immunoassay technique, EMIT). Although optimized for blood testing the assays and sample conditioning procedures were internally validated.

H-filter extraction

H-filter devices were fabricated as previously described.[17] The H-filter was contained in a custom manifold; inlet and outlet fluid lines were coupled *via* polyetheretherketone (PEEK) tubing (Upchurch Scientific, Oak Harbor, WA, USA) to computer-controlled syringe (250 μl) pumps (Kloehn, Las Vegas, NV, USA). The buffer and sample solutions were manually loaded into 1 ml sample loading tubes and pumped into the device at flow rates ranging from 500–5000 nl s^{-1}. These flow rates corresponded to Reynolds numbers ranging from 0.4 to 4.

SPRI experiments

Device substrates and surface patterning. The experimental method has been described in detail elsewhere.[20] Briefly, the sensing surface consisted of a glass slide with 1 nm/45 nm of Cr/Au patterned with a non-fouling Poly(ethylene glycol) (PEG) -terminated alkanethiol (Prochimia, Sopot, Poland) from the channel inlet to 22 mm downstream, then with a bovine serum albumin–phenytoin (BSA–PHN) conjugate (Fitzgerald Industries International, Inc., Concord, MA, USA, passively adsorbed from 10 mg ml^{-1} in phosphate buffered saline (PBS)), immediately adjacent to the end of the PEGylated region and for the remaining distance downstream. The microfluidic SPRI devices were assembled from poly(ethyleneterephthalate) (Mylar) or PMMA sheets (Fralock Inc., San Carlos, CA) laminated on top of the substrate. The device geometry consisted of three inlets leading to a single microchannel that directed flow across the sensing surface to the outlet. The common channel dimensions were ~62 μm in depth, ~3.6 mm in width, and ~60 mm in length.

Immunoassay procedure. The device was pre-filled with PBS and the assay was conducted by injecting specified samples into one of the three device inlets at 250 nl s^{-1} for a total volumetric flow rate in the common channel of 750 nl s^{-1} (unless otherwise indicated). Image data were collected using a custom-built portable SPRI instrument described in detail elsewhere.[21] The image integration time was 25 ms and 30-frame coadding was used. The incident angle of the LED source was adjusted prior to each experiment to set the reflectivity value near the bottom of the linear range of the SPRI curve (approximately 30% minimum reflectivity). An image collected at the start of the experiment was subtracted from subsequent images to highlight changes in reflectivity occurring during the course of the assay. Two regions of interest (ROIs) were designated for each sample flow stream, one in the non-fouling PEG region and one on the BSA–phenytoin detection surface (Fig. 2). The averaged intensities for each of the 50 × 50 pixel regions were calculated and plotted *versus* time. The intensities of the (non-fouling) PEG regions were used to control for bulk refractive index changes, relying on the PEG layer's ability to resist protein attachment.

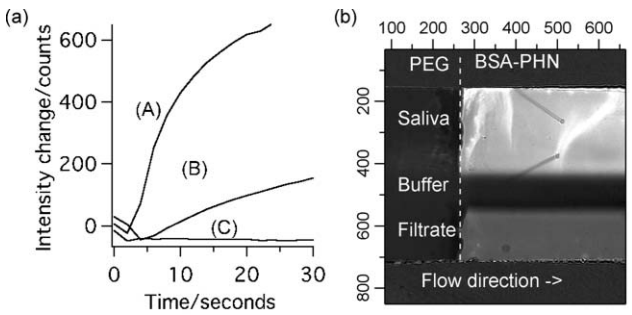

Fig. 2 (a) A plot of the reflectivity *versus* time for each sample stream: (A) unprocessed saliva, (B) filtered saliva and (C) buffer. (b) SPRI difference image of biosensor surface fouling. Flow is from left to right in a common channel. Bright areas indicate fouling to the patterned detection surface (antigens conjugated to BSA). In this case, the initial rate of intensity change for the WHS was 66 times the buffer control sample and the filtered saliva was 5.5 times the buffer control.

Ultrafiltration of saliva samples. The protein concentrators Vivaspin 2 and Vivaspin 6 (Sartorius, Goettingen, Germany) were used to fractionate filtered saliva samples. The molecular weights studied included 30, 50 and 100 kDa. The devices were pre-rinsed with deionized water (4× processed volume) to remove residual preservatives that had been shown to increase the bulk refractive index compared to buffer. Pooled filtrate was divided into 4 aliquots (untreated filtrate, 30, 50 and 100 kDa molecular weight cutoff (MWCO) Vivaspins). Samples were loaded into Vivaspin tubes and centrifuged (~3700*g*, 15 min, 20 °C). All four samples were assayed for: total protein (BCA), the size distribution of proteins and glycoproteins (SDS-PAGE), surface fouling (SPRI) and phenytoin concentration (RTS).

SDS-PAGE of saliva samples. Whole human saliva (WHS), filtrate and H-filter extracts were denatured in a 5× gel-loading buffer (62.5 mM Tris·HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% Bromophenol Blue, 5% 2-mercaptoethanol) by incubation at 95 °C for 5 min, then loaded into a 4–20% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was carried out at 130 V for 65 min in MES running buffer (Invitrogen). After electrophoresis, the gel was fixed, washed, oxidized, washed, and stained according to the Pro-Q Emerald Staining package instructions (Invitrogen). A Polaroid (667 film) picture was taken and then the gel was stained for proteins using the Rapid Ruby staining protocol (Invitrogen) and a second Polaroid picture taken. The grayscale pictures were scanned to create digital images of the stained gels.

Results and discussion

Preliminary surface fouling experiments

We have used SPRI as an example of a surface-sensitive detection technique, but the lessons learned here could apply to other surface-sensitive analytical methods. Fig. 2 illustrates an SPRI experiment in which a biosensor surface was exposed to an untreated WHS sample, membrane filtrate, and PBS. Average intensity values were calculated over specified regions of interest (ROIs) and used as a metric for evaluating surface fouling (slope of the reflectivity *versus* time) in the patterned region of a sample. The rate of accumulation is directly related to the concentration of interferents present in the sample matrix. The rate of accumulation plot (Fig. 2(a)) showed that the WHS exhibited very high levels of non-specific binding, indicating a need for a sample conditioning strategy for WHS samples prior to use with this biosensor system.

Sample conditioning step 1: membrane filtration

Membrane filters were studied as a method for removing interferents and reducing viscosity. We found that a 0.2 μm polyethersulfone (PES) with GD/X (a prefiltration stack of GMF 150 and GF/F glass microfiber media filter, Whatman, New Jersey, USA) removed large debris and a majority of the glycoproteins, resulting in a less viscous sample.[22] The membrane retained a minimal amount of the analyte and was capable of processing large (>1 ml) volumes of samples prior to the onset of clogging. Although filtering the sample significantly decreased surface fouling as compared to WHS (Fig. 2(a)), additional reduction was necessary.

Analysis by the BCA assay indicated that filtered samples still contained greater than 50% of the total protein found in WHS. Therefore, the sample was partitioned by molecular weight using commercial ultrafiltration devices of varying molecular weight cutoff values (30, 50 and 100 kDa). The BCA assay was used to measure total protein in each of the three ultrafiltrates and normalized to the total protein in the filtrate (sample loaded into Vivaspin).

The protein recovery as a percent of total protein measured in the filtrate, for the 30, 50 and 100 kDa MWCO devices were 26%, 27% and 75%, respectively. As expected, the total protein decreased with decreasing MWCO, although there was not a large difference between the 30 and 50 kDa separators. Next, the ultrafiltrates were evaluated using SPRI to determine the levels of non-specific surface fouling.[17] In both the 30 kDa and 50 kDa MWCO ultrafiltrate experiments there was little to no fouling (data not presented). However, the rate of surface fouling measured for the 100 kDa MWCO ultrafiltrate was significant (~14 times PEG control ROI). Based on these results, it was determined that the MWCO should be set somewhere between 50 and 100 kDa.

To further examine the molecular weight distribution of possible interferents, the ultrafiltrates were evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting electrophoretic patterns are presented in Fig. 3. Though one-dimensional gel electrophoresis of saliva has been extensively practiced,[23–25] this work compares saliva composition through sequential conditioning steps to characterize the interferent removal (both glycoprotein and protein) on a single gel.

The electrophoretic behavior of the MG1 mucins (Band 1) in saliva samples was typical, in that they appeared in the wells and could not enter the gel because of their large size.[26] The presence of the lower molecular weight mucins (Band 2, MG2, ~200 kDa) was detected with the Pro-Q Emerald, but they were not apparent after staining for protein (also reported by Habte *et al.*).[26] The gel showed MG2 was in both filtrate samples as well as 100 kDa MWCO Vivaspin ultrafiltrate and that the protein and glycoprotein components in the 100 kDa ultrafiltrate did not differ drastically from the filtered saliva sample. There was little if any indication of glycoprotein, or protein in the 30 and 50 kDa MWCO ultrafiltrate samples.

The presence of MG2 in the 100 kDa ultrafiltrate was somewhat surprising considering their large size (2× the MWCO). However, the linear polymer-like structure of the mucins (and their unusually high charge) could have allowed for their passing through the membrane at high hydrostatic pressures. Due to the presence of MG2 mucins in the 100 kDa ultrafiltrate and filtrate samples, it stood to reason that the mucins contributed to the fouling of the SPRI surface. As reported elsewhere,[27,28] the hydrophobic regions of mucins are responsible for complex formations with non-mucinous saliva components as well as for their sticky character.[26]

Sample conditioning step 2: H-filter extraction

While the filtration step successfully reduced the effective viscosity of the sample and removed large particulates from the WHS sample, SPRI results (Fig. 2) indicated that the filtrate still

exhibited non-specific surface adsorption when compared to the clean, protein-free buffer stream. The need for further sample pretreatment upstream of the SPRI surface was the impetus for H-filter extractions. Extractions were performed using PHY-spiked filtered saliva using the flow conditions determined from computational fluid dynamics modeling.[22] Filtration alone removed 43% (± 6%) total protein and 73% (± 12%) of total glycoprotein. Reference testing indicated 22% loss of analyte (± 3%). Additional processing *via* H-filter extraction further reduced the glycoprotein and protein concentrations to 3% and 8% (± 2%) compared with the WHS equivalent to previous results.[18] The phenytoin concentration was approximately 23% (± 3%) that measured in WHS and 30% the filtrate concentration. The results are summarized in Table 1. A more thorough description of the combined uncertainty in these measurements is described in detail elsewhere.[22] The recovery was lower than predicted by the 1D and 2D models (31.4% and 37.8% respectively).[22] The discrepancy may be attributed to the estimated value used for the diffusion coefficient (8.0×10^{-6} cm^2 s^{-1})[22,29] in the model as well as imperfect extractions arising from the use of filtered saliva as the sample input.

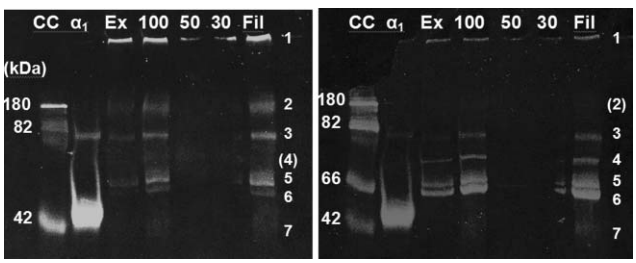

Fig. 3 SDS-PAGE of conditioned saliva. From left to right, the lanes contain: CandyCane™ Glycoprotein Molecular Weight Standard (CC), α_1-Acid glycoprotein control (α_1), H-filter extraction (Ex), Vivaspin ultrafiltrates (100, 50, 30) and saliva filtrate (Fil). The gel was first stained for glycoproteins (Emerald Pro-Q stain, left image) and then stained for all proteins (Ruby SYPRO®, right image). In addition to the unstained CandyCane marker, a pre-stained ladder was also used to aid in identifying bands (visible in a brighter, lower contrast photograph). At the very top of the gel, band 1 was the high molecular weight mucin (MG1). Band 2 was the lower molecular weight mucin, MG2. The secretory component of sIgA (band 3), serum albumin (band 4) and two isoforms of amylase (bands 5 and 6) were also noted. Band 7 is thought to be zinc-α2-glycoprotein.

SPRI of H-filter extracted saliva samples

A comparison of the SPR signal from antibody binding (needed for conducting a competitive immunoassay) to the signal from surface fouling (caused by the remaining interfering components present in the filtrate and H-filter extracted samples) is made in Fig. 4. Note that the filtrate was diluted by 70% so that it contained an analyte concentration equivalent to that in the H-filter extract. This was done to compare the effectiveness of the two fouling-reduction methods.

The intensity change that occurs in the H-filter extraction stream (Fig. 4(b), center stream), is in stark contrast to the diluted filtrate stream (bottom stream). Following a high initial binding rate (Fig. 4(a), 17–20 s) extract alone stabilizes, while the filtrate continues to non-specifically foul the surface. In this demonstration, the specific binding of the antibody (signal) gradually increases and continues to increase at a rate 3.6× that of extract. Further study is required to determine the relative adsorption rates of antibody and residual extract fouling in the competitive conditions (*e.g.* a mixture). The SPRI data indicated that the rate of non-specific accumulation in the ROI (BSA–PHN) for an extracted sample was much lower than that of the dilute filtrate. These experiments indicate that performing an H-filter extraction was more effective than diluting the filtrate sample to equivalent analyte concentration. In fact, filtrate samples diluted up to 10-fold still exhibited more surface fouling than samples conditioned by filtration and H-filter extraction.

Table 1 Assay results for WHS samples collected from several donors and conditioned using the 0.2 μm filter and flat H-filter extraction. Glycoprotein was measured using the PAS assay, protein was measured using the BCA assay ($n = 5$). The phenytoin recovery after each conditioning step was measured by the UW Clinical Chemistry Reference Testing Service. Values reported are percentages of the mucin, protein and analyte measured in the pooled WHS sample

	Mucin/ glycoprotein(%)	Protein(%)	Analyte (phenytoin)(%)
Filtrate	26.5	57.2	77.7
Extract	3.0	7.6	22.6

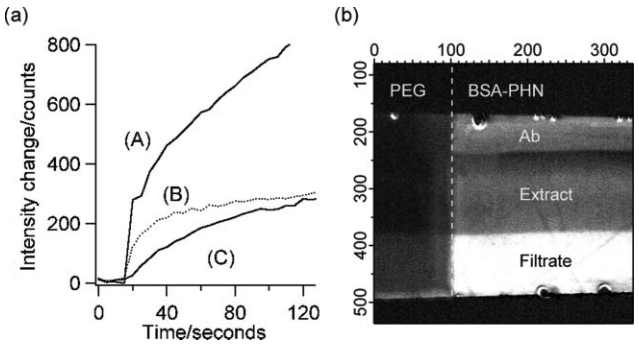

Fig. 4 (a) Rate data comparing specific and non-specific binding to the detection surface for three streams containing: (A) diluted filtrate, (B) H-filter extract and (C) PBS with 25 nM anti-phenytoin mAb, total volumetric flow rate was 300 nL s^{-1}; 8 min elapsed time. (b) SPRI difference image. Increasing intensity was due to specific binding (PBS + Ab) or fouling (filtrate and extract) and is presented to compare relative signals contributed by both processes.

Conclusions

A sample conditioning strategy for the extraction of a small molecule from saliva for use in a surface-binding-based biosensor has been described. Untreated saliva cannot be used in these biosensors because of excessive fouling of the detection surface, putatively by proteins and glycoproteins. The SDS-PAGE data in conjunction with the SPRI fouling experiments suggest that the presence of mucins (MG2), known for their adhesiveness and possibly some larger proteins (*e.g.* secretory component of IgA), were responsible for the fouling of the SPRI surface. The conditioning protocol removed 97% of mucins and 92% of total proteins while retaining 23% of the antiepileptic drug, phenytoin. Ultimately, a 23% recovery of the target analyte compared with 8% salivary protein for the entire

conditioning protocol translated to a three-fold enrichment of the analyte compared to direct dilution of the filtrate sample. The two-step sample preconditioning method was successful at removing interfering species and compared to current other methods requiring centrifugation, is conducive to integration and miniaturization on a card driven by positive displacement pumps. In addition, the protocol is compatible with continuous flow conditions, thereby simplifying on-card controls.

Acknowledgements

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