

Article

Ascorbic Acid Rejection Characteristics of Modified Platinum Electrodes: A Shelf Life Investigation

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Abstract: Ascorbic acid (AA) is the principle interferent present in brain extracellular fluid that can inhibit the ability of electrochemical sensors to selectively detect a particular analyte of interest. Considerable efforts have been made in recent times to develop highly selective membrane coatings to counteract the drawbacks associated with AA interference during *in vivo* monitoring. The primary objective of the work described within was to investigate the long term effect of storing such selective membranes, *i.e.*, Nafion® and Poly-*o*-phenylenediamine (PPD) under different conditions and how exposing them to repeated calibration protocols compromises the membranes ability to reject AA. Four different modified platinum (Pt) electrodes, Pt-PPD, Pt-Nafion® (5/2), Pt-Nafion® (1/2)-PPD, and Pt-Nafion® (2/1)-PPD, stored at 4 °C demonstrated deterioration of the polymers integrity when exposed to repeated calibrations. On the contrary, exposing the same four electrode types to single calibrations confirmed excellent retention of AA rejection characteristics. Pt-PPD electrodes were then exposed to varying storage conditions and calibrated against AA on day 1, day 56 and day 168. Storing the Pt-PPD electrodes at 4 °C/N₂ saturated glass container demonstrated retention of AA rejection characteristics after day 168. These results have clearly elucidated the optimum storage conditions for Pt-Nafion® and Pt-PPD modified electrodes.

Keywords: Ascorbic acid; Nafion®; Poly-*o*-phenylenediamine; membrane integrity; storage

1. Introduction

The mammalian brain has a large number of possible interfering species present at relatively high concentrations, (e.g., ascorbic acid (AA), uric acid (UA), and neurotransmitters including dopamine (DA) and serotonin (5-HT)). Due to its high abundance in brain extracellular fluid, AA is the principle electroactive interferent that can inhibit an electrochemical sensor's ability to selectively detect a particular analyte of interest. Possessing an $E_{1/2}$ between -100 to $+400$ mV vs. SCE, it is readily oxidised at platinum (Pt) electrodes through a $2e^-$ process that results in the production of L-dehydroascorbic acid [1]. Miele and Fillenz approximated an extracellular ascorbate concentration of *ca.* 500 μ M [2] which exists in anionic form at physiological pH and has diverse functions ranging from anti-oxidant to neuromodulator. Considerable efforts have been made in recent times to develop highly selective membrane coatings to counteract the drawbacks associated with AA interference during *in vivo* monitoring.

Routinely, Pt surfaces have been modified by a number of different methods including recast perfluorosulfonated ionomers and electropolymerised films. The perfluorinated ion exchange polymer Nafion[®], has been widely utilised as a permeable membrane in electrochemical sensor design demonstrating excellent permselective characteristics against anionic species including AA [3–6]. However, when dip adsorbed on Pt surfaces, Nafion[®] demonstrates cation exchange properties due to the hydrophilic ionic clusters of sulfonic acid groups that also give rise to its anionic rejection properties. Work undertaken in our research laboratory has described a novel procedure for coating Nafion[®] onto Pt electrode surfaces using a thermal annealing protocol [7]. The resulting Nafion[®] pre-coat layer completely eliminates anionic and cationic interferents from the disk surface but facilitates the diffusion of gaseous molecules such as nitric oxide (NO) and oxygen (O₂) across [8].

Poly-*o*-phenylenediamine (PPD) can be integrated into the design of electrochemical sensors to function either as a conducting polymer [9] or more commonly as an insulating polymer [10,11]. Electropolymerisation of the monomer *o*-phenylenediamine (*o*-PD) in pH 7.4, deposits PPD in its insulating form onto the electrode surface [12]. This insulating layer appears to provide selectivity by limiting access of larger molecules such as AA, DA, *etc.* to the electrodes surface. Literature states that different structures of the insulating form of PPD exist, a “ladder” like structure where the amino groups are condensed within the benzene rings adjacent to each other along the polymer chain [13] and an “open” 1,4-substituted benzenoid-quinoid structure [14]. Research undertaken by Losito and colleagues suggest that the “open” form of the polymer is the more dominant structure [15].

A number of previous reports have detailed the excellent permselective properties of electrochemical sensors that incorporate both Nafion[®] and PPD layers in their membrane design [6,16,17]. It is imperative that electrochemical sensors demonstrate efficient selectivity characteristics to enable reliable *in vivo* recordings to be performed. Additionally it is critical that these selectivity characteristics remain intact throughout long-term storage, allowing the researcher to manufacture and calibrate sensors on separate days to *in vivo* implantation. This forms the basis of the work described within. Investigations were undertaken to determine the effect of various different storage conditions on the longevity of Pt electrodes modified with various combinations of Nafion[®] and PPD layers.

2. Experimental Section

2.1. Reagents and Solutions

The Nafion[®] (5 wt % solution in a mixture of lower aliphatic alcohols and H₂O), L-ascorbic acid (AA; A.C.S. reagent) and *o*-phenylenediamine (*o*-PD, 99+%), sodium chloride (NaCl), sodium hydrogen phosphate (NaH₂PO₄) and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich Ireland Ltd. Parafilm was sourced from Lennox (Dublin, Ireland).

AA stock solutions (0.1 M) were prepared daily by dissolving 0.176 g in 10 mL of doubly distilled deionised H₂O. *In vitro* experiments were carried out in phosphate buffer saline (PBS) solution, pH 7.4 (0.15 M NaCl, 0.04 M NaH₂PO₄ and 0.04 M NaOH), which was deaerated with O₂-free N₂ for 20 min prior to commencing electrochemical measurements.

2.2. Sensor Manufacture

Bare Platinum (Pt) disk electrodes were made from Teflon insulated Platinum/Iridium (Pt/Ir 90%/10%) wire (127 µm bare diameter, 203 µm coated diameter (5T), Science Products GmbH, Hofheim, Germany) as previously described. Briefly, the electrodes were approximately 5 cm in length and were prepared by carefully cutting 5 mm of the Teflon insulation from one end of the wire. A gold electrical contact (Bilaney, UK) was soldered to this end of the wire to enable connection with the instrumentation. The other end of the wire acted as the active (disk) surface of the electrode.

Pt-PPD refers to the electropolymerisation of *o*-PD onto the bare Pt disk electrode which was carried out by applying a constant potential of +700 mV vs. SCE in a 300 mM solution made up in N₂ saturated PBS. The electrodes were polymerised for 30 min and the electrochemical cell was kept under a N₂ atmosphere as the monomer is readily oxidised in air.

Nafion[®] (5/2) refers to a previously described sensor incorporating 5 pre-coat, 2 applications of Nafion[®], annealed after each application at 210 °C which has been extensively characterised *in vitro* [7,8,18] and *in vivo* [19,20] for the electrochemical detection of NO.

Nafion[®] (1/2)-PPD refers to a previously described sensor incorporating 1 pre-coat, 2 applications of Nafion[®], annealed after each application at 210 °C, followed by electropolymerisation in *o*-PD for 30 min [21].

Nafion[®] (2/1)-PPD refers to a previously described sensor incorporating 2 pre-coats, 1 application of Nafion[®], annealed at 210 °C followed by 1 dip into 5% Nafion[®] solution, annealed at 210 °C and finally electropolymerised in *o*-PD for 30 min [21].

The previously reported pre-coat application method involves placing a fixed volume (5 µL) of Nafion[®] on a clock glass using a syringe which is allowed to air dry at room temperature for 5 min (repeated up to 5 times depending on the sensor type). A fresh application of Nafion[®] (one or two depending on the sensor type) is placed onto the concentrated pre-coat of Nafion[®]. The active surface of the electrode is dipped into this concentrated layer and then immediately removed and let air dry at room temperature for 2 min. The electrodes are then placed in the oven and annealed for 5 min at 210 °C. After the annealing process has been completed, the electrode is coated again (depending on number of pre-coats) using the same procedure *i.e.*, placing another application of Nafion[®] onto the concentrated

layer and dipped into this concentrated layer and then immediately removed and let air dry at room temperature for 2 min.

2.3. Storage Conditions

Enclosed container at 4 °C: Polymer modified electrodes were stored dry in a glass cell sealed tight with a lid and parafilm.

N₂ saturated PBS solution at 4 °C: Polymer modified electrodes were stored in a glass cell containing 10 mLs of PBS which had been purged for 20 min prior to storage. The cell was sealed tight with a lid and parafilm.

N₂ saturated enclosed container at 4 °C: Polymer modified electrodes were stored in a glass cell which had been purged with N₂ for 20 min prior to storage. The cell was sealed tight with a lid and parafilm.

Enclosed container at −20 °C: Polymer modified electrodes were stored dry in a glass cell sealed tight with a lid and parafilm.

2.4. AA Calibrations

All calibrations were performed in a standard three-electrode glass electrochemical cell which was constructed in house. A saturated calomel electrode (SCE) was used as the reference electrode and a large Pt wire served as the auxiliary electrode. All calibrations were performed at +900 mV *vs.* SCE to investigate the membranes suitability for incorporation into nitric oxide sensor designs. Following each AA calibration, the sensor tips were rinsed in deionised water to remove trace amounts of adsorbed salt and were returned to their respective storage conditions. To facilitate mixing, solutions were agitated using a magnetic stirrer for approximately 5 s following the addition of each aliquot. The current was then measured under quiescent conditions with a N₂ atmosphere maintained over the solution.

2.5. Instrumentation and Software

Constant potential amperometry (CPA) was performed as previously described. Briefly, all electrochemical experiments used a low-noise four channel biostat (ACM instruments; Cumbria, UK). Data acquisition was carried out with a logiQ M760TG notebook with an Intel dual core processor, an eight channel powerlab[®] 8/30 from (ADInstruments Ltd; Oxford, UK) and LabChart for Windows (v 6.1) software (ADInstruments Ltd., Oxford, UK). Scanning Electron Microscopy was performed on a Hitachi S-3200N.

3. Results and Discussion

3.1. Shelf-Life Studies—Effect of Repeated AA Calibrations

PPD modified Pt electrodes are extensively utilised to improve selectivity of the polymer layer of both electrochemical sensors [6,17,22–24] and biosensors [11,25–28] since extracellular concentrations of certain metabolites are considered low in relation to the excessive concentration of AA. Therefore it is imperative that the integrity of the polymer is retained following implantation. Moreover, the ability

of the polymer to selectively detect the analyte of interest must be verified prior to implant. The investigation of a polymers shelf-life is therefore a prerequisite prior to its utilisation in physiological determinations since sensors are manufactured and stored prior to deployment. Figure 1 illustrates the effect of storing Pt-PPD electrodes in an enclosed glass container at 4 °C over the course of 56 days. The electrodes were calibrated on day 1, 3, 7, 14, 28 and 56 with the maximum current recorded at 1000 μ M AA. In detail, day 3 (147 ± 10 pA, $n = 4$), day 7 (150 ± 53 pA, $n = 4$) and day 14 (590 ± 224 pA, $n = 4$) demonstrated no significant difference ($p > 0.05$) from day 1 (138 ± 19 pA, $n = 4$). However, day 28 (1847 ± 684 pA, $n = 4$) and day 56 (2707 ± 1031 pA, $n = 4$) revealed significant differences ($p < 0.05$).

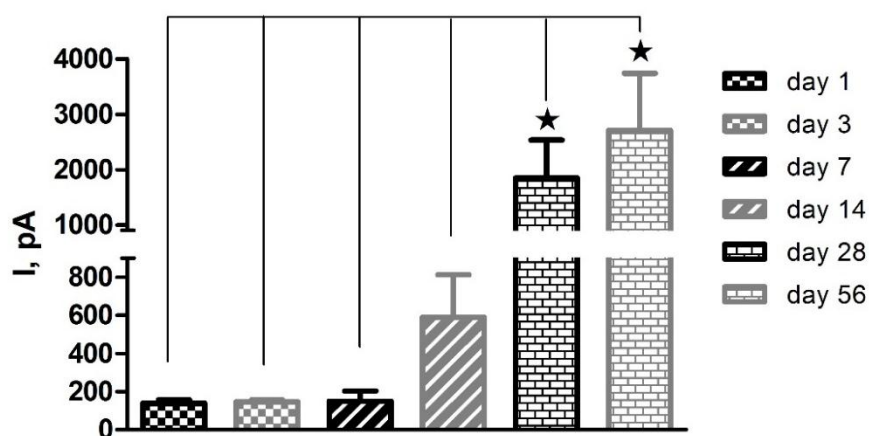


Figure 1. Comparison of the effects of repeated calibrations on the shelf life of platinum-poly-*o*-phenylenediamine (Pt-PPD) electrodes. Data represents maximum current recorded at 1000 μ M ascorbic acid (AA) for Pt-PPD ($n = 4$) electrodes.

The current-concentration profile in Figure 2 highlights the ability of the PPD polymer to facilitate the transport of AA at the lower concentrations. This feature diminishes with subsequent injections as the polymer pore size self-blocks the diffusion of incoming AA due to the entrapment of AA and its oxidised metabolite dehydroascorbic acid (DHA) within the PPD polymer matrix. This has been reported previously [29,30] and is characteristic of PPD polymers. It is interesting to note that linearity returns after day 56 in contrast to the self-blocking effect observed on day 1. The levels detected are still considerably lower than at a bare Pt electrode ($37,000 \pm 1000$ pA mM^{-1} , $n = 14$) but are considered too high for physiological applications. The augmented linear response after day 56 theorises that the overall integrity of the PPD polymer remains intact, however, the polymer pore size increases following storage and repeated calibrations. A plausible explanation for this can be attributed to the effect of repeated entrapment of the AA molecule within the pores of the PPD matrix over successive calibrations. Constant exposure to AA results in a detrimental effect due to damage inflicted on the PPD pores upon removal of the interferent from the polymer membrane following cessation of the calibration and re-storage. This removes the self-blocking effect observed on day 1 by facilitating the diffusion of larger concentrations of AA to the electrodes surface and removing the possibility of AA/DHA entrapment within the matrix. A similar phenomenon was reported by Geise and co-workers utilising reticulated vitreous carbon coated with PPD. After 60 days the response of a poly (1, 2-DAB) covered electrode to repeated calibrations of 1 mM acetaminophen was over 15% of the original bare electrode response [31].

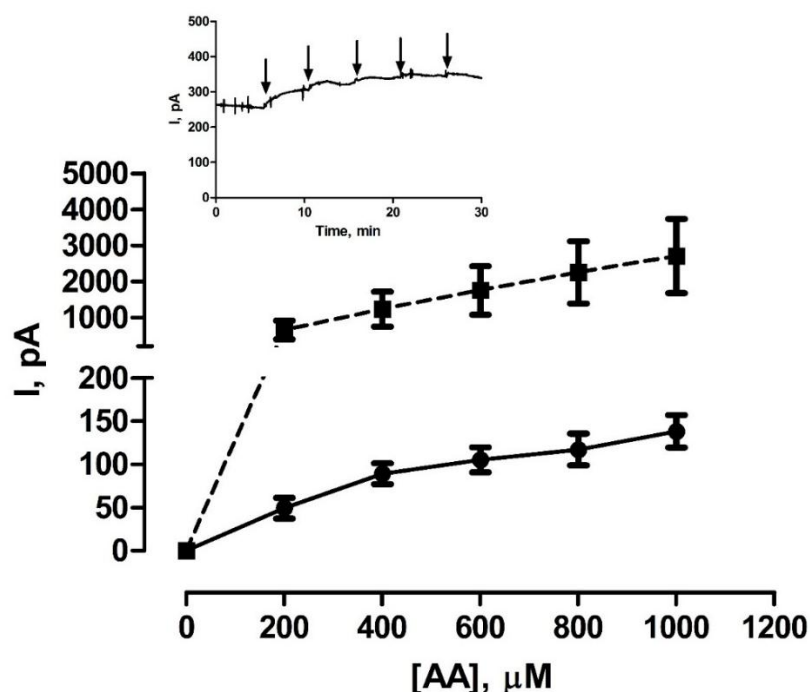


Figure 2. A current-concentration profile for 0–1000 μM AA on Pt-PPD electrodes on day 1 ($n = 4$, solid line) vs. day 56 (4 $^{\circ}\text{C}$ storage, $n = 4$, dashed line) at +900 mV vs. saturated calomel electrode (SCE). Electrodes calibrated on day 1, 3, 7, 14, 28, and 56. (Inset) Typical raw data trace. Arrows indicate injection of AA aliquot.

Table 1 details the permselective characteristics of the four different types of Pt modified electrodes utilised throughout this study. All electrode types were exposed to the same storage conditions and calibration parameters. Pt-Nafion[®](5/2) sensors incorporate an annealed form of the perfluorinated sulfonic acid ionomer on the electrode surface which has demonstrated excellent rejection characteristics previously [8]. In the past Nafion[®] has been employed in electrochemical sensor design for its dual ability as both a cation exchange polymer [32–35] and an anionic rejection layer [4–6]. However, the described Nafion[®] pre-coat method displays both anionic and cationic rejection characteristics while facilitating the diffusion of gaseous molecules such as NO and O₂ to the electrode surface. The extremely selective nature of this film can be attributed to the annealing procedure which reduces the permeability of the film by increasing the crystallinity of the film [36,37]. Similar to the Pt-PPD electrode, the maximum current recorded at 1000 μM AA on day 3 (260 ± 211 pA, $n = 4$), day 7 (592 ± 328 pA, $n = 4$), day 14 (1254 ± 902 pA, $n = 4$) and day 28 (1598 ± 1099 pA, $n = 4$) demonstrated no significant difference ($p > 0.05$) from day 1 (131 ± 29 pA, $n = 4$), though, day 56 (2707 ± 1031 pA, $n = 4$) did reveal a significant difference ($p < 0.05$). It is possible that storing the sensor over time and repeatedly exposing them to calibrations has resulted in cracking of the Nafion[®] membrane leading to an increased permeability to AA. This has been reported previously by Mercado *et al.*, who demonstrated that curing Nafion[®] at a high temperature reduces cracking somewhat, but does not eliminate it entirely [38]. It is worth noting that their work relates to subcutaneously implanted sensors, however, a similar phenomenon can be attributed to the work described within. The process of calibrating the sensor in electrolyte and subsequently storing the electrode compromises the integrity of the Nafion[®] modified surface resulting in eventual cracking of the crystalline structure. A possible explanation is the adsorption

of salts from the electrolyte augments the permeability of the polymer to AA following frequent exposure. Mercado *et al.* refer to the calcification of Nafion[®] polymers following implantation, resulting in an increase in the membranes permeability to interferent molecules. Due to the hydrophilic sulfonic groups and the hydrophobic fluorocarbon backbone within Nafion[®], it is conceivable that this contributes to calcification. Therefore it is plausible that this translates to the results described within, whereby salts from the PBS electrolyte adsorb on the Nafion[®] surface after repeated exposure, compromising its integrity over time.

Table 1. Shelf-life data for various platinum (Pt)-modified electrodes. Electrodes calibrated on day 1, 3, 7, 14, 28, and 56. Data represents maximum current recorded at 1000 μ M ascorbic acid (AA) for each electrode type ($n = 4$). “★” denotes level of significance against day 1. “ns” denotes no significant change.

Day	Pt-PPD			Pt-Nafion [®] (5/2)			Pt-Nafion [®] (1/2)-PPD			Pt-Nafion [®] (2/1)-PPD		
	Mean I (pA)	SEM	Stats	Mean I (pA)	SEM	Stats	Mean I (pA)	SEM	Stats	Mean I (pA)	SEM	Stats
1	138	19		131	29		−59	10		2	10	
3	147	10	ns	260	211	ns	141	107	ns	8	1	ns
7	150	53	ns	592	328	ns	2583	944	★	473	156	★
14	590	224	ns	1254	902	ns	2910	1000	★	1328	408	★
28	1847	684	★	1598	1099	ns	3299	1049	★	2231	594	★★
56	2707	1031	★	3239	1175	★	6539	1504	★★	4434	665	★★★

Previously, a number of groups have investigated the simultaneous combination of Nafion[®] and PPD on carbon fiber electrodes in the expectation of improving sensor performance [6,16,17,39]. Two variations of this polymer combination were investigated and the results are presented in Table 1. Briefly, for Pt-Nafion[®](1/2)-PPD modified sensors the maximum current recorded at 1000 μ M AA on day 3 (141 ± 107 pA, $n = 4$) demonstrated no significant difference ($p > 0.05$) from day 1 (-59 ± 10 pA, $n = 4$). In contrast, the remaining day 7 (2583 ± 944 pA, $n = 4$), day 14 (2910 ± 1000 pA, $n = 4$) and day 28 (3299 ± 1049 pA, $n = 4$) did reveal a significant difference ($p < 0.05$), as did day 56 (6539 ± 1504 pA, $n = 4$, $p < 0.01$). The negative current below baseline levels on day 1 can be attributed to zero response from AA aliquots and the background current drifting below the previously recorded baseline value over the time scale of the calibration. Similarly, for Pt-Nafion[®](2/1)-PPD modified sensors the maximum current recorded at 1000 μ M AA on day 3 (8 ± 1 pA, $n = 4$) demonstrated no significant difference ($p > 0.05$) from day 1 (2 ± 10 pA, $n = 4$). However, day 7 (473 ± 156 pA, $n = 4$, $p < 0.05$), day 14 (2910 ± 1000 pA, $n = 4$, $p < 0.05$), day 28 (2231 ± 594 pA, $n = 4$, $p < 0.01$) and day 56 (4434 ± 665 pA, $n = 4$, $p < 0.0001$) all resulted in significant differences from day 1. These investigations suggest that modifying Pt electrodes with a combination of Nafion[®]/PPD yielded a less stable membrane which may be due in part to the process of electropolymerising PPD onto a non-uniform Nafion[®] pre-coat surface as opposed to a bare Pt surface. Friedemann *et al.* reported that Nafion[®] must be applied prior to the electropolymerisation of PPD because if applied in the reverse order, the selectivity profile is greatly diminished. It was suggested that the alcohols in which Nafion[®] is dissolved was removing the PPD layer and compromising functionality [6]. It is apparent from Figure 3 that the annealed Nafion[®] pre-coat layer on both designs is disproportionate which theoretically, may result in an inefficient subsequent PPD electropolymerisation process. This is in stark contrast to

the PPD layer grown on bare Pt which can be controlled more efficiently due to the self-limiting characteristic associated with the polymer during the electropolymerisation process. A number of groups report the thickness of PPD polymers to be between 10 and 30 nm [12,40,41]. Similarly, Nafion[®] has been electrodeposited onto carbon electrode surfaces as a method of incorporating a uniform reproducible layer to ameliorate issues associated with dip coating and annealing [3,32,42]. However, despite an improvement in Nafion[®] membrane uniformity and thickness, selectivity against cationic species including the catecholamines was insufficient.

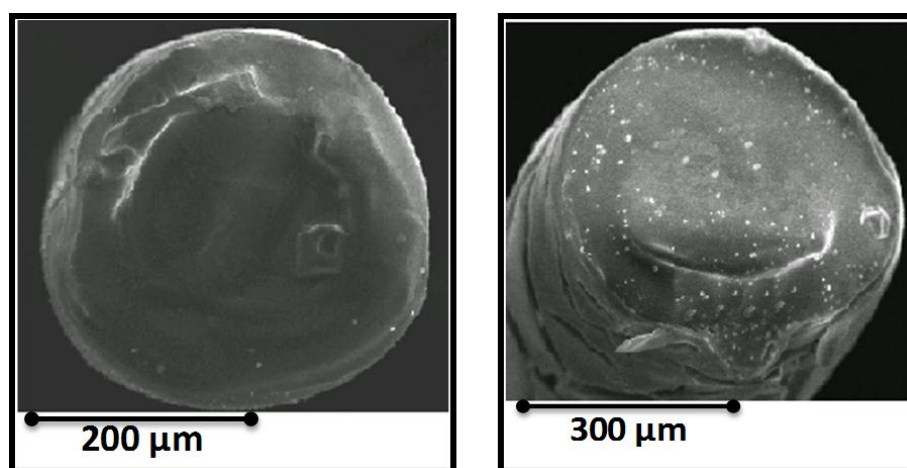


Figure 3. Scanning Electron Microscopy (SEM) of **(Left)** Pt-Nafion[®](1/2)PPD modified electrode, 200 μm dimensional bar **(Right)** Pt-Nafion[®](2/1)PPD modified sensor, 300 μm dimensional bar.

We hypothesise that this weakened PPD layer deteriorates more rapidly following repeated exposure to calibrations and storage, more than likely due to a compromised PPD electropolymerisation process. The exposed pre-coats of Nafion[®] will undergo cracking as described earlier resulting in larger AA currents detected over time. Based on these findings an alternative method of calibration was investigated which better represents shelf-life studies and removes the discrepancies observed with repeated exposures.

3.2. Shelf-Life Studies—Effect of Single AA Calibration

To confirm the detrimental impact of repeated calibrations on the integrity of the respective polymer modified electrodes, it was decided to investigate the effect of a single AA calibration after 56 days storage. Sensors were initially calibrated on day 1 as described previously and stored in an enclosed container at 4 $^{\circ}\text{C}$ for 56 days. A second calibration was thus performed on day 56. Figure 4 illustrates current concentration profiles for 0–1000 μM AA on Pt-PPD electrodes on day 1 and day 56 respectively. There is essentially no change in AA detection with the maximum current recorded at 1000 μM AA on day 56 (24 ± 2 pA, $n = 4$) demonstrating no significant difference ($p > 0.05$) from day 1 (19 ± 4 pA, $n = 4$).

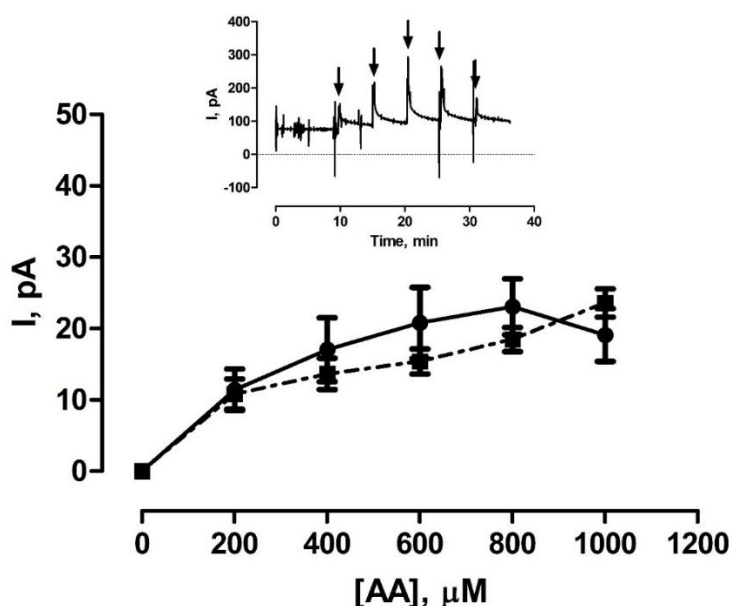


Figure 4. A current-concentration profile for 0–1000 μM AA on Pt-PPD electrodes on day 1 ($n = 4$, solid line) vs. day 56 (4 $^{\circ}\text{C}$ storage, $n = 4$, dashed line) at +900 mV vs. SCE. Electrodes calibrated on day 1 and 56. (Inset) Typical raw data trace. Arrows indicate injection of AA aliquot.

Similar findings were observed for the remaining sensor types which are illustrated in Figure 5. In detail, the maximum current recorded at 1000 μM AA on day 56 for Pt-Nafion[®](5/2) sensors (-5 ± 1 pA, $n = 4$) was not significantly different ($p > 0.05$) from day 1 (-2 ± 1 pA, $n = 4$). As alluded to earlier the negative current below baseline levels can be attributed to the background current falling below the previously recorded baseline value over the course of the calibration. Pt-Nafion[®](1/2)-PPD and Pt-Nafion[®](2/1)-PPD sensors displayed maximum AA currents of 60 ± 30 pA, $n = 4$ and 3 ± 1 pA, $n = 4$ respectively on day 56. These levels were not significantly different ($p > 0.05$) than those recorded on day 1 (6 ± 1 pA, $n = 4$) and (1 ± 1 pA, $n = 4$) respectively. Collectively these findings support the theory that repeated exposure to calibration conditions and subsequent storage compromises the integrity of the various polymers. This theory is further reinforced by findings from a third calibration performed after 168 days on the respective sensors incorporating Nafion[®]. There was still negligible detection of AA at Pt-Nafion[®](5/2) sensors (-22 ± 3 pA, $n = 4$), Pt-Nafion[®](1/2)-PPD (117 ± 40 pA, $n = 4$) and Pt-Nafion[®](2/1)-PPD (-32 ± 7 pA, $n = 4$) after this extended period. There is substantial improvement in the AA rejection characteristics of all sensor types when compared against the repeated exposure detailed in Table 1. These findings postulate that the longevity of these polymer modified electrodes is in fact dictated by the frequency of exposure to calibration protocols and the interferent molecule as opposed to the duration left on the shelf. This bodes well for the manufacture and storage of these sensors for *in vivo* investigations and eventual commercialisation.

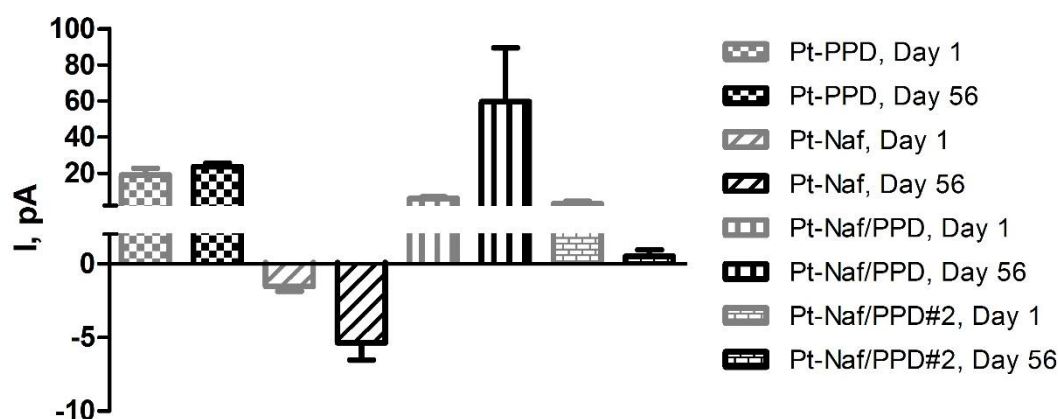


Figure 5. Effect of a single calibration on the shelf life of various Pt electrodes (Pt-PPD, Pt-Nafion[®](5/2), Pt-Nafion[®](1/2)-PPD, and Pt-Nafion[®](2/1)-PPD. Data represents maximum current recorded at 1000 μ M AA for each electrode type ($n = 4$).

3.3. Effect of Various Storage Conditions on Shelf Life of Pt-PPD Electrodes

The work described thus far involved storing Pt-PPD sensors at 4 $^{\circ}$ C in an enclosed glass cell container. A popular protocol employed by a number of different research groups involves storing sensors in PBS at 4 $^{\circ}$ C [29,43,44], however, based on findings from this study it appears that these conditions may deteriorate the polymers. To investigate this, Pt-PPD sensors were exposed to a number of different storage conditions and the results are detailed in Table 2.

Table 2. Shelf-life data for Pt-PPD electrodes exposed to various storage conditions (4 $^{\circ}$ C, 4 $^{\circ}$ C/N₂ saturated PBS, 4 $^{\circ}$ C/N₂ saturated, and -20° C). Electrodes calibrated on day 1, 56, and 168. Data represents maximum current recorded at 1000 μ M AA for each electrode ($n = 4$). “*” denotes level of significance against day 1. “ns” denotes no significant change.

	4 $^{\circ}$ C			4 $^{\circ}$ C/N ₂ Saturated PBS			4 $^{\circ}$ C/N ₂ Saturated			-20° C		
Day	Mean I (pA)	SEM	Stats	Mean I (pA)	SEM	Stats	Mean I (pA)	SEM	Stats	Mean I (pA)	SEM	Stats
1	19	4		18	7		251	27		139	23	
56	24	2	ns	1123	121	***	295	105	ns	804	302	*
168	3490	1276	*	4057	122	***	222	131	ns	1706	615	*

As discussed previously for Pt-PPD electrodes stored in an enclosed container at 4 $^{\circ}$ C, there is essentially no change in the AA levels detected with the maximum current recorded at 1000 μ M AA on day 56 (24 ± 2 pA, $n = 4$) demonstrating no significant difference ($p > 0.05$) from day 1 (19 ± 4 pA, $n = 4$). However, extending the investigations out to 168 days (3490 ± 1276 pA, $n = 4$) resulted in a significant difference ($p < 0.05$) being observed. This considerable increase in AA detection suggests that re-storing the PPD modified electrodes subsequent to the previous calibrations has greatly affected the polymer layer. A possible explanation might be due to drying of the layer, producing a brittle composition which gradually weakens over time.

To examine if storing the Pt-PPD electrodes dry had an adverse effect on polymer integrity, a separate set were stored in N₂ saturated PBS at 4 $^{\circ}$ C over the same time course. The maximum currents recorded at 1000 μ M AA on day 168 (4057 ± 122 pA, $n = 4$) and day 56 (1123 ± 121 pA, $n = 4$) were significantly

different ($p < 0.0001$) from day 1 (18 ± 7 pA, $n = 4$). This clearly highlights the fact that storing the PPD modified electrodes in solution greatly compromises the interference rejection characteristics and shortens the shelf-life dramatically. This is supported by work undertaken by Gyurcsanyi *et al.*, whereby they evaluated the effect of different storage conditions on the stability of the PPD size exclusion layers under dry and wet conditions. They reported that none of the dry stored electrodes showed measurable current in the presence of ascorbic acid and acetaminophen, however, these studies were only conducted over a 15 day period [45]. Further to this, Sasso and colleagues investigated the effect of storing PPD coated electrodes in 0.1 M, pH 6.5 phosphate buffer at 4 °C. The polymer film degradation was followed for 62 days and the interference current increased linearly at a rate of 4.26×10^{-2} µA/day [46].

Based on this rationale, it was decided to investigate the effect of N₂ saturating the glass container for 20 min prior to enclosing the electrodes and storing them at 4 °C. There was a dramatic improvement in the longevity of the Pt-PPD electrodes stored at these conditions. Maximum currents recorded at 1000 µM AA on day 168 (222 ± 131 pA, $n = 4$) and day 56 (295 ± 105 pA, $n = 4$) were not significantly different ($p > 0.05$) from day 1 (251 ± 27 pA, $n = 4$). This significant improvement in shelf-life postulates a contribution of O₂ to the gradual deterioration of the PPD polymer layer associated with previous storage conditions. Findings from our laboratory strongly support this theory and the PPD electropolymerisation protocol routinely utilised stringently ensures that O₂ interference is maintained at zero. We hypothesise that a tightly regulated O₂ free polymerisation will ensure smaller pore sizes scattered throughout the PPD layer guaranteeing lower levels of AA diffuse across to the electrode surface over extended periods.

Finally, a set of Pt-PPD electrodes were stored at −20 °C to determine the effect of freezing on the polymer matrix. The maximum currents recorded at 1000 µM AA on day 168 (1706 ± 615 pA, $n = 4$) and day 56 (804 ± 302 pA, $n = 4$) were significantly different ($p < 0.05$) from day 1 (139 ± 23 pA, $n = 4$). It is obvious from these results that storing the electrodes at such a low temperature has had a detrimental effect on the rejection characteristics of the polymer layer and should be avoided.

4. Conclusions

The body of work described within clearly demonstrates that exposing various polymer modified electrodes to repeated AA calibration protocols compromises their shelf-life over a 56 day period. Contrary to this, a single AA calibration performed post manufacture prior to storage, demonstrated no significant difference from a second calibration performed 56 days later. Further, a number of different storage conditions for Pt-PPD electrodes were investigated over an extended storage period. It was clearly evident that storing the electrodes dry, in a N₂ saturated glass container at 4 °C ensured the polymers integrity was retained after 168 days. These findings are extremely positive and highlight the ability to manufacture and store various polymer modified electrodes suitable for *in vivo* deployment at a later date. Such insight is invaluable if considering commercialising the sensor technology for industrial deployment. Elucidating the optimum conditions for storage and transport are a prerequisite for a successful company.

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Author Contributions

Andrea Wynne carried out all of the experiments detailed within this article. Niall Finnerty compiled the paper based on this data.

Conflicts of Interest

The authors declare no conflict of interest.

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