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Talanta 67 (2005) 617–624
Talanta
www.elsevier.com/locate/talanta

Optimization of a portable microanalytical system to reduce electrode fouling from proteins associated with biomonitoring of lead (Pb) in saliva

Wassana Yantasee, Charles Timchalk, Karl K. Weitz, Dean A. Moore, Yuehe Lin *

Pacific Northwest National Laboratory, 902 Battelle Blvd, P.O. Box 999, Richland, WA 99352, USA

Received 14 February 2005; received in revised form 14 March 2005; accepted 14 March 2005
Available online 6 June 2005

Abstract

There is a need to develop reliable portable analytical systems for on-site and real-time biomonitoring of lead (Pb) from both occupational and environmental exposures. Saliva is an appealing matrix since it is easily obtainable, and therefore a potential substitute for blood due to existing reasonably good correlation between Pb levels in blood and saliva. The microanalytical system is based on flow-injection/stripping voltammetry with a wall-jet (flow-onto) microelectrochemical cell. Samples that contain as little as 1% saliva can cause electrode fouling, resulting in significantly reduced responsiveness and irreproducible quantitations. In addition, incomplete Pb release from salivary protein can also yield a lower Pb response than expected. This paper evaluates the extent of in vitro Pb-protein binding and the optimal pretreatment for releasing Pb from the saliva samples. Even in 50% by volume of rat saliva, the electrode fouling was not observed, due to the appropriate sample pretreatment and the constant flow of the sample and acidic carrier that prevented passivation by the protein. The system offered a linear response over a low Pb range of 1–10 ppb, low detection limit of 1 ppb, excellent reproducibility, and reliability. It also yielded the same Pb concentrations in unknown samples as did the ICP-MS. These encouraging results suggest that the microanalytical system represents an important analytical advancement for real-time non-invasive biomonitoring of Pb.

Keywords: Lead; Electrochemical sensor; Flow injection; Saliva; Protein; Stripping voltammetry; Electrode fouling; Passivation

1. Introduction

Biomonitoring enables quantitative evaluation of human exposures to both occupational and environmental hazards, including heavy metals [1,2]. Of all heavy metals, lead (Pb) is one of the most problematic. The conventional approach for biomonitoring of Pb is accomplished by making a blood Pb determination since blood is the accepted biological matrix for assessing Pb exposure and the basis for the current biological exposure index (BEI) for Pb [3]. However, collecting blood samples is highly invasive and often not suitable for newborns and infants. Non-invasive samples like saliva are easily to be collected, making them an appealing matrix as blood substitute. Our research group [3,4] and others [5,6] have reported a good correlation between the saliva and blood Pb levels in rats which suggests the feasibility of utilizing a "spot" saliva sample to predict internal dose as proposed in our previous work [3,4].

The analytical techniques for determining Pb concentration in biological samples include ion chromatography, atomic absorption spectroscopy (AAS), or inductively coupled plasma mass spectrometry (ICP-MS). These analyses are generally done at centralized laboratories, resulting in lengthy turn around time. They are also expensive and require significant labor and analytical resources [7]. Thus there is a growing need to develop reliable, portable, and cost-effective analytical instruments for on-site, real-time monitoring of trace metals in individuals. Electrochemical sensors based on stripping voltammetry appear to be a promising technique

* Corresponding author.
E-mail address: yuehe.lin@pnl.gov (Y. Lin).

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doi:10.1016/j.talanta.2005.03.039

for determining aqueous heavy metal concentrations because they usually are sensitive, compact, low cost, easily integrated into field-deployable units, and can be automated [8–10].

There are two major obstacles that prevent the wide applications of electrochemical sensors for analyzing chemical species in biological samples. The first one is the lack of understanding of the binding between the target chemicals and the surface-active species in the biological samples [11]. For instance, since Pb is suggested to be extensively bound to high molecular weight molecules such as proteins [7], the low electrochemical response to known concentrations of Pb in rat saliva may be caused by the incomplete sample digestion and release of Pb from saliva protein [4,11]. Thus, understanding of the saliva composition, the binding of Pb by the saliva protein, and release of Pb via acid treatment of the samples will greatly improve the Pb quantitation by electrochemical sensors.

The second obstacle is related to significant signal reduction, caused by the electrode fouling [11–16]. Biological samples contain surface-active species (i.e., protein) that can hamper electrochemical sensing by causing interference and electrode passivation [11]. Adsorption of the protein at the solid–liquid interface is widely known even at very low saliva content in the samples [17–19]. The Compton's group [14,16] has found that when all other conditions remain constant, additions of artificial saliva (0.1–0.9% by volume) in the sample causes a significantly reduced Pb signals. The authors attributed the signal reduction to the increasing passivation (covering layer) of the electrode surface by the surface-active glycoproteins in the saliva. Electrode fouling normally causes the fluctuation in baseline current and signal depression, leading to inaccurate quantitation of the target species. Electrode fouling also requires frequent surface cleanings, which are labor- and time-consuming.

In response to the need for on-site biomonitoring of heavy metals, especially Pb, using non-invasively collected saliva, a microanalytical system for the assay of Pb in saliva has been developed in our lab. This microanalytical system is compact and portable, requires small sample volumes (in microliters), and offer rapid analysis. Having realized that getting biological samples in the form that can take full advantage of the instrument's capability is the most challenging task of the analysis [11], this manuscript focuses on further characterizing the dynamics of saliva protein and Pb interactions (binding and release) such that samples can be prepared in a way that minimizes the fouling and optimize the electrochemical analysis. Rats were initially used as the saliva donors instead of humans, since the results in rats will facilitate the initial validation of the technology, which involves exposing animals to known concentrations of Pb. Future studies will extend this evaluation to human saliva. The electrochemical parameters were optimized and the figures of merits (detection limits, reproducibility, and linear calibration range) were determined. The results were compared with those from the ICP-MS to demonstrate that the system can accurately quantify Pb in saliva. It is anticipated that this new technique and device will potentially have broad applications for assessing both occupational and environmental Pb exposures.

2. Experimental

2.1. Microanalytical system

The detailed fabrication of the microelectrochemical cell has been published elsewhere [7,10]. A schematic diagram of the unit is presented in Fig. 1(A). The microanalytical system in Fig. 1(B) consists of a computer-controlled micropump (MilliGAT pump, Global FIA, Fox Island, WA), an injection valve with a 50 μL sample loop, interconnecting PTFE tubing, and a microelectrochemical cell. The microelectrochemical cell integrates three electrodes and is based on a wall-jet (flow-onto) design [20,21]. A laser-cut Teflon gasket was sandwiched between two PEEK blocks to form a radial flow-cell, and 1-mm diameter strips of platinum (Pt) and silver (Ag/AgCl) were used as the auxiliary and reference electrodes, respectively. The solution flowed onto the Hg-film working electrode (glassy carbon) by a computer-controlled micropump and exited through the groove from the outlet that was positioned directly across the Pt auxiliary electrode. The three electrodes were connected to a hand-held bipotentiostat (model 1232, CH Instrument Inc., TX), which

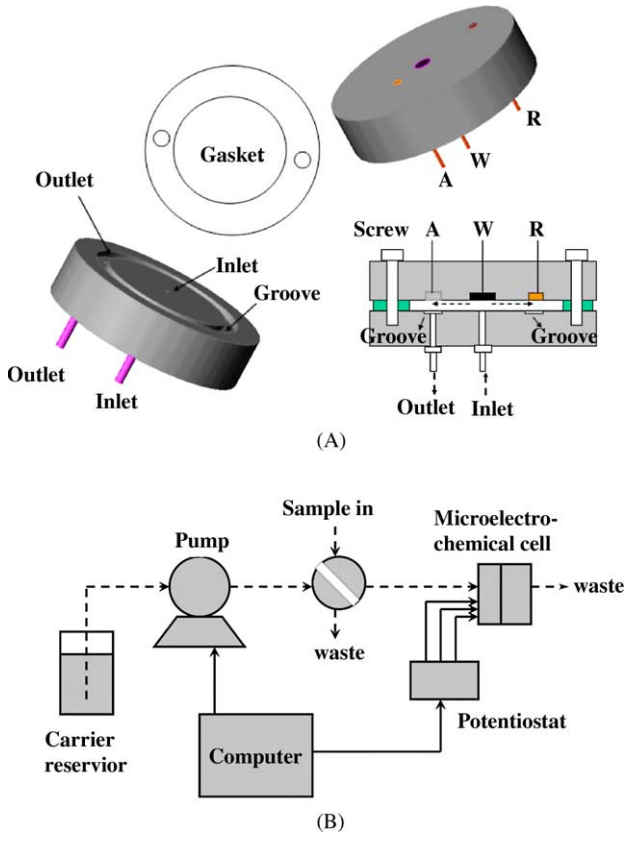

Fig. 1. Schematic of (A) the microelectrochemical cell having 1.8 cm × 2.4 cm of total size and (B) the microanalytical system, dash lines represents liquid lines and solid lines represent cable lines.

was also connected to a computer for instrumental control and data recording.

2.2. Collection of rat saliva

Saliva specimens were collected from naïve male Sprague Dawley rats (~10 animals, each weighed from 0.33 to 0.37 kg) as previously described [4]. In brief, the rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (87 mg/kg):xylazine (13 mg/kg). In addition, each rat was given an i.p. injection of the cholinergic agonist pilocarpine (1 mg/kg) to induce salivation. Saliva was then collected from each rat, using a glass capillary tube, for approximately 30 min (1–2 mL saliva). Additional saliva was collected from the same rats after allowing them 1–2 days of recovery. For the in vitro Pb binding analysis the saliva specimens were not pooled; however, for the acid treatment studies and Pb measurements by the microanalytical system the saliva specimens were pooled. All saliva was stored frozen (−80 °C) until the Pb analysis was conducted.

2.3. In vitro Pb binding analysis

The extent of in vitro Pb saliva protein binding was determined using saliva obtained from naïve male Sprague-Dawley rats. Saliva was collected utilizing ultra-clean methods as previously described above. The saliva specimens were spiked with Pb-acetate to achieve 50 ppb Pb(II). The Pb-acetate was incubated in saliva at a ratio of 1:36 (Pb-acetate:sample) by volume in order to minimize sample dilution. The binding assay was carried out in prewashed (10% (v/v) HNO_3/HCl) glass tubes rinsed in ultra-pure water to remove potential Pb contamination. The assay was conducted at 37 °C in a shaking water bath. Initial studies were likewise conducted to determine an optimum incubation time to insure that steady state has been obtained. Based on the results of Kaplan et al. [22], it was anticipated that <1 h was required to achieve optimum binding. Next, aliquots of spiked saliva were subjected to ultrafiltration as previously described [23]. In brief, the saliva ultrafiltration was done utilizing the MPS-1 micropartitioning system (Amicon Corp., Danver, MA) at 37 °C and the ultrafiltrate was weighed and analyzed for Pb by ICP-MS. The extent saliva protein binding was determined by comparing the ratio of Pb concentrations in the saliva with the respective filtrates.

Samples of saliva that had been incubated with Pb-acetate in vitro were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Saliva was acid digested prior to ICP-MS analysis as follows. The specimens were carefully weighed into a tarred advanced composite vessel (ACV), and 1 mL each of ultrapure Millipore water (18.2 MΩ-cm) and concentrated HNO_3. The Teflon ACV vessels were capped and sealed into numbered stainless steel Parr Bomb sleeves, then transferred to a preheated oven and maintained at 140 °C for a minimum of 3 h. Upon removing and cooling of the samples, each vessel was opened and rinsed with ~2.0 mL of ultrapure water; the procedure was repeated three times. The contents of the vessel and rinses were transferred to a polypropylene centrifuge tube and the solution volume was brought up to 14.0 mL with ultrapure water. All glassware was prewashed in a leaching solution (10% (v/v) HNO_3/HCl) to remove potential Pb contamination.

A VG Plasma Quad, model PQ2 ICP-MS (VG Instruments Inc., Cherry Hill Drive, MA) was used for Pb analysis with a scanning mode acquisition: the parameters were set at 10.24 dwell (millisecond), 19 channels/amu, PC Detector mode, 0.5 time/sweep (sec), and selected mass range of 99.6–210.4. Mass 208 was selected for the Pb analysis. Most samples were diluted to few ppb level of Pb (up to 100-fold dilution) to minimize matrix effect with 2% (v/v) HNO_3, which also was used as rinse and background solutions. All sample and standard solutions contained 2.5 ppb of Thallium (Tl-205), 2 ppb of Iridium (Ir-193), and/or 2 ppb Rhodium (Rh-103) as internal standards. Three to five Pb standard solutions were analyzed along with the samples to establish a calibration curve that covered the concentration range of Pb(II) in the samples (e.g., from 0.1 to 5 ppb, $R^2 = 0.999$). The analysis time per mass was 3 s, and a total of three replicates per sample were run.

2.4. Acid treatment study

One milliliter of rat saliva was spiked with a given volume (50–60 μL) of 2000 ppb Pb(II) solution, prepared by diluting atomic absorption standard solution from Aldrich Co. (comprised of 1000 mg/L Pb(II) in a 1–2% HNO_3 solution) with ultrapure Millipore water. The mixture was then incubated for 10 min in a 30 °C water bath. Next, the mixture was spiked with a given volume (7–157 μL) of concentrated HNO_3 or concentrated HCl (trace element grade, Sigma-Aldrich). At this point, the saliva fraction was approximately 90% (by volume) of the total solutions, the as-prepared concentration of Pb was 100 ppb and the acid concentrations were 0.1, 0.5, 1.0, or 2.0 M. The mixture was centrifuged at room temperature at the relative centrifugal force (RCF) of 15,200 × g for 15 min. The supernatant was removed and stored frozen (−80 °C) until the Pb analysis by ICP-MS was conducted using the procedure mentioned in the in vitro Pb binding analysis section, but without further acid digestion. Prior to the ICP-MS analysis, the samples were diluted 100-fold with ultrapure water resulting in samples with acid concentrations ranging from 0.001 to 0.02 M. The effect of the matrix (from 0.001 to 0.02 M HNO_3) on Pb signals by ICP-MS was measured and found to be negligible.

2.5. Pb analysis by microanalytical system

To quantify trace amounts of Pb in rat saliva samples, the samples were prepared in the similar fashion as in the acid treatment study, but with an optimal acid condition (1.0 M HCl) and varied spiked Pb concentrations. The supernatants were diluted two-fold with 10 ppm Hg (II) in ultrapure water. The carrier was prepared to have the same matrix (5 ppm

Table 1
Typical operating parameters

Differential pulse voltammetry parameters	
Initial potential	−0.8 V
Final potential	−0.45 V
Amplitude	0.05 V
Pulse width	0.05 s
Pulse period	0.2 s
Microanalytical system parameters	
Flow rate	1 μL/s
Carrier	5 ppm Hg(II)/0.5 M HCl
Sample	Pb (II) in 50% saliva (in same matrix with carrier)
Deposition	90 s of carrier at −1.0 V, followed by 45 s of sample at −1.0 V
Cleaning	60 s at +0.7 V in 1 μL/s of carrier

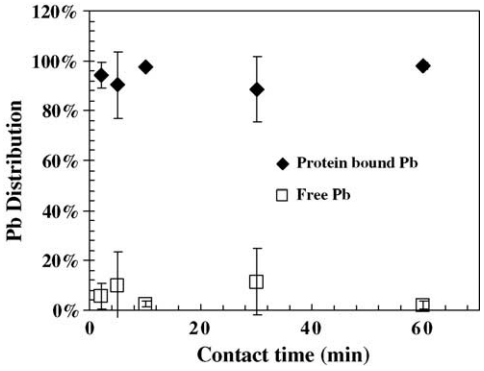

Fig. 2. The liquid- and solid-phase distributions of 50 ppb of lead in rat saliva (pH 8.5) as a function of contact time.

Hg(II)/0.5 M HCl) with the feed samples. The carrier was stored in a reservoir and the sample was injected into the system at a sample inlet port prior to each analysis. Both carrier and sample were delivered into the microanalytical system by a computer-controlled micropump. Differential pulse anodic stripping voltammetry (DPASV) was used for quantitation of Pb in the specimens. The typical operating parameters of DPASV and the microelectroanalytical cell are summarized in Table 1.

Prior to the analysis, the working electrode was regenerated daily by polishing with 35% (v/v) HNO_3, followed by water, acetone, and water, respectively. Next, the film of Hg was first deposited on the electrode surface at −1.0 V for 10 min in 1 μL/s of carrier on the first run and for only 90 s for the subsequent runs. Then the Pb- and Hg-containing saliva sample was flowed over the electrode surface for 45 s, while Pb(II) and Hg(II) were simultaneously reduced and deposited. After the deposition period, the flow was stopped, and the potential at −0.8 V was applied for 10 s quiet period, followed by scanning the potential of the working electrode from −0.8 V to −0.45 V also under the no-flow condition. For the electrode cleaning, the carrier was pumped in at the flow rate of 1 μL/s, while a positive potential of 0.7 V was applied for 60 s. The electrode was then ready for analysis of the next specimen. The sensor was normally reliable for a whole analysis day (over 50 runs). For a new-day analysis, the two PEEK blocks were taken apart, and the working electrode was regenerated as previously described. Only one regeneration was normally required per day. All water was ultrapure (18.2 MΩ-cm resistivity). All reagents were of highest purity grade as possible. Unless specified otherwise, all glassware was rinsed with 2% HNO_3 prior to use.

3. Results and discussion

3.1. Nature of Pb binding with surface-active species in saliva

In our previous reports [3,4], Pb(II) concentrations in saliva samples analyzed by a similar microanalytical system was consistently lower than that obtained by ICP-MS. This lower responsiveness may be caused by the incomplete sample digestion and release of Pb from saliva protein. Without fully understanding the dynamics of Pb-protein binding in saliva, sample pretreatment was carried out by diluting the sample five-fold with 0.5 M HCl. More recent studies have been conducted in our lab to better understand the binding activity and saliva components. These data provide new insight on an alternative sample pretreatment protocol that will take full advantage of the microanalytical system. The specific rat saliva was analyzed for total protein and amylase activity using protocols described elsewhere [24,25]. The protein content was 0.66 ± 0.28 mg/mL ($N = 5$) and the amylase activity was 18.1 ± 1.7 U/mL ($N = 5$), where N represents the number of saliva samples tested. This large molecular-weight amylase is believed to be one of the key Pb-binders [7].

To better understand Pb-protein binding, the extent of Pb distribution (in solution and in solid phases) was measured at different incubation times (2–60 min) after the saliva samples were spiked with Pb to obtain 50 ppb Pb(II). The saliva pH was measured to be 8.5 ± 0.2 ($N = 5$). The kinetics of Pb adsorption to salivary protein is presented in Fig. 2. The Y-error bars represent standard deviation of four samples. The kinetic study demonstrates that > 95% of Pb adsorbed onto the salivary protein within 2 min of Pb exposure. Thus an incubation time of 10 min was chosen for subsequent studies to ensure the complete Pb adsorption. These data suggest that saliva Pb is highly bound to available protein.

Complete release of Pb bound to the protein is very important for accurate Pb quantitation in the liquid phase. To release the protein-bound Pb, samples of Pb-spiked saliva (100 ppb Pb) were treated with a range of acid concentrations. Table 2 shows the Pb concentrations (measured by the ICP-MS) in supernatants after different acid treatments as well as the percent Pb(II) recovered. All acid concentrations, including a concentration as low as 0.1 M, resulted in a significant release of Pb (94%) from the saliva proteins. At 0.1 M acid level, the pH is likely to be lower than the pK_a of the protein, leading to protonation of the protein. At such conditions, an excess molar ratio of proton (H^+) per protein-bound

Table 2
Effects of acid treatments on the release of saliva-bound Pb, n represents the number of analysis

Pb spiked (ppb)	Acid treatment	Final Pb in supernatant (ppb)	% Pb recovered
103.3 ± 0.7 (n = 2)	0.1 M HNO_3	97.3 ± 6.7 (n = 2)	94
	0.5 M HNO_3	99.2 ± 5.7 (n = 2)	96
	1.0 M HNO_3	102.0 ± 3.1 (n = 2)	99
	2.0 M HNO_3	101.6 ± 3.9 (n = 2)	98
	1.0 M HCl	103.8 ± 3.2 (n = 2)	100

Pb(II) ($\sim 5 \times 10^{-7}$ M) ensures the completion of the Pb release. Both HCl and HNO_3 at the same concentration (1.0 M) were comparable in releasing 100% of the bound Pb. Due to the incompatibility of ICP-MS and HCl, HNO_3 was used for the samples prepared for the ICP-MS analysis. On the other hand, for the proper function of Ag/AgCl electrode, HCl was used for the samples prepared for the microanalytical system in order to constantly provide the required chloride ions.

3.2. Sample pretreatment for microanalytical system

Although 0.1 M acid appeared to be sufficient in releasing protein-bound Pb, in the perspective of electrochemical detection, sample treatment with 0.1 M or 0.5 M acid was found to be ineffective to prevent signal reductions when the samples were subjected to the Pb analysis by the microanalytical system (data not shown). This is likely due to the incomplete removal of some electroactive species that caused the high background currents or the formation of the passivation layer [19]. Therefore, 1.0 M acid was used to spike the samples prior to centrifuging. However, a 0.5 M HCl solution was found to be optimal for the operation of the Hg-film electrode; at 1.0 M and 2.0 M HCl, poor reproducibility of the Pb signals were observed (not shown). This may be attributed to poor Pb and Hg deposition due to some hydrogen gas production at −1.0 V. Thus the 1.0 M-acid-treated samples were diluted two-fold with a 10 ppm Hg solution resulting in samples containing 50% saliva/5 ppm Hg(II)/0.5 M HCl/trace level of Pb(II). The carrier was prepared analogously to the samples (without saliva and lead) to contain 5 ppm Hg(II) in 0.5 M HCl. Finally, the speed of centrifuging was also found to play an important role; RCF of 2100 × g was not as effective as RCF of 15,200 × g to remove the impurity in the saliva, resulting in poor Pb signals (not shown).

3.3. Pb analysis by microanalytical system

In spite of the great potential of electrochemical sensors for biomonitoring of trace metals, limited number of works has been done on minimizing the electrode fouling in blood and saliva [12–16]. Coating of the mercury-film electrodes with polymers like Nafion [26,27] has been reported to improve the sensitivity and resistance to electrode fouling in passivation media by excluding negatively charged organic species [28,29]. However, the lack of reproducibility of the Nafion film coating often causes the poor intersample reproducibility. In addition to Nafion coating, the Compton's group has investigated "sonoelectroanalysis" by exploiting ultrasound during the analyte deposition period in order to minimize electrode fouling in saliva samples [13–16]. The momentum from the applied sound wave promotes high turbulent flow and causes cavitation activity (e.g., collapse of cavities) at the solid–liquid interface. This cavitation at the surface and the shear force when the electrode is flushed are believed to remove organic species from the electrode surface allowing the deposition of the target analyte to proceed. Analogously, we propose that, under the right conditions and with appropriate sample pretreatment, constant flow-through of the samples in the microanalytical system during the deposition period can also remove the organic species, thereby eliminating the fouling without the need of polymer membrane coating.

To minimize a formation of the passivation layer by the salivary protein, a glassy carbon electrode was used as the working electrode since the protein adsorption is less severe at carbon electrodes than on gold or platinum electrode [30]. The film of Hg was first deposited on the electrode surface, followed by the deposition of Pb in the Pb-containing saliva sample at an optimal time (45 s). Like acoustic streaming, the flow through of the samples also increases the mass transport, thereby reducing the deposition time (to only 45 s) and increasing the sensitivity [31–32]. After the stripping and the electrode cleaning at a positive potential (0.7 V), some Hg-film was also removed along with Pb and protein. Thus the film required replenishing first by deposition of Hg(II) in the carrier for 90 s prior to subjecting the electrode to the sample for another 45 s of deposition. This demonstrates the major advantage of the microanalytical system, in which the interchanging of solution streams can be conveniently controlled without interruption and easily automated. The volume of sample and reagent required are also minimal (i.e., 50 μL of saliva and 150 μL of carrier per run).

3.4. Calibration curve

Fig. 3 shows comparison of representative Pb signals (found at −0.6 V) measured using 5 ppb Pb(II) in carrier without or with saliva (50%, v/v). When all conditions were held constant, the Pb signals without or with saliva remained unchanged, demonstrating no negative effects of saliva on the Pb detections. This suggests that the calibration curve can be constructed from standard solutions having the same matrix

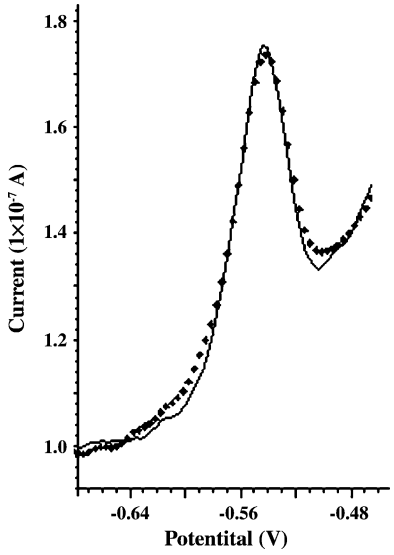

Fig. 3. Representative voltammograms of 5 ppb Pb(II) in carrier (5 ppm Hg/0.5 M HCl) with 50% saliva (dotted line) and without saliva (solid line). Other conditions are as in Table 1.

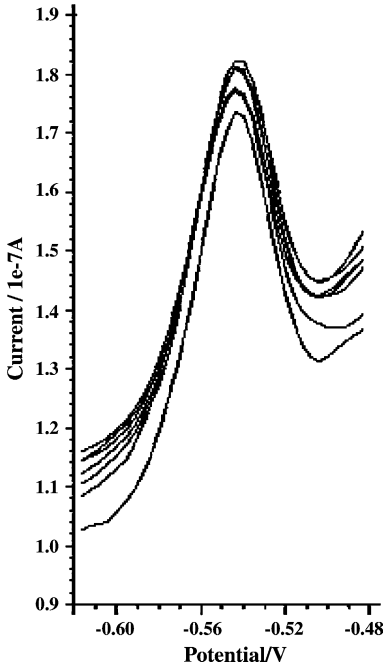

Fig. 5. Reproducibility of 5 ppb Pb(II) signals (peak areas) (after two-fold dilution) from seven consecutive runs with %R.S.D. of 5.0. Other conditions are as in Table 1.

with the samples but without saliva. Fig. 4 shows such a calibration curve, in which the signal (area in volt-ampere) is a linear function of Pb concentration in the solution ranging from 1 to 10 ppb ($R^2 = 0.99$), which is a reasonable range for a portable sensor system for salivary Pb monitoring [3].

3.5. Reproducibility

In order to measure the reproducibility of the Pb measurements, whole saliva was spiked with 10 ppb Pb(II) then diluted two-fold with the carrier solution and subjected to the microanalytical system for seven consecutive measurements. Fig. 5 shows the Pb voltammograms from the seven runs. With a 50% saliva sample, the system yielded a highly reproducible signal for Pb; areas from the seven runs were 2.22×10^{-9}, 2.22×10^{-9}, 2.15×10^{-9}, 2.15×10^{-9}, 1.99×10^{-9}, 1.97×10^{-9}, 2.21×10^{-9} V A, respectively. This corresponded to a relative standard deviation (%R.S.D.) of 5.0% and a measured Pb concentration (corrected for dilution) of 9.2 ± 0.5 ppb. The reproducibility from day-to-day operations was also determined. When used to measure 10 ppb of Pb(II) on three separate days, the freshly prepared Hg-film electrode (after regeneration with HNO_3, acetone, and water) yielded Pb peak areas that were 1-, 1.01-, 1.13-fold relative to that of day 1. These factors were used to scale all peak signals on a given day to the day that the calibration curve was measured.

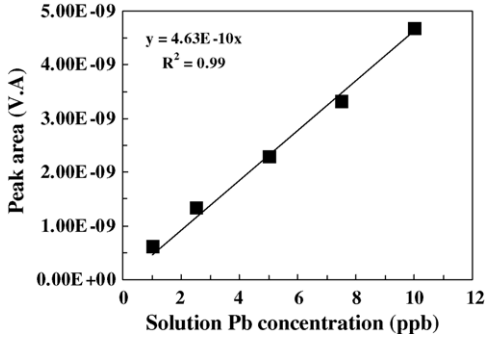

Fig. 4. Calibration curve for the Pb analysis measured using the Pb standard solutions containing same matrix with the samples (5 ppm Hg/0.5 M HCl). Other conditions are as in Table 1.

3.6. Detection limits

The detection limit was determined as the concentration of Pb that would give a signal three times the background noise. The background noise was measured using the optimal conditions previously described but without Pb in the solution. Three times the background noise was measured to be 5×10^{-10} volt-ampere, which corresponded to a detection limit of 1 ppb Pb(II).

3.7. Analysis of unknown samples

To assess the accuracy of saliva Pb quantitation by the microanalytical system, three unknown samples (prepared by spiking lead into saliva) were analyzed and the results were compared with those from the ICP-MS. The results are summarized in Table 3. The microanalytical system yielded the results that were highly comparable to the ICP-MS, suggesting the great potential of the system for on-site biomonitoring

Table 3
Comparison of Pb concentrations in unknown saliva samples by the microanalytical system and ICP-MS, *n* represents number of analysis

Unknown #	Pb peak area (V A) (dilution = 2)	Pb conc. (ppb) by microanalytical system	Pb conc. (ppb) by ICP-MS
1	4.58E-10 4.25E-10	2.2 ± 0.1 (*n* = 2)	2.2 ± N/A (*n* = 1)
2	1.29E-09 1.19E-09	6.1 ± 0.3 (*n* = 2)	6.2 ± 0.2 (*n* = 2)
3	2.48E-09 2.37E-09	11.9 ± 0.4 (*n* = 2)	11.6 ± 1.4 (*n* = 4)

of Pb as opposed to the in-lab analysis by the ICP-MS. This is also the first time that Pb in up to 50% by volume of real saliva in the samples can be quantified accurately by an electrochemical technique, compared to 2.5% of saliva as previously reported [14,16]. Reducing the fraction of saliva (and hence protein content) in the samples by using large dilution factors is not desirable because it compromises the analysis of the real samples having inherently low Pb content [11].

4. Conclusions

In response to the growing need for biomonitoring of heavy metals, a microanalytical system has been developed that will potentially improve the quality and efficiency of on-site and real-time biomonitoring of heavy metals. Saliva is very attractive as a biomonitoring matrix since it can be easily and non-invasively collected. However, saliva poses a challenge on the electroanalysis of heavy metals because of the lack of understanding of the metal–protein binding and the fouling of the electrode caused by the surface-active species in saliva. In vitro studies were carried out to understand Pb protein-binding and releasing using rat saliva.

With the proper pretreatment of the samples and the optimal operating conditions of the microanalytical system, 50% (v/v) saliva in the samples did not cause the electrode fouling and the highly reproducible Pb analyses were achieved. The microanalytical system was reliable, required low saliva sample (50 μL) and minimal cleaning (1 min), offered rapid analysis (3 min total analysis time) and low detection limit, as well as allowed interchanging of reagents and sample streams without interruption. In addition, the system is very compact in size and can be automated, making it suitable for field-deployable analysis.

Having many advantages, this new microanalytical system, when used with properly pretreated biological samples, will potentially have broad applications for quantitation of both occupational and environmental Pb exposure. Additional research on the mode of action for Pb transport from blood into salivary glands, and subsequently secreted into the saliva is needed. In addition, in vivo studies comparing the pharmacokinetics of Pb saliva clearance in rodents and human will be needed to determine the limitations of saliva as a biomonitoring matrix. The results will be reported in due course.

Acknowledgement

The work was supported by grant 1 R01 ES010976-01A2 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIEHS. The work was performed in part at the Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by the DOE' s Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory (PNNL). PNNL is operated by Battelle Memorial Institute for the U.S. Department of Energy (DOE). The authors thank Dr. Guodong Liu for helpful discussion and Angela D. Woodstock for rat saliva collection

References

[1] J.M. Christensen, Sci. Total. Environ. 166 (1995) 89.
[2] L. Friberg, C.G. Elinder, J. Work Environ. Health 19 (Suppl. 1) (1993) 7.
[3] C. Timchalk, T.S. Poet, A.A. Kousba, J.A. Campbell, Y. Lin, J. Toxicol. Environ. Health, Part A 67 (2004) 635.
[4] C. Timchalk, T.S. Poet, Y. Lin, K.K. Weitz, R. Zhao, K.D. Thrall, AIHAJ 62 (2001) 295.
[5] A.Y.S. P'an, J. Toxicol. Environ. Health 7 (1981) 273.
[6] N. Mobarak, A.Y.S. P'an, Toxicology 32 (1984) 67.
[7] Y. Lin, R. Zhao, K.D. Thrall, C. Timchalk, W.D. Bennett, M.D. Matson, Proc. Soc. Opt. Eng. (SPIE) 3877 (1999) 248.
[8] J. Wang, Y. Lin, L. Chen, Analyst 118 (1993) 277.
[9] J. Wang, Analytical Electrochemistry, VCH Publishers, Inc., New York, 1994.
[10] Y. Lin, C.A. Timchalk, D.W. Matson, H. Wu, K.D. Thrall, Biomed. Microdev. 3 (2001) 331.
[11] J. Wang, J. Electroanal. Chem. 139 (1982) 225.
[12] J.L. Hardcastle, G.G. Murcott, R.G. Compton, Electroanalysis 12 (2000) 559.
[13] J.L. Hardcastle, R.G. Compton, Electroanalysis 14 (2002) 753.
[14] J.L. Hardcastle, C.E. West, R.G. Compton, Analyst 127 (2002) 1495.
[15] C.E. Banks, R.G. Compton, Analyst 129 (2004) 678.
[16] C.E. West, J.L. Hardcastle, R.G. Compton, Electroanalysis 14 (2002) 1470.
[17] S. Roy, J.H. Kim, J.T. Kellis, A.J. Poulose, C.R. Robertson, A.P. Gast, Langmuir 18 (2002) 6319.
[18] P.B. Welzel, Thermochim. Acta. 382 (2002) 175.
[19] D.T. Kim, H.W. Blanch, C.J. Radke, Langmuir 18 (2002) 5841.
[20] C.E. Bohs, M.C. Linhares, P.T. Kissinger, Curr. Sep. 13 (1994) 6.
[21] F. Zhou, J.T. Aronson, M.W. Ruegnitz, Anal. Chem. 69 (1997) 728.
[22] M.L. Kaplan, A.G. Jones, M.A. Davis, L. Kopito, Life. Sci. 16 (1975) 1545.

[23] C. Timchalk, R.J. Nolan, Toxicol. Appl. Pharmacol. 144 (1997) 268.
[24] T.S. Poet, H. Wu, A.A. Kousba, C. Timchalk, Toxicol. Sci. 72 (2003) 193.
[25] Salimetric 2005, Saliva α-amylase assay kit, www.salimetrics.com/amylasekitinsert.htm.
[26] C.M.A. Brett, D.A. Fungaro, J.M. Morgado, M.H. Gil, J. Electroanal. Chem. 468 (1999) 26.
[27] C.M.A. Brett, D.A. Fungaro, Talanta 50 (2000) 1223.
[28] S. Jaenicke, R.M. Sabarathinam, B. Fleet, H. Gunasingham, Talanta 45 (1997) 703.
[29] B. Hoyer, T.M. Florence, G.E. Batley, Anal. Chem. 59 (1987) 1608.
[30] G. Sanna, M.I. Pilo, P.C. Piu, N. Spano, A. Tapparo, R. Seeberb, Electroanalysis 14 (2002) 1512.
[31] R.G. Compton, J.C. Eklund, F. Marken, Electroanalysis 9 (1997) 509.
[32] C. Agra-Gutiérrez, R.G. Compton, Electroanalysis 10 (1998) 603.