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Determination of stability characteristics for electrochemical biosensors via thermally accelerated ageing

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ABSTRACT

Biosensors are devices that are prone to ageing; this phenomenon can be characterized as a decrease in signal over time. Biosensor stability is of a crucial importance for commercial success and as biosensors are presently being applied to an increasing and variety of applications. Stability characteristics related to shelf life, reusability and/or continuous use stability are often poorly investigated or unreported in literature, yet are important factors. Instability or ageing can be accelerated at an elevated temperature; Arrhenius (exponential) and linear models were investigated in order to propose a novel method for rapid ageing characteristics determination. Linear correlation proved more suitable with higher coefficients of determination than exponential correlation. Degradation rate is linearly dependent on temperature and by utilizing the proposed models, long term shelf life of a biosensor can be determined in 4 days and continuous use stability in less than 24 h. Reusability studies are found to correlate poorly due to the unpredictable nature of biosensor handling. Basic constructed screen printed electrode glucose oxidase biosensors were used as a model biosensor in order to propose models for shelf life, reusability and continuous use stability.

1. Introduction

Biosensors are currently being exploited in several very different fields and applications ranging from basic biochemical/environmental analysis [1–4], food [5,6] and industrial processing, biosecurity [7], pharmaceutical [8] to personalized diagnostics [9–12] and medical research [13–15], with more than a few commercial products available on the market [16]. Stability of a biosensor is of a crucial importance, be it a biosensor for monitoring malolactic fermentation in wine [17], magnetostrictive detection of *Salmonella typhimurium* [18] or blood glucose monitoring [19]. The view on ageing is industry and product specific – a battery producer would view ageing as a decrease in energy capacity retention [20], food and cosmetic industry would refer to bacterial growth or ingredient oxidation [21], while in the pharmaceutical industry it is not only the depletion of the active ingredient but also the occurrence and increase in the concentrations of impurities that is considered as an effect of ageing [22]. In the face of the widely available reports on the stability of the biological components of biosensors i.e. for enzymes [23,24] and antibodies [25–27], less than a couple of these collective reports feature the stability of biosensors as a whole system.

Biological products of any kind are prone to ageing and biosensors are no exception. What is more, the ageing of a biosensor, or product stability, is an important limitation to commercial success [28]. Biosensor ageing is referred to as a decrease in sensitivity over a period of time. Ageing mechanisms of a biosensor or decay in sensitivity are complex and affect each layer and reagent used. Biosensor ageing is therefore the sum of total changes in the functionality of the complex, be it the biological component, be it an enzyme [23,24] or antibody layer [25–27], signal mediator (e.g. Prussian Blue [29,30]) or protective membrane (e.g. Nafion) [31]. The purpose of present work is not to investigate the ageing phenomena and mechanisms due to the complexity of the system, but rather provide a simplistic protocol and model for quick assessment of ageing characteristics for biosensor devices.

Arrhenius was among the first scientist that mathematically recorded and physically justified the effect of temperature on chemical and biochemical reactions, including protein denaturation, oxidation, hydrolysis and other phenomena and mechanisms of ageing. Currently, Arrhenius type extrapolation for rapid tests at elevated temperatures is the most commonly used method to determine long time, low temperature ageing stability of a certain product [32].

Despite the frequent use of the Arrhenius model for stability studies, it is often criticized as the exponential fitting is rarely optimal.

Abbreviations: SPE, screen printed electrode; WE, working electrode; RE, reference electrode; CE, counter electrode; PB, Prussian blue; m, mass; V, volume

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In the food industry, Schaal oven stability test is often conducted [33–37]. Relying on the assumption of a linear correlation of accelerated ageing e.g. a test object incubated at 60 °C for one day equates to the amount of ageing that corresponds to a month stored in a 20 °C environment [21].

Biosensor ageing is a relatively well investigated field; some studies have been reported, although most remain commercial secrets [28]. The vast majority, if not all biosensor stability research is focused only on single use and shelf-life estimations, therefore, lacking the findings of "in use" ageing that is essential for novel applications such as use in semi and/or permanently integrated biosensor devices for environmental monitoring, bioprocessing and other on line or point of care use. It has been empirically noted that biosensor ageing is strongly dependent on the application and handling. Applying these differences, three scenarios were investigated in the scope of the present work: 1) well known shelf-life estimation, 2) reusability (repetitive usage and storage) and 3) continuous use (online detection of ageing while the sensor is in use). All measurements were tested with both Arrhenius (exponential) and modified Schaal (linear with several measurement points); the best fitting accelerated ageing temperature model option was selected in order to find the most suitable protocol that can be applied when studying and determining the stability and usability of a biosensor over a period of time due to age.

2. Materials and methods

2.1. Chemicals and materials

All reagent used were of analytical grade unless and commercially sourced from Sigma Aldrich (Darmstadt, Germany) unless otherwise stated: phosphate buffered saline (PBS) 100 mM, pH 7.4 tablets, iron (III) chloride, glutaraldehyde, glucose oxidase (GOx) from *Aspergillus niger* VII S (195 U mg 1) (E.C. 1.1.3.4), Albumin from bovine serum(BSA), Nafion 117, hydrochloric acid (Merck Millipore, Germany), potassium ferricyanide (Baker, Holland), D(+)-glucose anhydrous (VWR, Belgium)). Reverse osmosis (RO) water was prepared using Elga system (Elga LabWater, UK). Experiments were conducted using screen printed electrodes SPE (3 mm diameter graphite working electrode, graphite counter electrodes and sliver reference electrode) purchased from ECOBIO lab (Florence, Italy), m-stat potentiostat (PalmSens, Netherlands) and a QX3 digital microscope (Digital Blue, USA).

2.2. Fabrication of glucose oxidase biosensors

Prussian blue (PB) modified screen printed electrodes (SPEs) consisting of three electrodes: working electrode [WE], counter electrode [CE] and reference electrode [RE], were taken and the WEs were carefully modified in defined steps to produce a biosensor for glucose detection. Using a modified Ricci et al. [29,38] PB modification method, potassium ferricyanide and ferric chloride were separately diluted to 0.1 M concentrations in 10 mM aqueous solution of hydrochloric acid and then mixed together in a ratio of 1:1, forming Prussian blue. 10 µL of freshly prepared mixture was deposited onto working electrodes, fully covering the WE and incubated in the darkness for 15 min, followed by rinsing with 10 mM hydrochloric acid and copious amounts of deionized water. PB electrodes were then baked in the oven for 1 h at 100 °C. After cooling down to room temperature, 2.5 µL of 1% (V/V) glutaraldehyde (diluted with deionized water from 25% stock) was then deposited onto the surface of the working electrode and left at room temperature in the darkness to dry. For the final sensor layer, a cocktail consisting of 0.1% (V/V) Nafion 117 (diluted in distilled water from 5% (V/V) stock), 0.24 IU glucose oxidase (from powder, dissolved in deionized water) and 5% (m/V) BSA (dissolved in deionized water) was prepared. 3 µL of this cocktail was then deposited onto the surface of the WE and left to dry at 8 °C at 40% relative humidity overnight.

2.3. Shelf-life determination

For determining the shelf-life of the fabricated biosensors, 45 glucose oxidase biosensors were individually vacuum packed and each set of 15 was stored at different temperatures: room temperature (controlled, stable at 22 °C), 40 °C and at 50 °C. After every 24-h period, three biosensors from each of the three different temperatures were taken, unpacked and their enzyme activity response to glucose substrate was monitored (amperometrically at 0.06 V) at 0 and 1.5 mM glucose concentrations logged at 10 Hz for 100 s – in 20 mL of PBS or 1.5 mM glucose in PBS, respectfully. Each measurement was made in triplicate. The experiment lasted 96 h.

2.4. Reusability determination

The study to determine the reusability of the glucose biosensors, individually wrapped screen printed electrodes were divided into 5 groups of 3 and stored at different temperatures (fridge at 4 °C, incubator at 20 °C, oven at 35 °C, 45 °C and at 55 °C). After every 24 h, biosensors were taken out and their responses in 20 mL of PBS at glucose concentrations of 0 and 1.5 mM were logged for 100 s (0.06 V at 10 Hz). This experiment lasted 196 h.

2.5. Continuous use stability determination

For biosensor continuous usability determination, the experimental setup was conducted in an incubator; an individual sensor was submerged in 20 mL of 1.5 mM solution of glucose in PBS. Timed measurements were logged every 5 s for 24 h or until the stabilization of the signal fell to 0.

2.6. Equations and temperature accelerated ageing models

In cases for the determination of shelf-life and reusability, the signal time data logged between the 90th and 95th second was averaged to give a single data point:

$$\bar{I}_a = \frac{\sum_{n0}^{n1} I_i}{n} \tag{1}$$

Where $\bar{I}_a$ is the mean signal, n_0 and n_1 denote the time brackets of the signal averaged (90 and 95 s), I_i a signal at a given time point and **n** the number of measurements (5 s at 10 Hz, hence 50 measurements). As each measurement was repeated three times ($\bar{I}_a$, $\bar{I}_b$ and $\bar{I}_c$, respectfully), the mean of those was considered as data point, $\bar{I}$.

$$\bar{I} = \frac{\bar{I}_a + \bar{I}_b + \bar{I}_c}{3} \tag{2}$$

In this manner, two values were determined – $\bar{I}$ at 0 mM and at 1.5 mM, marked $I_{x,0}$ and $I_{x,1.5}$, respectfully. $I_{x,0}$ was subtracted from $I_{x,1.5}$, yielding one signal for one sensor for one time point. However, as three sensors are tested in parallels, the standard deviation (S, Eq. (3)) is also calculated and used for plotting and further calculations.

$$S = \sqrt{\frac{\sum (I_i - \bar{I})^2}{N - 1}} \tag{3}$$

where I_i is a discrete measurement, $\bar{I}$ the average of the string of measurements and N the number of data points.

100% of the signal at time point 0 is considered the signal of a fresh, stabilized biosensor before any decay is detectable. In case of continuous use ageing experiment, linear sections of the raw data are used directly for further calculations.

Two models have been investigated – one following Arrhenius ageing model (Eq. (4a)) (exponential regression of data points – Eq.

(4b)) and the other investigating linear regression of data points (Eq. (5)). Here, I stands for the signal from an electrochemical biosensor at a certain time and temperature, I_0 initial signal, k_T temperature dependent degradation coefficient (k_T 's of both models in Eqs. (4) and (5) are not the same) and t is time.

$$lnI = lnI_0 - k_T t \tag{4a}$$

$$\frac{I}{I_0} = e^{-k_T t} \tag{4b}$$

$$\frac{I}{I_0} = k_T t \tag{5}$$

For each experiment, Arrhenius and linear regression of data points of each temperature is calculated – intersecting the ordinate at 100%. Coefficients of determination (R^2) of each data string for both regression models are compared and the one with highest R^2 values was chosen, yielding k_T values (referred to as degradation coefficients). Degradation coefficients are subsequently plotted against the temperatures, undergoing another linear regression analysis (Eq. (6)). This equation is then used for calculating a degradation coefficient for any discrete temperature within the range of validity of the model.

$$k_T = pT + r \tag{6}$$

where p and r values are characteristic for linear regression of the temperature dependence of degradation coefficient and depend on the biosensor and conditions of use only. Inserting k_T from Eq. (6) into Eq. (5) yields Eq. (7) that is ultimately used for time and temperature dependent biosensor signal prediction.

$$I = I_0((pT + r)t) \tag{7}$$

3. Results and discussion

3.1. Fabrication and characterization of glucose oxidase biosensors

Screen printed electrodes have been surface modified into glucose biosensors by Prussian blue modification [38], addition of glutaraldehyde and a cocktail consisting of glucose oxidase, BSA and Nafion. For each fabrication step, a cyclic voltammetry test was conducted between −0.5 and 0.5 V (Fig. 1). While unmodified electrode has no redox peaks in its voltagramme, Prussian blue modified electrode already shows a strong peak at 0.06 V, which is then enhanced by further modifications towards the finished glucose oxidase biosensor.

The protocol that was used for the fabrication of model glucose biosensors is basic and is therefore more prone to ageing. Such sensors

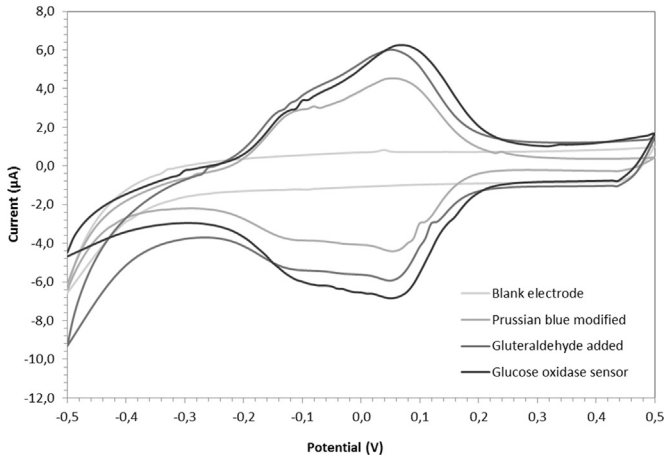


Fig. 1. Cyclic voltammogram of the SPE glucose oxidase enzyme biosensor (−0.5 to 0.5 V), Measurement conducted in phosphate buffer pH 7.4 (0.1 mol^{-1} KCl). Bare working electrode (dotted line), PB modified working electrode (dashed line) and PB-GOx WE (full black line).

are less stable, which elevates the ageing effect, but does not affect the applicability of the models investigated with other biosensors. In fact, more stable biosensors might exhibit better correlations and hence more reliable results of the accelerated ageing tests [28].

As the cyclic voltammetry of the fabricated glucose biosensor shows a peak potential at 0.06 V, this potential was selected to be held for amperometric measurements. Calibration curve was constructed using solutions of glucose in 50 µL droplets of different, known concentrations in PBS, measured in triplicates. The model glucose biosensor was found to have the limit of detection at 0.006 mM with linear range up to 3 mM. Response time t_{90} is 58 s, whilst the intra- and inter-sensor reproducibility's were found to be 4.0% and 5.7%, respectively.

3.2. Accelerated ageing experiments

Three tests have been conducted: 1) shelf-life estimation, 2) reusability and 3) continuous use. For each test and temperature group, data points have been plotted against time followed by linear or exponential fitting. In this way, temperature dependence of ageing is established (Table 1).

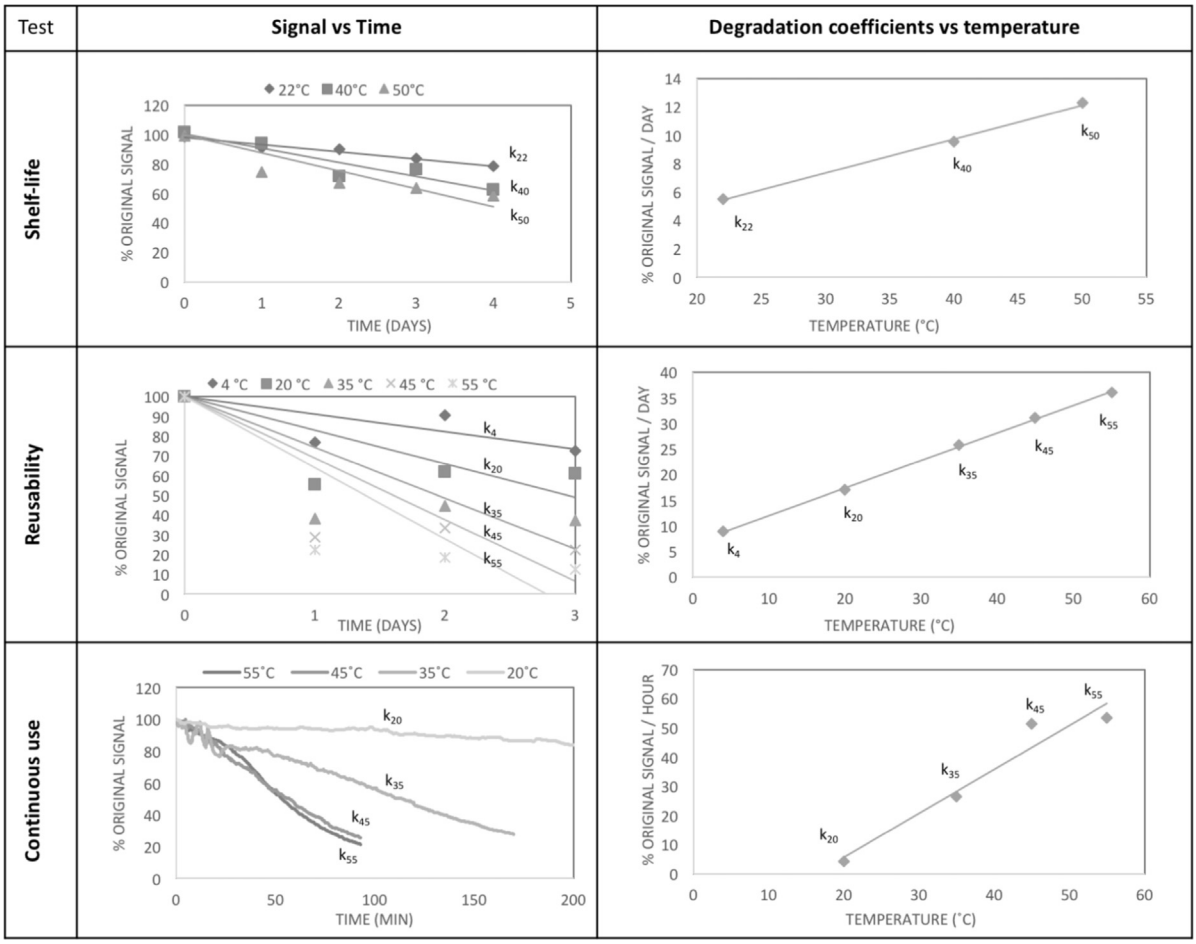
Data points are plotted versus time, followed by linear and exponential regression analysis. Linear regression was selected for further analysis as it yielded higher coefficients of determination in significant studies - shelf-life and continuous use (see Supplementary data, Table S1). Results of the shelf-life estimation experiment have been plotted followed by linear approximation. A statistical test of the regression yielded the p-values of 0.003, 0.018 and 0.029 for the incubations at 22, 40 and 50 °C, respectively. The same statistical test was performed for the continuous use experiments yielding the p-values of 0 for all the incubation temperatures. Both Arrhenius and linear regression models fail to sufficiently approximate the reusability phenomena that is therefore excluded from further calculations.

Shelf-life studies show good reproducibility and linearity of the regression analysis – linear fitting shows higher coefficients of determination than exponential (see Supplementary Data). Since biosensors are individually vacuum packed before storage at elevated temperatures and only used once, ageing can be attributed to the gradual degradation of the enzyme on the working electrode, excluding or limiting other environmental factors (e.g. evaporation, contamination, oxidation etc.). Degradation coefficients can be calculated for any temperature point as the correlation is well characterized, enabling the estimation of the degradation in time given in days and or minutes/seconds. For instance, a packed glucose biosensor on a shelf at room temperature (approx. 20 °C) will degrade 20% in 4 days (see Supplementary data, Table S2). Stability of glucose biosensors tested is not market leading as a very basic and poorly protected biosensors were used in order to elevate the effect of ageing for the investigation of ageing characteristics.

Continuous use ageing studies yield even better results with outstanding linearity of regression analysis. Similarly, to the shelf-life estimation, continuous use stability can also be determined for any temperature. A continuously used glucose biosensor at 20 °C can be expected to degrade 20% in only a little more than 3.5 h (see Supplementary data, Table S3). Compared to shelf-life, this is a 7-fold shorter period. We can therefore conclude that ageing of biosensors is not only affected by the temperature but to other factors like submersion, activity of an enzyme and the potential that is applied to the electrode.

Results of reusability show very poor reproducibility and neither linear nor exponential fitting is suitable – despite the misleading linearity of the plot of degradation coefficient versus temperature (see Supplementary data, Table S3). The reason behind the poor reproducibility is the third aspect of ageing that is irreproducible per se – handling. Scratches, contact with different materials and temperature instability during handling all contribute to the differences in ageing of biosensors.

Table 1
Accelerated aging studies. For each test and temperature group, data points have been plotted against time followed by linear fitting (Signal vs time). [Hence, temperature dependence of ageing is established (Degradation coefficients vs temperature)].



Degradation coefficients can be easily calculated for any temperature as a result of this study; however, some limitations apply. As a certain water content is always present within the layers of the biosensor (originating from air moisture or the final step of biosensor fabrication – incubation at 40% relative humidity) freezing can cause degradation [39,40]. Elevated temperatures beyond 60 °C can, on the other hand, cause rapid degradation of the enzyme. Hence, the validity of the proposed model can be estimated to be between the freezing point and around 60 °C, depending on the enzyme or biological compound used.

Digital microscopy pictures shown in Fig. 2 depict the glucose biosensor working electrode magnified 10 and 60 times through the stages of its life span. It was regimentally observed that when the screen printed electrode is modified into a glucose biosensor, a glassy film is easily seen covering the carbon surface of the circular working electrode. Single use of a biosensor causes no visibly detectible damage to the glazy layer, which changes after the continuous use experiments – the surface is rougher and thinner, the glassiness of the film deteriorates, however the film over the carbon surface is still visible. Reusability studies have caused much more damage of the modified working electrode surface of glucose biosensors; for example, Fig. 2d exposes a deep scratch on the surface of the biosensor, a result of repetitive handling. The random nature of such events translates into poorly correlating results.

4. Conclusions

Biosensors containing a biological component (e.g. glucose oxidase) are bound to age in the same way to other biologicals like food and biopharmaceuticals [21,22]. Ageing of biosensors is seen as a deterioration of signal at a certain concentration of measured analyte. Ageing is dependent on time, handling or mode of use and temperature. Elevation of the latter accelerates the ageing, following the Arrhenius hypothesis. Shelf-life studies are conducted often, however rarely published [28]. Such studies are sufficient for single-use disposable sensors, disclosing little information regarding ageing characteristics for long-term use. Here, in this study we were able to confirm the theory that electrochemical biosensors, regardless of the application, age faster at elevated temperatures. Biosensors can vary significantly between types, labels and fabrication protocols, meaning that the presented results do not apply universally. However, the proposed methodology, procedures and models for determining sensor degradation using accelerated ageing by elevated temperatures are applicable for variety of enzyme biosensors and biosensing techniques.

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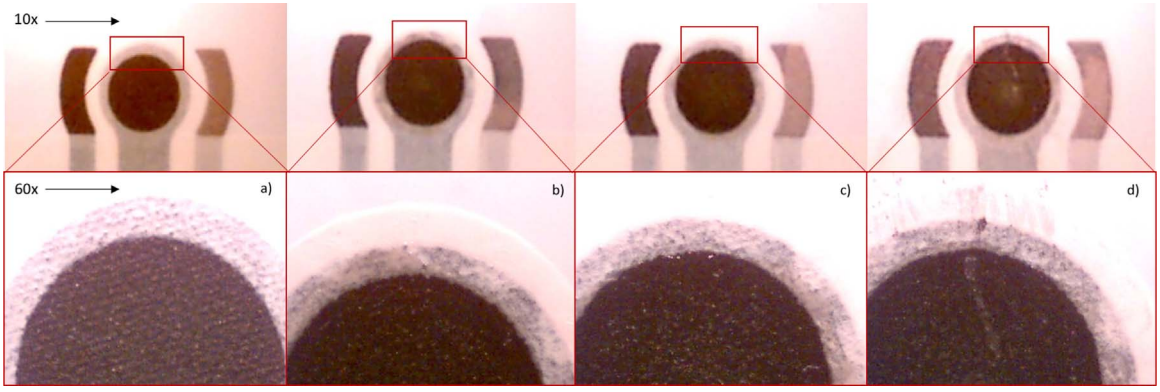

Fig. 2. Microscopy image of used glucose oxidase SPE biosensors (10× and 60× magnification). Biosensor aging: a) Unused, unmodified – blank screen printed electrode. b) Freshly prepared glucose biosensor. c) Glucose sensor after continuous use study. d) Glucose biosensor after reusability study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.04.011.

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