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Evaluating the ability of energy dispersive X-ray analysis to monitor binding oil content of carbon paste electrodes exposed to biofouling agents

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ABSTRACT

Physiological conditions can alter the performance of electrochemical sensors through complex interactions arising at the tissue matrix and electrode interface. Understanding this relationship is a prerequisite to the eventual deployment of these sensors *in vivo*. Herein, we investigate the ability of energy dispersive X-ray (EDX) analysis to monitor silicone oil levels within carbon paste electrodes (CPE). We correlate these findings with scanning electron microscopy (SEM) images and cyclic voltammetry (CV) data. EDX analysis reported significant variations in silicone oil content when the CPE was immersed in protein (bovine serum albumin (BSA)), lipid (phosphatidylethanolamine (PEA)), surfactant (Triton®X (TX)) and brain tissue over a 28-day period. Moreover, the greatest effect occurs within the first 24 h of exposure. Protein adsorption appears to hinder the X-rays emitted during SEM imaging resulting in reduced silicone oil measured by EDX. SEM images and voltammetric profiles support this finding. Brain tissue homogenate appears to reduce silicone levels at a slower rate than PEA and TX which corroborates voltammetric data. Notwithstanding this, the surface morphology determined by SEM imaging suggests comparable surface alterations for the three treatments over the 28 days. Finally, we report the impact that continuously cycling CPEs in protein and lipid has on the silicone oil content. There was a significant improvement recorded over a 3.5h period when compared to EDX analysis performed on CPEs stored in the solutions for the same period. Collectively, the data provided within support the utility of EDX analysis as a valid and simple surface analytical technique that can be employed to follow the removal of silicone oil from CPEs.

1. Introduction

Since Ralph Adams seminal report in 1958 detailing a novel paste electrode composed of carbon powder and an organic binding liquid [1], researchers worldwide have identified carbon paste electrodes (CPEs) as their preferred substrate for the development of a broad range of electrochemical sensors. They are widely available, cost effective and their ease of modification makes them useful as highly selective sensors [2]. However, one of the most quoted disadvantages of CPEs is that their operational success depends on the practical experience of the user [3]. Each prepared electrode is individual, due to the uneven distribution of carbon and binding liquid and the irregular surface formed. They are best described as an ensemble of carbon particles dispersed in and linked by a pasting liquid [4]. Heretofore, paraffin oils [4–6] and silicone oils [7–13] are the most popular binders utilised in the design of CPEs for implantation in biological tissue [14]. Primarily, the role of the binding agent is to mechanically bind the graphite particles in the carbon paste composite. However, it can also govern the properties of the CPE and typical characteristics required include chemical inertness, high viscosity, limited aqueous solubility and organic solvent immiscibility [5]. Notwithstanding this, it is generally accepted that the carbonaceous material is the main component responsible for the electrochemical behaviour, whereas the binder plays a minor role [15]. Mikysek and colleagues reported the measurement of ohmic resistance and redox peak separation to determine the desired ratio between various carbon and pasting liquid moieties which they describe as being the pivotal characteristic in optimising the carbon paste composition [15,16].

CPEs have been routinely used over the years for the recording of neurochemicals in rodent brain tissue and their altered surface morphology and performance characteristics have been reported [14,17–20]. The material-tissue interaction that arises following implantation of an electrochemical sensor *in vivo*, is one of the primary considerations when developing viable, long term implantable sensors.

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Living tissue contains a broad range of potential poisons in the form of lipids, proteins and surfactants. These can limit diffusion and adsorption at the electrode surface [14], thereby disrupting the voltammetric signal produced by CPEs. This is primarily due to the lipophilic nature of biological tissue which has been reported to remove the hydrophobic oil from the CPE surface [6], thus altering the morphology and electrochemical characteristics. Triton®X (TX) is a non-ionic surfactant [21] that has been routinely used to mimic physiological conditions due to its hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic or hydrophobic group. Moieties containing hydrophilic heads and hydrophobic tails can form lipid bilayers which make up the cell walls surrounding almost all living organisms. CPEs functionalised with low concentrations of TX have been shown to improve their electrochemical activity [21–23] by forming a monolayer [22] of surfactant on the electrode surface, but higher concentrations result in fouling of the substrate [24]. Similarly, Kauffmann and colleagues described the incorporation of the amphiphile hexadecyl sulfonic acid into CPEs, producing a functionalised electrode with extremely useful analytical characteristics [25,26]. However, it was O'Neill and colleagues that identified the interaction with lipids alters the carbon paste composition to a carbon powder, through the leaching of binding oil from the paste cavity [27,28]. This is a well-established characteristic that provides CPEs with their excellent long term stability *in vivo* due to the removal of fouling proteins from the electrode surface [8]. Likewise, Kaufmann et al. reported a comparable erosion effect on the CPE surface caused by surfactants that exhibited a certain repelling effect and avoided electrolysis under certain extreme conditions [5,25,26].

Protein adsorption is another important consideration for *in vivo* sensors, in particular CPEs. Bovine serum albumin (BSA) is a large globular protein which is readily purified from bovine blood, and is often used to mimic protein concentrations in laboratory scenarios [29,30]. It has been utilised previously to investigate the effect of protein adsorption on electrochemical sensor performance [7,24,31]. BSA is particularly detrimental to CPE surfaces due to its adsorption hindering access to electron transfer sites [7], however, it is not believed to interact with the binding oil present. On the other hand, phosphatidylethanolamine (PEA) is a phospholipid found in all living cells and has been reported to remove silicone oil from CPEs upon contact. Kane et al. reported that the reversal of protein adsorption on CPEs in the presence of a lipid-protein matrix was caused by the removal of the pasting oil from within the paste [7]. It is this surface cleaning characteristic that has been attributed to the electrodes long term functionality under physiological conditions. Heretofore, electrochemical measurements have been the principal method of confirming this leaching phenomenon [8,27,28], however, more direct methods of tracing the binding oil content are an attractive proposition.

Herein, we present an investigation into the ability of energy dispersive X-ray (EDX) analysis to function as a simple analytical tool to monitor the removal of binding oil from CPEs. EDX is a technique used in conjunction with scanning electron microscopy (SEM) that utilises X-rays generated when the electrons interact with the sample. Each element has an X-ray energy and wavelength facilitating the elemental composition of a sample to be identified. CPEs were exposed to solutions of surfactant (TX), lipid (PEA), protein (BSA) and brain tissue homogenate for 0–28 days prior to analysis. EDX analysis was coupled with SEM and voltammetry data to substantiate the findings. In addition, the impact of continuous cycling in protein and lipid for a 3.5h period was investigated using EDX analysis and cyclic voltammetry data. A myriad of studies exist that report the use of EDX to confirm the chemical composition of CPE surfaces targeted towards physiological monitoring [32–34]. However, to the best of our knowledge, this is the first report detailing the ability of EDX analysis to monitor the silicone oil content of CPEs following long-term exposure to biofouling agents routinely found under physiological conditions.

2. Materials and methods

2.1. Chemicals and solutions

All reagents used *i.e.*, sodium chloride (NaCl), sodium hydroxide (NaOH), sodium hydrogen phosphate (NaH_2PO_4), graphite powder (particle size ≪20 μm, 1.9 g/cm^3), silicone oil (0.96 g/mL, ((-Si$(CH_3)_2$O-)n, α-Methyl-ω-methoxypolydimethylsiloxane)), bovine serum albumin (BSA), phosphatidylethanolamine (PEA) and Triton®X-100 (TX) were purchased from Sigma Aldrich Chemical Co. (Dublin, Ireland). Cyclic voltammogram (CV) investigations were performed in phosphate buffered saline (PBS); NaCl (0.15 M), NaOH (0.04 M) and NaH_2PO_4 (0.04 M) made up in deionised water. 1% solutions of BSA, PEA and TX were made up in deionised water. Homogenised brain tissue was obtained from a euthanised Wistar rat and made up in deionised water.

2.2. Electrode manufacture and treatments

CPEs were manufactured from Teflon®-insulated silver (Ag) wire (200 μm bare diameter 8 T, Advent Research Materials; Oxford, UK) as described previously [35,36]. A 5 cm length of Teflon®-insulated Ag wire was cut. Approximately 1 mm of the Teflon® insulation was removed from one end, exposing the bare Ag wire. Using a tweezers, the Teflon® was gently moved along the length of the wire, exposing a 1 mm cavity at the opposite end of the electrode. The cavity was packed with carbon paste (0.71 g graphite powder (≪20 μm, 1.9 g/cm^3) and 250 μL silicone oil (0.96 g/mL)). The exposed Ag wire at the opposite end was soldered into a gold clip (Fine Science Tools GmbH, Heidelberg, Germany). A bare Ag wire with the same diameter was used as a plunger, to ensure that the paste was compactly packed. The surface was then levelled by gently rubbing it on a clean, flat surface. Individual sets of CPEs (n = 4) were stored in either 1% BSA, PEA, Triton®X (TX) and homogenised brain tissue (BT) at 4 °C for 1, 3, 7 and 28 days. Once the electrodes were removed from the treatment solution, they were rinsed in deionised water prior to imaging or electrochemical analysis (100 cycles). A control set of electrodes were also stored at 4 °C for the same duration. Further sets of CPEs (n = 4) were cycled in BSA or PEA for 400 cycles, *ca.* 3.5 h or stored for the same duration and then cycled. Imaging analysis was then performed on these CPEs. A separate set of electrodes were exposed to BSA, BT and TX for 24-h and subsequently polished on a Buehler microcloth (Buehler, Illinois, USA) to determine the impact of removing any adhered biofouling layer. SEM and EDX analysis were subsequently performed on these electrodes.

2.3. SEM and EDX analysis

A 5 mm long section was cut from the active end of the CPE. The Teflon® was carefully removed from the bottom 2 mm, and the exposed Ag wire was angled to 90° and placed onto 12 mm carbon adhesive tabs (Agar Scientific), mounted on 15 mm × 6 mm specimen stubs (Agar Scientific), so that the modified surface was *ca.* 90° to the mount. The stubs were placed in the sputter coater and a vacuum was applied for 30 min. Sputter coating was performed, under argon, with an Au/Pd target, until a thickness of 8 nm was obtained. SEM images presented are from one CPE. On average, five sites of interest on the electrode surface were investigated for EDX analysis. Subsequently, the mean silicone oil content was calculated for the respective treatment days.

2.4. CV investigations

All CVs were performed in a standard three-electrode glass electrochemical cell containing 20 mL PBS as previously described [35]. The PBS was purged with nitrogen (N_2) gas (BOC Ireland) for 20 min to eliminate any interference from the reduction of oxygen that might be

observed in the CV. A saturated calomel electrode (SCE) was used as the reference electrode and a large Pt wire served as the auxiliary electrode. All CPEs were cycled over the potential range −700 mV to +800 mV *vs*. SCE @100 mV/s. CPEs that were stored in the respective solutions were rinsed in deionised water prior to cycling in PBS. The average voltammogram obtained for each set of treated electrodes ($n = 4$) were overlaid for the respective days and presented graphically. The capacitance current was extrapolated at +0.4 V for cycle 100 on each day of treatment ($n = 4$) to facilitate quantitative comparisons. This potential was chosen due to a reproducible and stable current being evident in the voltammograms.

2.5. Instrumentation, software and data analysis

SEM and EDX analyses were carried out using a Hitachi S-3200-N with a tungsten filament electron gun. This has a maximum magnification of 200,000×, a resolution of 3.5 nm and accelerating voltage of 20 kV. This microscope was equipped with an Oxford Instrument INCA x-act EDX system with silicon drift detector. Voltammetric experiments were performed using a low-noise potentiostat (ACM Instruments, Cumbria, UK) and converted using an A/D converter (PowerLab, ADInstruments, Oxford, UK). The CV signals were recorded using eChem software (v2.1.16, eDAQ Ltd., Sydney, Australia) running on a Dell computer or laptop. The mean silicone oil content of the untreated CPE was calculated from five sites of interest around the electrode surface and normalised to 100%. Each EDX result obtained (averaged from five sites) on the respective treatment day was compared against this normalised value. The significance of variation observed during investigations was determined using One-way ANOVA. Two-way ANOVA was used to determine the difference in variation between CPEs, stored and cycled in BSA or PEA, with respect to cycle number/time. Treatment protocol (cycled *vs*. stored) and cycle number/time were set as fixed factors. Student's *t*-test for unpaired observations were used for EDX analysis where appropriate. Two-tailed levels of significance were used with $p \ll 0.05$ considered to be significant. Analysis was performed using GraphPad Prism® version 5.01 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Effect of surfactant exposure

The effect of immersing CPEs in a 1% solution of Triton®X is detailed in Fig. 1. It is clear from the SEM images that exposure to this concentration is detrimental to the CPE surface creating a concave morphology which recesses backwards into the cavity over the course of the treatment protocol. This is particularly evident from day 1 onwards and when compared against an untreated CPE (See Supplementary Fig. SM1). The EDX image displayed in Fig. SM4 illustrates evidence of silicone oil, although its quantity has been reduced when compared to the EDX of a freshly modified CPE (see Fig. SM1). The silicone oil content was determined using EDX analysis on each day of exposure and compared against day 0 CPE which was not stored in the surfactant. In all instances, an average silicone oil % was calculated from five sites on the CPE surface. The average silicone oil content from a day 0 CPE was normalised to 100% and treated electrodes were compared with this. One-way ANOVA identified a significant variation in oil content ($F(4, 19) = 13$, $p \ll 0.0001$, $n = 4$) recorded across day 0 (100 ± 25%), day 1 (29 ± 4%), day 3 (18 ± 1%), day 7 (0.7 ± 0.2%) and day 28 (0.08 ± 0.01%). Furthermore, a decreasing trend in oil content ($F(3, 15) = 38$, $p \ll 0.001$, $n = 4$) was observed across day 1–28 with Bonferroni *post hoc* analysis identifying a significant difference between all days except 7 *vs*. 28. These findings suggest a continual decrease in silicone oil over the 28 days of exposure, however, it appears that all silicone oil had in fact been removed from the CPE by day 7.

The voltammograms presented in Fig. 1B demonstrate a clear indication of surface alterations over the respective days of treatment and corroborate the SEM images detailed in Fig. 1A. The observed morphological changes and disrupted voltammetric profiles can be attributed to the well-established leaching phenomenon caused by the surfactant on the CPE surface [14,27]. The leaching of the silicone oil from the paste culminates in a concave morphology and a more powder like surface resulting from a higher carbon: oil ratio at the electrode/solution interface. Fig. 1C describes the capacitance current recorded from CPEs at +0.4 V *vs*. SCE over the different days of TX storage. A significant variation in the capacitance current was identified by one-way ANOVA ($F(4, 29) = 91$, $p \ll 0.0001$, $n = 4$) which is closely correlated with the EDX data. This supports the EDX techniques ability to accurately monitor silicone oil removal from within the paste. An increasing variation in capacitance current ($F(3, 15) = 9$, $p \ll 0.01$, $n = 4$) was recorded for day 1–28 and Bonferroni *post hoc* analysis identified a significant difference for days 3 and 28 with respect to day 1. This is in stark contrast to the control set of electrodes stored at 4 °C for the same duration (see Fig. SM6). Since the silicone oil is non-conducting within the carbon paste, the increased carbon: oil ratio may result in a more conductive surface and the higher currents obtained. The large capacitance currents recorded over the respective days of exposure may be representative of this change in surface composition. These findings support previous reports from O'Neill and colleagues, whereby the electrochemical characteristics of ascorbic acid at surfactant treated CPEs demonstrated faster electron transfer and a shift to lower oxidation potentials [6,27]. Additionally, the well-established ferrocyanide redox couple revealed improved peak separation at the modified CPEs due to increased electron transfer at the more conductive powder like surface [6,27]. Notwithstanding this, it is important to consider that an overall increase in the electrodes surface area, caused by the switch to a more concave like morphology, may impact on the capacitance currents also.

3.2. Effect of phospholipid exposure

Next we investigated the effect of exposing CPEs to the phospholipid PEA using SEM and EDX analysis coupled with CV investigations. Fig. 2A details the SEM images over the respective days of PEA exposure. The impact on the CPE morphology is similar to TX with clear modifications evident. The surface has been considerably altered, particularly from day 3 onwards, to a concave like morphology which may be attributed to the leaching of the binding oil. As previously, the EDX image in Fig. SM3 had a silicone component but is greatly reduced when compared to the untreated CPE surface. The oil content was analysed (see Fig. 2A) using one-way ANOVA and there was a significant variation ($F(4, 19) = 11$, $p \ll 0.001$, $n = 4$) recorded across day 0 (100 ± 25%), day 1 (22 ± 2%), day 3 (18 ± 1%), day 7 (18 ± 1%) and day 28 (13 ± 2%). Furthermore, a decreasing trend in oil content ($F(3, 15) = 4$, $p \ll 0.05$, $n = 4$) was observed across the exposure days 1–28 and Bonferroni *post hoc* analysis identified a significant difference between day 1 and 28. As alluded to earlier, this confirms that the decrease in silicone oil content is dependent on the duration of exposure.

Fig. 2B overlays the CVs recorded for the respective days of PEA exposure with obvious differences observed between the voltammetric profiles. Fig. 2C identifies a significant variation in capacitance currents recorded at +0.4 V over days 0–28 ($F(4, 29) = 50$, $p \ll 0.0001$, $n = 4$). Furthermore, an increasing variation in capacitance current ($F(3, 15) = 12$, $p \ll 0.001$, $n = 4$) was recorded for day 1–28 and Bonferroni *post hoc* analysis identified a significant difference between day 1 and day 28 which corroborates EDX analysis. As described for TX investigations, the larger capacitance currents are in contrast to those recorded for controls (see Fig. SM6), however, they coincide with the lower silicone oil content and alterations in surface morphology. These findings corroborate previous reports that exposing CPEs to lipids

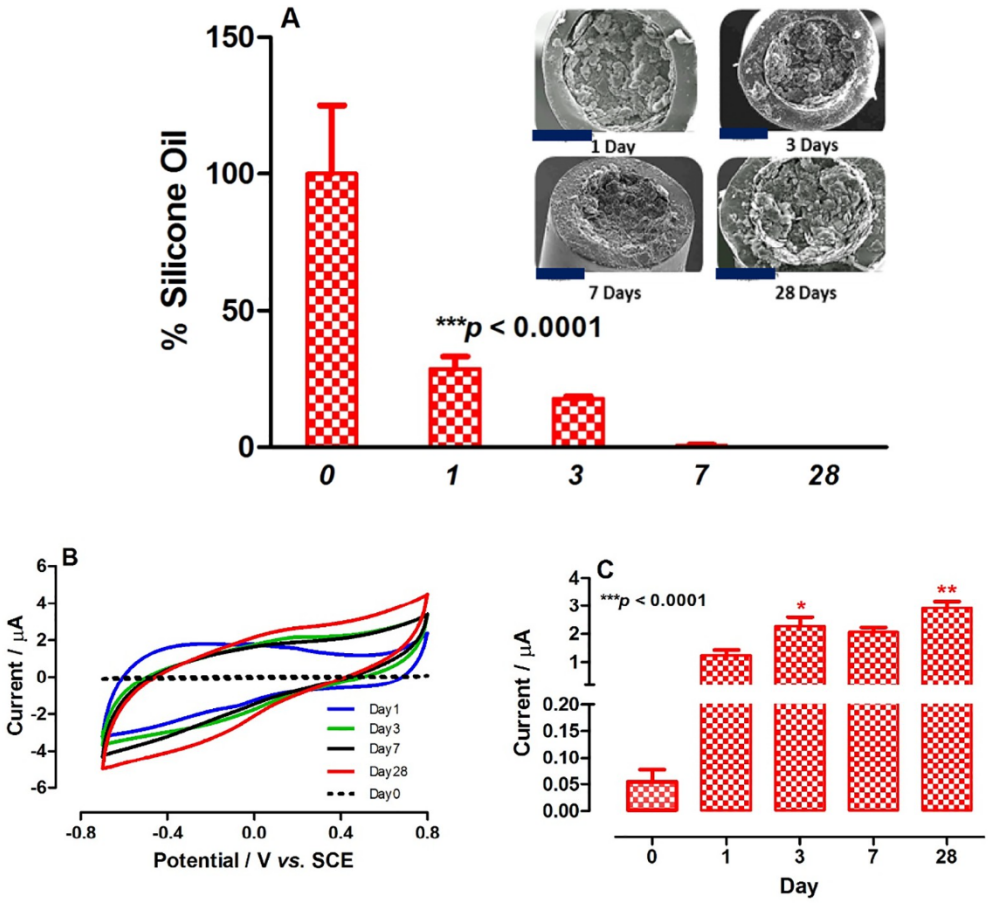

Fig. 1. Effect of exposing carbon paste electrodes (CPEs) to 1% Triton®X (TX) treatment for 0–28 days using (A) energy dispersive X-ray (EDX) analysis and (Inset) scanning electron microscopy (SEM). SEM scale bar (100 µm) depicted using blue box in each image. Data represented as mean % silicone oil content ± standard error mean (B) average effect of 0–28 days of TX exposure on CVs of CPEs (n = 4) and (C) average capacitance currents recorded at +0.4 V *vs.* saturated calomel electrode (SCE) from CPEs (n = 4) stored in TX for 0–28 days. Data represented as mean current ± standard error mean. * denotes level of variation (ANOVA) or significant difference (Bonferroni, with respect to day 1).

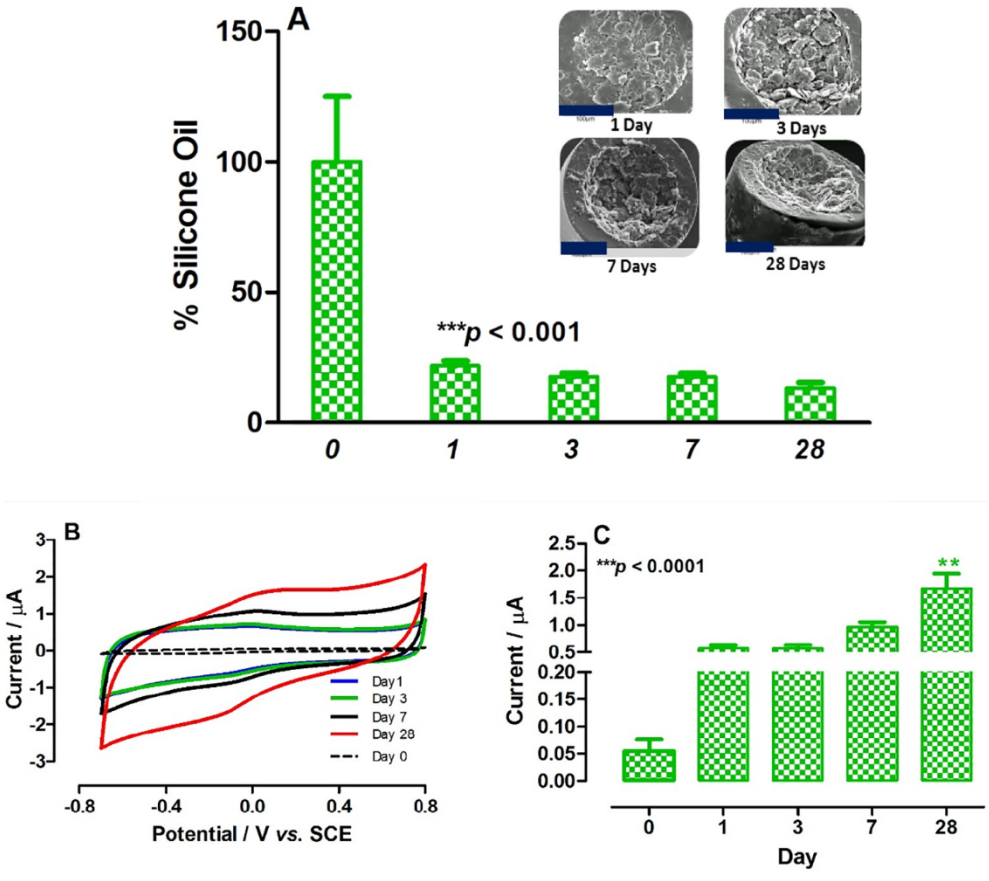

Fig. 2. Effect of exposing CPEs to 1% phosphatidylethanolamine (PEA) treatment for 0–28 days using (A) EDX analysis and (Inset) SEM. SEM scale bar (100 µm) depicted using blue box in each image. Data represented as mean % silicone oil content ± standard error mean (B) average effect of 0–28 days of PEA exposure on CVs of CPEs (n = 4) and (C) average capacitance currents recorded at +0.4 V *vs.* SCE from CPEs (n = 4) stored in PEA for 0–28 days. Data represented as mean current ± standard error mean. * denotes level of variation (ANOVA) or significant difference (Bonferroni, with respect to day 1).

removes the silicone oil from the carbon paste, producing a carbon powder electrode that contributes to their improved stability during long term recordings *in vivo* [8,27,28]. Moreover, this provides further support for the potential utility of EDX as a reliable tool for determining the loss of silicone from carbon paste following prolonged exposure to lipid environments.

3.3. Effect of brain tissue homogenate exposure

The effect of brain tissue homogenate is described and detailed in Fig. 3. Although not an exact representation of the physiological environment, exposing an electrochemical sensor to brain tissue serves as a good indication of the effect of *in vivo* conditions on the sensors performance [37]. The complex nature of the tissue homogenate encompasses proteins and lipids at concentrations that would be encountered *in vivo*. Fig. 3A (inset) illustrates the effect of brain tissue exposure on the CPE surface morphology. As alluded to with previous treatments, the electrode appeared intact after 24 h of exposure and resembled an untreated CPE (see Fig. SM1). Thereafter, the surface became concave, which infers a loss of silicone oil from the electrode surface. After 28 days stored in brain tissue, the electrode appeared totally dried out and had pulled away from the Teflon® support. There was evidence of residual brain tissue on the paste and Teflon® surfaces, although the electrodes were thoroughly rinsed prior to imaging. It was possible that removing the electrode from the homogenised solution may have removed some paste from the electrode surface. Fig. SM5 illustrates the EDX of the electrode surface after storage in brain tissue. There is still evidence of silicone oil, however, this quantity has been reduced when compared to the EDX of a freshly modified CPE. Using one-way ANOVA, Fig. 3A details a significant variation in oil content ($F(4, 19) = 7$, $p \ll 0.01$, $n = 4$) was recorded across day 0 (100 ± 25%), day 1 (44 ± 14%), day 3 (21 ± 1%), day 7 (20 ± 3%) and day 28 (16 ± 1%). It is interesting to note that the initial drop on day 1 was not as drastic as previous treatments. This is an interesting finding since brain tissue homogenate is the closest representation of *in vivo* conditions. Moreover, one-way ANOVA analysis of the exposure days 1–28 identified a non-significant variation in oil levels ($F(3, 15) = 3$, $p \gg 0.05$, $n = 4$). Bonferroni *post hoc* analysis identified no significant difference ($p \gg 0.05$) between any of the individual treatment days. This suggests prolonged exposure to physiological tissue fails to impact on the carbon to binding oil ratio, however, SEM images identify an altered surface morphology.

Electrochemical investigations described in Fig. 3B illustrate improved retention of the voltammetric profiles over the course of the 28 days of exposure despite increasing capacitance currents still observed and highlighted in Fig. 3C. One-way ANOVA indicated a significant variation in currents recorded at +0.4 V over the 28 days ($F(4, 29) = 133$, $p \ll 0.0001$, $n = 4$). Furthermore, an increasing variation in capacitance current ($F(3, 15) = 63$, $p \ll 0.001$, $n = 4$) was recorded for day 1–28 and Bonferroni *post hoc* analysis identified significant differences for all exposure days with respect to day 1 (see Fig. 3C). The capacitance currents were considerably lower after 28 days of exposure which is in contrast to those recorded for TX and PEA. This suggests a lower carbon: oil ratio at the electrode/solution interface which corroborates the reduced silicone oil removal highlighted by EDX analysis. Notwithstanding this, a linear increase in capacitance current is observed over the 28 days and demonstrates a comparable trend with increasing silicone oil loss from the CPE. Once again the capacitance currents are considerably higher than control values detailed in Fig. SM6 but are more comparable than PEA and TX findings. Collectively this infers a slower reduction in silicone oil content coupled over the 28 days with lower capacitance currents which suggests a reduced leaching effect of binding oil in homogenised brain tissue. Furthermore, correlation across all of the analyses support the ability of EDX analysis

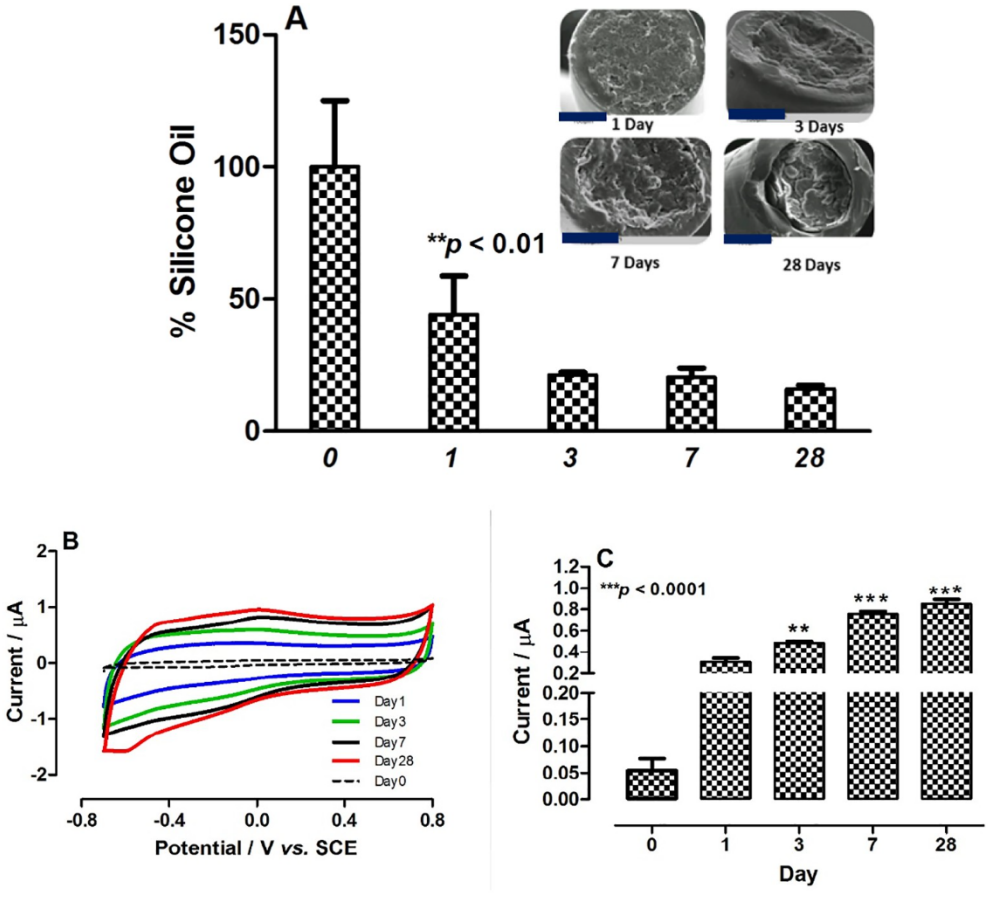

Fig. 3. Effect of exposing CPEs to brain tissue homogenate (BT) treatment for 0–28 days using (A) EDX and (inset) SEM. SEM scale bar (100 µm) depicted using blue box in each image. Data represented as mean % silicone oil content ± standard error mean (B) average effect of 0–28 days of BT exposure on CVs of CPEs ($n = 4$) and (C) average capacitance currents recorded at +0.4 V *vs*. SCE from CPEs ($n = 4$) stored in BT for 0–28 days. Data represented as mean current ± standard error mean. * denotes level of variation (ANOVA) or significant difference (Bonferroni, with respect to day 1).

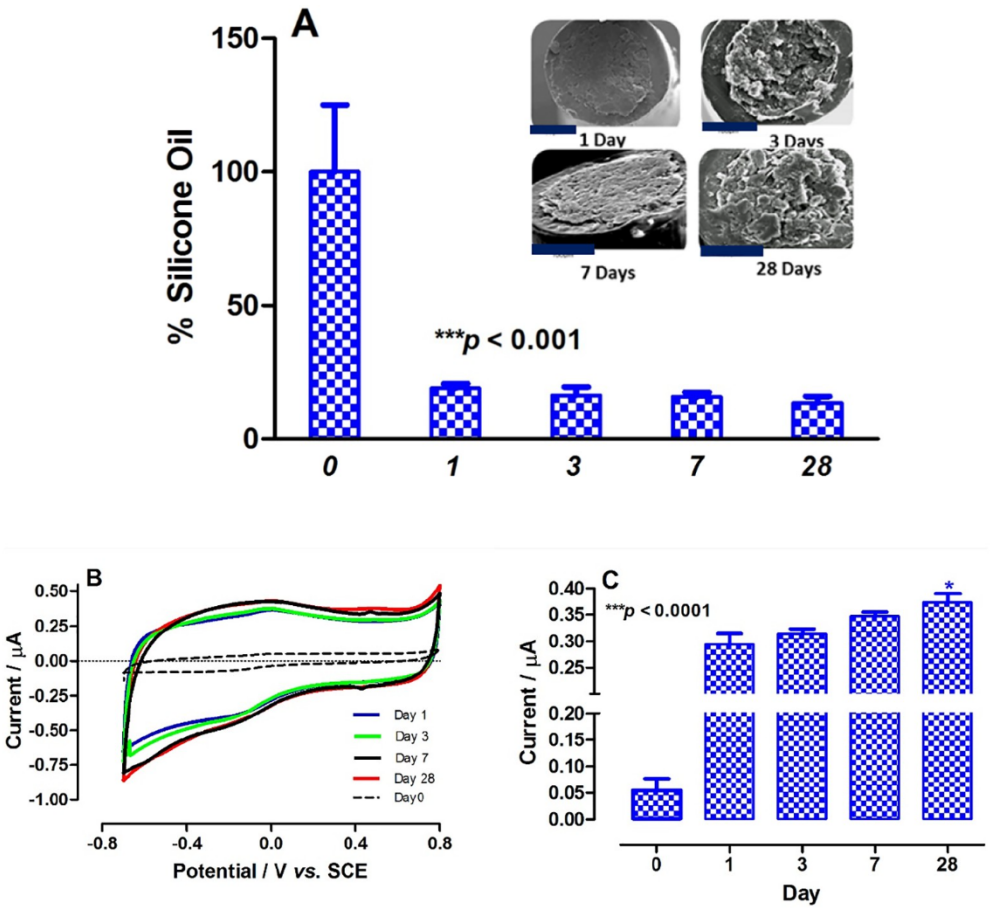

Fig. 4. Effect of exposing CPEs to 1% bovine serum albumin (BSA) treatment for 0–28 days using (A) EDX and (inset) SEM. SEM scale bar (100 µm) depicted using blue box in each image. Data represented as mean % silicone oil content ± standard error mean (B) average effect of 0–28 days of BSA exposure on CVs of CPEs ($n = 4$) and (C) average capacitance currents recorded at +0.4 V *vs*. SCE from CPEs ($n = 4$) stored in BSA for 0–28 days. Data represented as mean current ± standard error mean. * denotes level of variation (ANOVA) or significant difference (Bonferroni, with respect to day 1).

to monitor silicone oil content reliably.

3.4. Effect of protein exposure

Fig. 4 illustrates the effect of storing CPEs in 1% BSA for 1, 3, 7 and 28 days. After day 1, the SEM images in Fig. 4A (inset) suggest retention of the surface integrity and the electrode appeared unaltered with similar morphology to an untreated CPE (see Fig. SM1). Over subsequent days the CPE surface appeared to remain intact, however, deposits of adsorbed protein are visible on the electrode surface. Proteins demonstrate a tendency to inhibit electron transfer processes through the blocking of active sites present on the CPE surface [38]. EDX analysis presented in Fig. 4A suggests considerable reduction in silicone oil content despite the SEM images portraying a consistently smooth surface. One-way ANOVA identified a significant variation ($F(4, 19) = 11$, $p \ll 0.001$, $n = 4$) recorded across day 0 (100 ± 25%), day 1 (19 ± 2%), day 3 (16 ± 3%), day 7 (16 ± 2%) and day 28 (13 ± 2%). The large drop in silicone levels was somewhat surprising since exposing the CPEs to protein was not expected to induce leaching of the binding oil from the carbon paste. One-way ANOVA analysis of the exposure days identified a non-significant variation in oil concentration recorded across day 1–28 ($F(3, 15) = 1$, $p \gg 0.05$, $n = 4$). Furthermore, Bonferroni *post hoc* analysis identified no significant difference ($p \gg 0.05$) between any of the individual exposure days. This suggest continuous exposure to protein fails to decrease the level of binding oil over the 28 days of exposure. The immediate fouling of the electrode surface upon BSA exposure is a well-documented phenomenon [39,40], however such a dramatic decrease in binding oil content was unexpected.

The CVs in Fig. 4B detail the effect on CPE voltammograms. Interestingly, there was no substantial increase in the capacitance current recorded across all days of exposure, inferring that the carbon paste has remained intact and retained its properties. This was further supported by Fig. 4C. One-way ANOVA analysis indicated a significant variation in currents recorded at +0.4 V over days 0–28 ($F(4, 29) = 36$, $p \ll 0.0001$, $n = 4$). Furthermore, an increasing variation in currents ($F(3, 15) = 6$, $p \ll 0.05$, $n = 4$) was recorded for days 1–28 and Bonferroni *post hoc* analysis indicated a significant difference at day 28 with respect to day 1. Notwithstanding this, the capacitance currents remained relatively comparable over the 28 days of exposure and only *ca* 10 fold greater than control currents illustrated in Fig. SM6. These findings suggest that prolonged exposure to BSA fails to significantly impact on the carbon: oil ratio within the carbon paste. This is in contrast to the EDX analysis provided. A plausible explanation is an adsorptive effect of the protein that blocks active sites on the CPE surface contributing to reduced electron transfer [7]. Furthermore, this may impede the X-rays and subsequent detection of the binding oil is compromised. EDX analysis uses X-rays emitted from a depth that depends on how deep the secondary electrons are formed. Depending on the sample density and incident beam, this is usually from 0.5 to 2 µm in depth [41]. Under these circumstances, EDX analysis demonstrates limitations in silicone oil evaluation unless utilised in combination with voltammetric investigations. These findings warranted further study.

3.5. Effect of polishing CPE surface following exposure to biofouling agents for 24 h

In light of the BSA findings, it was decided to polish the electrode surface after overnight storage in a selection of contaminants, prior to EDX analysis. The purpose of this was to remove any adhered layer from the CPE surface that may be impacting on the emitted X-rays and confirm our hypothesis. Meticulous care was taken to try to remove the

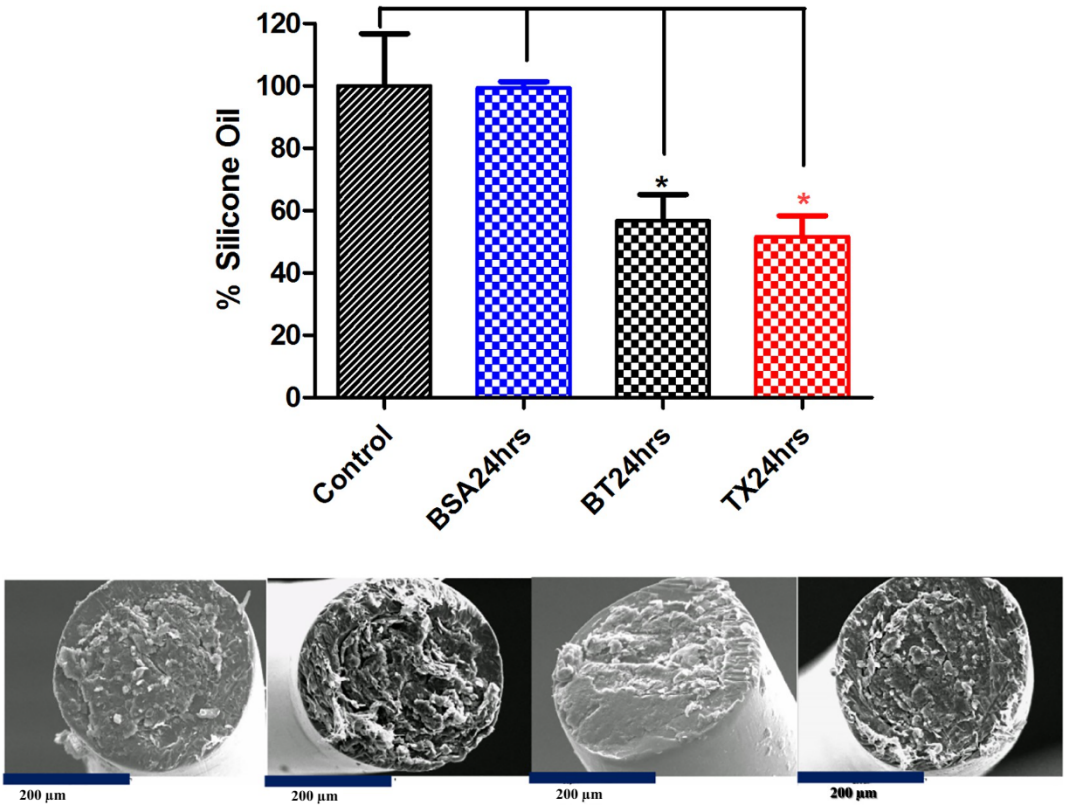

Fig. 5. (top) Effect of polishing control CPEs and those exposed to 1% BSA, BT and 1% TX treatment for 24 h using (A) EDX and (bottom) respective SEM images. * denotes level of significance (unpaired *t*-test) with respect to control.

adhered surface layer only and leave the carbon paste unaltered, however, it is important to consider that in some instances paste may have been removed from the surface exposing fresh paste underneath. SEM images detailed in Fig. 5 illustrate the impact of polishing on the electrode surface with minor discrepancies noted between treatments. As previously, the average silicone oil content from a polished, control CPE was normalised to 100% and treated electrodes were compared against (see Fig. 5). The silicone oil content for the respective treatments were as follows; CPE (100 ± 17%), CPE-BSA (99 ± 2%), CPE-BT (57 ± 8%) and CPE-TX (52 ± 7%). It was interesting to note that the binding oil content of BSA treated electrodes demonstrated no significant difference ($p \gg 0.05$) to controls following polishing of the CPE surface. In contrast, significant differences were identified between CPEs exposed to BT ($p \ll 0.05$) and TX ($p \ll 0.05$) compared to controls. These findings lend strong support to our assumption that the adhered BSA impedes emitted X-rays that resulted in the reduced content measured in earlier EDX investigations. However, it is also apparent from these results that adhered BT and TX impacted on the emitted X-rays in earlier studies through the formation of a passivating layer which needs to be considered. Notwithstanding this, polishing the CPE surfaces resulted in significant differences from control and BSA exposed CPEs which confirms that the reduced oil content in BT and TX exposed CPEs can be attributed to its leaching from the carbon paste. These findings support the ability of EDX analysis to accurately measure the binding oil content of CPEs.

3.6. Effect of continuous cycling in protein and phospholipid on silicone oil content

Recently, we reported the benefits of continuously cycling functionalised carbon paste-based pH sensors in BSA and PEA solutions over storing them in the same solutions [36]. The pH sensitive oxidation peak demonstrated improved electron transfer and peak resolution over successive cycles. We postulated that the constant application of a redox potential prevents a protein or lipid layer from building up on the surface and is more reflective of how a peripheral tissue sensor would operate in clinical practice [36]. Fig. 6A describes the effect of cycling CPEs in BSA and PEA for 3.5 h (400 cycles) against storing them for the same duration. As described previously, the untreated CPE was normalised to 100%. There was no significant difference ($p \gg 0.05$) in binding oil content recorded between control CPEs and CPEs that were cycled in PBS for 400 cycles. For comparative purposes, the respective currents recorded at each 50th cycle for the PBS cycled CPEs are provided in Figs. 6B and C.

Unpaired student *t*-tests identified a significant decrease in silicone oil content for CPEs stored in BSA solution for 3.5 h compared to untreated electrodes (100 ± 4% *vs.* 46 ± 4%, $n = 4$, $p \ll 0.0001$). This effect was almost completely reversed when CPEs were cycled in the 1% BSA solution over the same time period (100 ± 4% *vs.* 90 ± 9%, $n = 4$, $p \gg 0.05$). Moreover, this translated into a significant improvement between the two treatment conditions (90 ± 9% *vs.* 46 ± 4%, $n = 4$, $p \ll 0.01$). Two-way ANOVA analysis (see Fig. 6B) identified a cycle number/time dependent increase in capacitance current recorded at +0.4 V for CPEs stored in BSA for 3.5 h (effect of cycle number, $F(7, 34) = 48.69$, $p \ll 0.0001$, $n = 4$). This effect was attenuated when CPEs were continuously cycled in BSA over the same period (effect of continuous cycling, $F(1, 34) = 188.46$, $p \ll 0.0001$, $n = 4$) and the attenuation was also cycle number/time dependent (cycle number x treatment protocol, $F(7, 34) = 10.23$, $p \ll 0.0001$, $n = 4$). Bonferroni *post hoc* analysis identified significant differences at cycle numbers 200, 250, 300, 350 and 400. Interpretation of these results suggest that the different BSA treatment protocols resulted in cycle number/time dependent differences in capacitance currents. The impact of storing CPEs in BSA for 3.5 h is evident in the increasing currents observed up to cycle 300. Subsequently the currents plateaued. The initial increase may be indicative of protein adsorbing on the

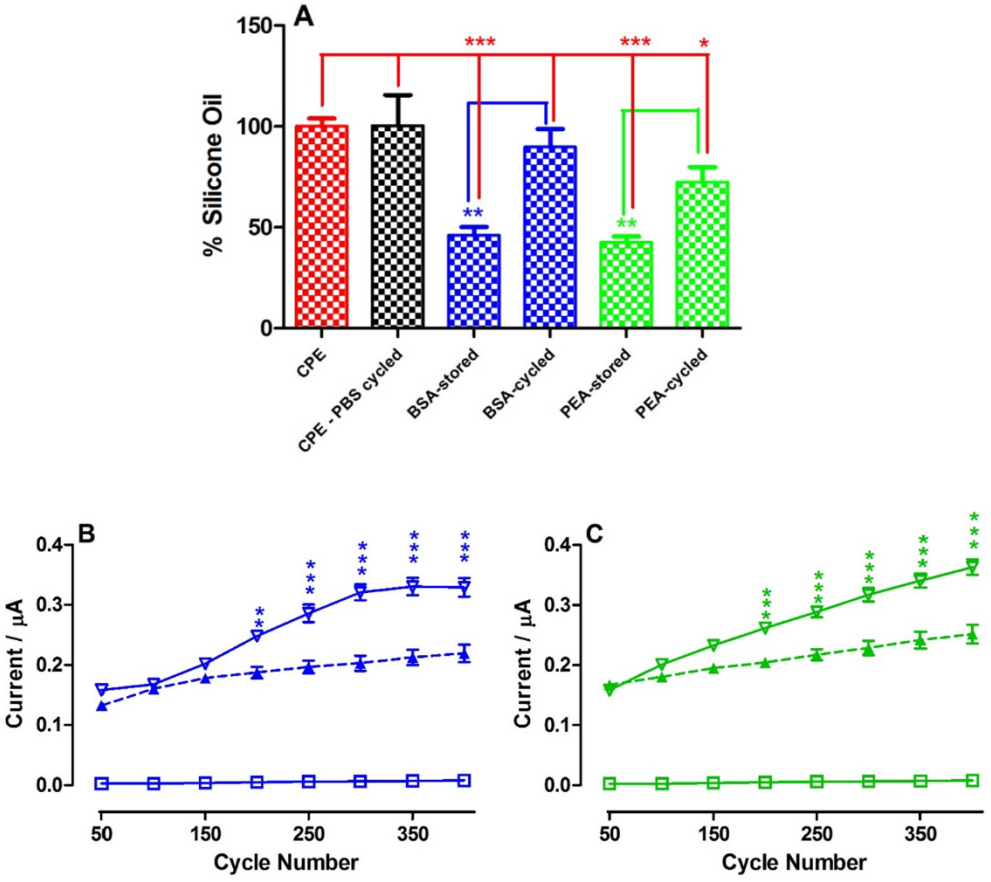

Fig. 6. Comparing the effect of storing and continuously cycling CPEs in PBS (black trace), 1% BSA (blue traces) and PEA (green traces) on (A) silicone oil content using EDX analysis and capacitance currents recorded at +0.4 V *vs*. SCE using CV for (B) BSA and (C) PEA treated CPEs. Open triangle – stored, closed triangle – cycled and open box – cycled in PBS. * denotes level of significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

electrode surface which corroborates the reduced silicone oil content measured using EDX. Linear regression highlighted a current increase of 5.7×10^{-4} µA/cycle or 1.1×10^{-3} µA/min ($r^2 = 0.95$) for this set of CPEs ($n = 4$). This increasing current was significantly attenuated in the cycled electrodes because the application of the redox potential reduces the adsorption of the protein on the electrode surface. Linear regression analysis identified a slower current increase of 2.3×10^{-4} µA/cycle or 4.5×10^{-4} µA/min ($r^2 = 0.93$) for these cycled electrodes ($n = 4$). This assumption was further substantiated by the higher level of silicone oil detected using EDX which was comparable to that of an untreated CPE. These findings demonstrate two things; (1) further support that the EDX technique can measure silicone oil levels reliably and (2) further support for our assumption that prolonged exposure to BSA impedes the X-rays generated.

Similarly, for PEA investigations detailed in Fig. 6A, unpaired *t*-tests identified a significant decrease in silicone oil content for CPEs stored in the phospholipid (100 ± 4% *vs*. 43 ± 3%, $n = 4$, $p \ll 0.0001$) over the 3.5h period. Once again, this effect was ameliorated when the CPEs were continuously cycled over the same duration, however, a significant difference was still recorded against the untreated electrode (100 ± 4% *vs*. 72 ± 7%, $n = 4$, $p \ll 0.05$). Notwithstanding this, a significant improvement in silicone oil retention was observed following continuous cycling (72 ± 7% *vs*. 43 ± 3%, $n = 4$, $p \ll 0.001$). Two-way ANOVA analysis (see Fig. 6C) highlighted a cycle number/time dependent increase in capacitance current for CPEs stored in PEA for 3.5 h (effect of cycle number, $F(7, 48) = 56.35$, $p \ll 0.0001$, $n = 4$). This effect was reduced when CPEs were continuously cycled in PEA over the same period (effect of continuous cycling, $F(1, 48) = 160.09$, $p \ll 0.0001$, $n = 4$) and the attenuation was also cycle number/time dependent (cycle number x treatment protocol, $F(7, 48) = 9.59$, $p \ll 0.0001$, $n = 4$). Bonferroni *post hoc* analysis identified significant differences at cycle numbers 200, 250, 300, 350 and 400. It is obvious from Fig. 6C that the capacitance current increases linearly after storage in the phospholipid. This is either a result of the adsorbed lipid removing the binding oil or increasing electron transfer kinetics. Linear regression analysis identified a current increase of 5.7×10^{-4} µA/cycle or 1.1×10^{-3} µA/min ($r^2 = 0.99$) for the stored CPEs ($n = 4$). Once again, this increasing capacitance current was attenuated by continuously cycling the CPEs in PEA supporting our previous assumption that the application of a redox potential prevents the adsorption of contaminants on the electrode surface. This was further supported by linear regression analysis which identified lower current increases of 2.4×10^{-4} µA/cycle and 4.8×10^{-4} µA/min ($r^2 = 0.99$) for the stored and cycled electrodes respectively. It is interesting to note that the PEA treated CPEs demonstrated excellent linearity ($r^2 = 0.99$) over the entire recording period for both stored and cycled electrodes. BSA electrodes deviated from linearity towards the latter part of the cycling, for both treatment protocols, suggesting reproducibility in electrode morphology and conditioning. Again this is reflected in the EDX analysis for both treatment protocols whereby decreased silicone oil content is reported for lipid exposure.

4. Conclusion

We presented a thorough investigation into the effect that long term exposure to physiological contaminants has on the silicone oil content of CPEs. EDX analysis, coupled with SEM and voltammetry, was utilised to determine the binding oil content of CPEs following prolonged exposure to lipid, surfactant, brain tissue homogenate and protein. In summary, all treatments significantly reduced the silicone oil content measured using EDX analysis. Evidence suggests that BSA adsorption on the electrode surface appears to impede X-rays and subsequent quantification of mixing oil, supported by SEM images and voltammetric profiles. Polishing the BSA exposed CPE surface appears to remove this

passivating layer and returns silicone oil content back to control levels. In contrast, PEA, Triton®X and brain tissue homogenate remove the mixing oil from the carbon paste, causing considerable morphological changes and increasing capacitance currents. Furthermore, we demonstrated the ability of continuous cycling to negate the effect of BSA adsorption on the CPE surface, and to reduce the leaching observed when stored in PEA. The EDX findings corroborate previous voltammetry reports that PEA exposure continuously removes the binding oil from the carbon paste, where BSA exposure exerts its effect by blocking active sites on the CPE surface and fouling the surface with multi-layer coatings. Our findings support the utility of EDX analysis as a valid and simple surface analytical technique that can be employed in parallel with electrochemical methods to follow the removal of silicone oil from CPEs, while the corresponding micrographs provide information on electrode surface morphology.

Declaration of Competing Interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jelechem.2019.113237.

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