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Proposed model for shelf-life prediction of stabilised commercial enzyme-based systems and biosensors

K. McAteer [a,*], C.E. Simpson [a], T.D. Gibson [b], S. Gueguen [c], M. Boujtita [c], N. El Murr [c]

[a] *Applied Enzyme Technology Ltd., 175 Woodhouse Lane, Leeds, LS2 2AR, UK*
[b] *Enzyme Biotechnology Group, Irene Manton Building, University of Leeds, Leeds, LS2 9JT, UK*
[c] *Electrochemistry Group, LAIEM (CNRS-UPRSA 6006), Faculté des Sciences, Université de Nantes, 2 rue de la Houssinière, BP 92208, 44322 Nantes Cedex, France*

Abstract

The ability to use model systems to predict the shelf-life of biological products is an extremely important area for enzyme manufacturers and the diagnostic industry in particular. Biosensor devices where the biological recognition is carried out by enzymes, antibodies or some other relatively unstable material are also subject to deactivation and therefore have a finite lifetime on storage. This shelf stability is obviously of the highest importance to biosensor manufacturers, however the experimental protocols used to predict the shelf-life of these devices are generally well guarded company secrets and little attempt has been made to standardise such methods. This paper presents a generic model system that is able to estimate shelf-stability to a high degree for first order enzyme deactivations and may be used as a rough guide for screening enzyme based systems, including biosensors where the order and mechanism of deactivation is unclear. Dehydrated glucose oxidase in unstabilised and stabilised forms, solution stabilised antibody–enzyme conjugate and glucose oxidase biosensors are presented as experimental examples. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enzymes; Shelf-life; Biosensor devices

1. Introduction

Enzyme based systems and biosensors are becoming more and more important as analytical tools in both the medical and food industries. One limitation to commercial success is the stability of enzyme based analytical systems and biosensor devices and the methods used to determine or estimate the working lifetime after manufacture. The ability to predict the activity or ‘working shelf stability’ of an enzyme based system or biosensor is extremely important for diagnostic and biosensor producers and may be likened to the need for enzyme producers to know the shelf-life of their product.

In previous publications we have demonstrated the effective stabilisation of biosensor components using combinations of polyelectrolytes and polyalcohols added as soluble enzyme additives [1–4]. The effect of the additives is to produce a stable microenvironment around the enzyme being used, thereby enhancing shelf stability on storage. The type of polyelectrolyte and polyalcohol combination used in

* Corresponding author

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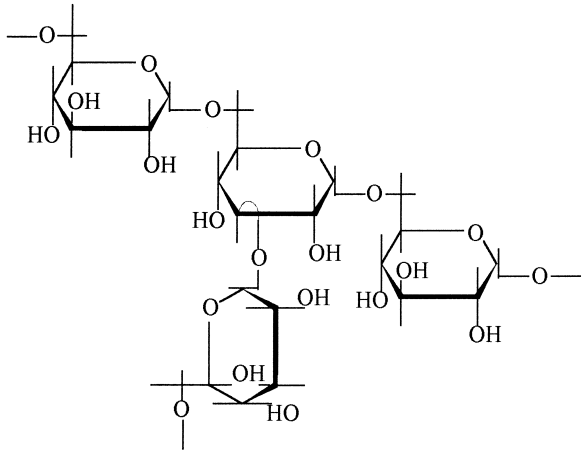
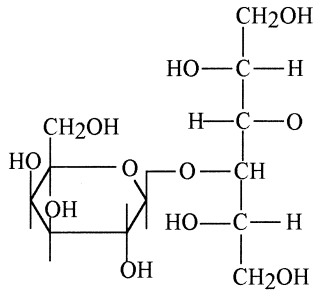
this study was chosen in view of its generic nature in stabilising a range of different enzymes in the dehydrated state [5,6]. Diethylaminoethyl (DEAE)–dextran is a cationic polyelectrolyte commercially available from a number of different suppliers, with an average molecular weight of 10^6 Da. The dextran molecule is substituted at the 2 position to give the cationic groups depicted in Fig. 1. The polyalcohol, lactitol, is a partially reduced from of the disaccharide lactose and is chemically, 4-*O*-β-D-galactopyranosyl-D-glucitol again this is shown in Fig. 1. It is a non-reducing polyhydroxyl compound, very soluble in water (over 4 M solutions can be prepared) and produces a neutral solution. On dehydration, the DEAE–dextran/lactitol combination produces a glass-like film which resolubilises fairly easily.

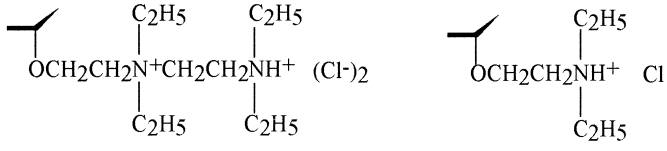
The methodology of using accelerated degradation protocols to predict shelf-life of bioproducts has been used in many instances [7,8], however little attempt at standardisation of experimental regimes has been developed. Each producer appears to follow their own particular model. The following procedure outlines a suggested model for the prediction of the storage times required for the enzyme activity to drop to a predetermined level. It is suggested that the model has limitations, particularly with respect to the order of degradation of the enzyme component of the biosensor device under trial. Very accurate predictions can only be obtained for first order deactivation kinetics, however even with mixed order deactivations of complex systems having multiple components a rough estimate may be able to be given. The model has



Structure of Dextran Backbone



Lactitol



DEAE-Dextran Derivative

Fig. 1. Molecular structure of some stabilising additives. The molecular structures of dextran, showing the types of groups occurring in the substituted cationic polymer, DEAE–dextran and lactitol (4-*O*-β-D-galactopyranosyl-D-glucitol). Combinations of these molecules have been found to stabilise dehydrated enzymes in a generic manner. The pK_a values of the tertiary nitrogens contained in DEAE–dextran are 9.2 for the single substitution in the 2 position, 5.5 for the tandem substitution and the quaternary nitrogen of the tandem group pK_a is around 14.

been applied to a dehydrated glucose oxidase preparation in both unstabilised and stabilised form, enzyme label stability of a solution stabilised antibody–enzyme complex and to an unstabilised glucose oxidase modified carbon paste sensor.

2. Materials

Glucose oxidase (GOx) from *Aspergillus niger* (EC.1.1.3.4) was purchased from either Biozyme (250 U mg^{-1}) or Boehringer Mannheim (256 U mg^{-1}) and horseradish peroxidase (HRP-4), isoenzyme C (250 U mg^{-1}, EC.1.11.1.7) were obtained from Biozyme Diethylaminoethyl (DEAE)–dextran (average molecular weight—10^6 Da) was obtained from Amersham Pharmacia Biotech. Lactitol was obtained from Zyma (UK). Goat anti-human IgA–alkaline phosphatase conjugate (Sigma, A-3063, Lot No: 016H8825), glutaraldehyde and bovine serum albumin (BSA-Fraction V) were obtained from Sigma. Ferrocene was obtained from Strem Chemicals, USA. Carbon powder and paraffin oil was obtained from Fluka Chemicals. Castor oil from obtained from Merck, Germany. Proprietary stabilisation reagents for the solution stabilisation of the IgA conjugate were prepared from formulations obtained from Applied Enzyme Technology.

3. Methods

3.1. Preparation of dehydrated stabilised glucose oxidase

Lactitol and DEAE–dextran, were dissolved in 25 mM sodium phosphate buffer, pH 7.0, at stock concentrations of 40% w/v and 10% w/v, respectively, with the DEAE–dextran being neutralised with sodium hydroxide solution before buffer was added. Glucose oxidase was also dissolved in 25 mM sodium phosphate buffer, pH 7.0, after which the stabiliser solutions were added to the enzyme solution to give a final concentration of 5% w/v lactitol and 0.5% w/v DEAE–dextran respectively. This solution was then aliquotted into UV grade polystyrene cuvettes in 20 μl quantities and then dried in a vacuum oven at 35°C, 0.1 mbar for 4 h. Once dry, the cuvettes were then incubated over fresh silica gel as desiccant at 4°C, 25°C, 50°C and 70°C and several cuvettes were assayed as zero time point references.

3.2. Unstabilised dehydrated glucose oxidase

The dehydrated unstabilised enzyme was prepared in exactly the same way as above but the stabilisers (lactitol and DEAE–dextran) were replaced by phosphate buffer before the drying step. The cuvettes so produced were treated at the same temperatures as the stabilised preparations.

Periodically, cuvettes from both the stabilised and unstabilised preparations, at each storage temperature were removed and allowed to equilibrate to room temperature for 1 h before being tested. A 'Trinder' assay method was used to test the activity of the dried enzyme using 0.4 mM 4-aminoantipyrine, 25 mM phenolsulphonic acid (Na salt) and 2 units ml^{-1} horseradish peroxidase (type 4) [5,9]. The production of the red quinoneimine dye was measured at 505 nm and the rate of reactions were calculated.

3.3. Preparation of solution stabilised goat anti-human IgA–alkaline phosphatase conjugate

The goat anti-human IgA–alkaline phosphatase (IgA-AP) stock solution obtained from Sigma was diluted 1 in 15 from the stock solution into 50 mM Bis–Tris buffer pH 6.5. Solution deactivation was carried out by a further 100 fold dilution of the 1 in 15 dilution into preheated aliquots of buffer, using a temperature range of 30°C to 67°C for the unstabilised samples and 45°C to 67°C for the stabilised

samples. The samples were assayed for enzyme label activity at regular intervals by taking 20 μl aliquots and adding to 5 mM 4-nitrophenyl phosphate in 100 mM 2-amino-2-methyl-1-propanol buffer pH 10.5. The rate of formation of the yellow colour (1 max 405 nm) due to the production of 4-nitrophenol corresponded to the activity of the alkaline phosphatase activity. The stabilisation of the conjugate was done by adding a proprietary stabilisation solution developed by Applied Enzyme Technology, Leeds, UK.

3.4. Glucose oxidase modified CPE

The carbon paste electrodes used were prepared to a preselected patented protocol [10] using glucose oxidase purchased from Boehringer and BSA, glutaraldehyde being used to chemically cross-link the proteins via the amino groups present and ferrocene being added as the redox mediator. The method is briefly described here: The active enzyme powder (1 g quantity) was prepared by dissolving bovine serum albumin (50 mg) in 0.667 ml of distilled water, after which 50 units of glucose oxidase was added. Following dissolution of the enzyme, 0.330 ml of a 0.25% w/w glutaraldehyde solution was added to form a cross-linked gel. The enzyme mixture was stirred for 10 min, after which the 1 g of carbon powder was slowly added and further stirred to allow homogenisation. The mixture was first lyophilised at −20°C in a freeze drier (Camlab) and then further dried in a vacuum desiccator. Once dry, the active carbon powder was crushed in a mortar and pestle, sieved and then mixed with castor oil containing ferrocene (proprietary formulation). The resultant paste was then packed into a plastic tube (cartridge) with an internal diameter of 0.3 cm forming a macro carbon paste sensor (working electrode). An active surface area of 0.07 cm^2 at the bottom of the plastic cartridge is in continuous contact with the analyte solution during the analysis procedure. A graphite rod (0.29 cm diameter) was inserted in one end of the cartridge to form an electrical contact with the sensing element and the measurement device.

All measurements were performed at a working electrode of +0.9 V vs. Pt with a 20 s sampling time, using a Multicapteur MC2 developed at the University of Nantes [11,12]. A number of working electrode cartridges were prepared and stored in equal amounts at 35, 45 and 55°C storage temperatures. Periodically two cartridges were removed and tested with a glucose solutions and the result shown in Fig. 6.

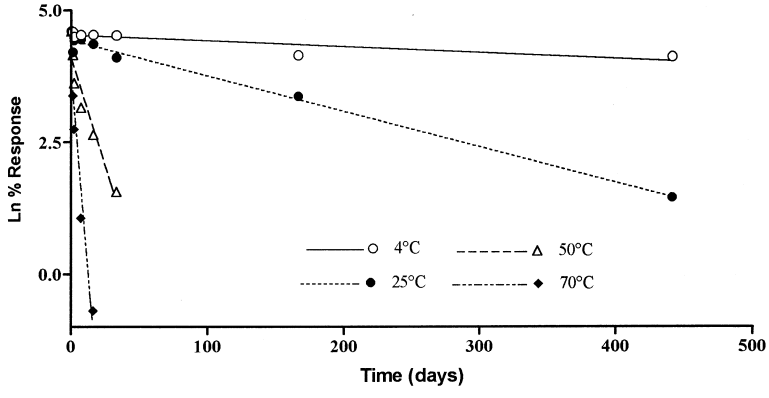


Fig. 2. Semi-log plot of deactivation of unstabilised, dehydrated glucose oxidase preparation. The plot of ln (% Response) vs. Time for stabilised, dehydrated glucose oxidase stored at 4°C, 25°C, 50°C and 70°C, gives an approximately linear relationship. This indicates the deactivation approximates to first order and therefore should substitute well into the predictive model described. The slopes these graphs gave values of K_T at the different temperatures of incubation, allowing a further plot of ln K_T vs. $1/T$ in Fig. 3.

4. Model for shelf-life prediction

In the development of an accurate and viable prediction model, it is assumed that the deactivation process of the enzyme system in question is a first order process and that no intermediates are formed. This is perhaps an oversimplification in real terms, but the assumption allows the use of an *Arrhenius* type plot and provides a basis for simple deactivation profiles which at least approximate to first order kinetics:

$$\ln A = \ln A^{\circ} - K_T t \tag{1}$$

where A is the measured activity of the enzyme either as units per milligram or remaining response, expressed as a percentage of the initial enzyme activity; t is the storage time measured in days; A° is the activity measured at $t = 0$ and K_T is the constant of deactivation calculated for a particular temperature.

The dependence of K_T on temperature (T in Kelvin) can be described as:

$$\ln K_T = (\text{Intercept}) + (\text{Slope})\left(\frac{1}{T}\right). \tag{2}$$

Therefore, by knowing the initial activity of the enzyme at a particular storage temperature and choosing the required enzyme activity level, an estimation of the storage time required to reach that activity can be made using:

$$t = \frac{(A^{\mathrm{d}})(A^{\circ})}{(K_T)(100)} \tag{3}$$

where A^{d} is the decrease in activity; A° is the activity at $t = 0$; K_T is the obtained deactivation constant and t is the estimated storage time.

To use this prediction model with the enzyme forms such as in a dry stabilised preparation or in a solution stabilised form, Eq. (3) has to be modified due to the enzyme activity being presented as a percentage with respect to the initial activity measured.

This results in the following equation:

$$t = \frac{(A^{\mathrm{d}})}{(K_T)(100)} \tag{4}$$

where A^{d} here represents the chosen percentage activity drop.

The models outlined in Eqs. (3) and (4) have been applied to a dehydrated stabilised glucose oxidase preparation, the enzyme label stability of a solution stabilised antibody–enzyme complex (goat anti-human IgA–alkaline phosphatase conjugate) and to an unstabilised glucose oxidase modified carbon paste sensor.

5. Results

5.1. Unstabilised and stabilised dehydrated glucose oxidase

The semi-log plot of deactivation vs. time for glucose oxidase stored in the unstabilised, dehy-

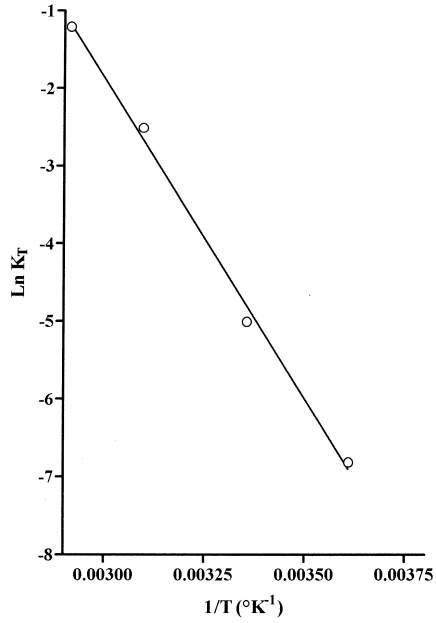


Fig. 3. Plot of ln K_T vs. $1/T$ for dehydrated, unstabilised glucose oxidase. Graph of ln K_T vs. $1/T$ (K), to estimate the K_T value for specific storage temperatures, to give prediction of the shelf-life of dehydrated unstabilised glucose oxidase at any temperature of incubation.

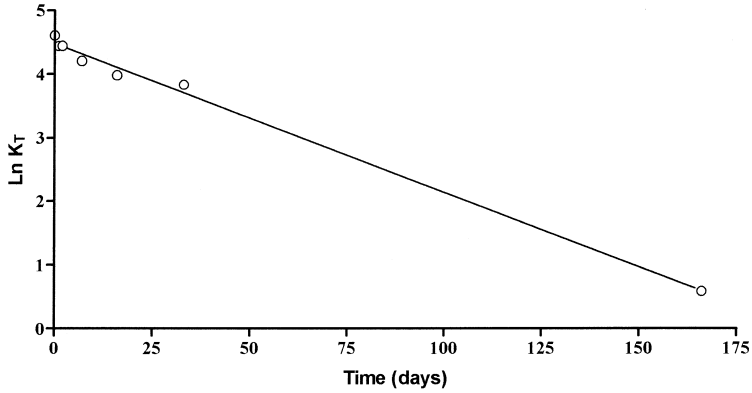


Fig. 4. Semi-log plot of deactivation of unstabilised, dehydrated glucose oxidase preparation stored at 37°C as the model test. The deactivation of dehydrated, unstabilised glucose oxidase is shown at a temperature of 37°C. The deactivation profile approximates quite well to first order kinetics in this case, with an r^2 value of 0.995.

drated form gave the results observed in Fig. 2. From this graph the K_T values can first be calculated for each temperature and these may then be expressed graphically. Plotting the natural log of the deactivation constant against the reciprocal of the temperature results in linear relationship with a slope with an r^2 value = 0.996, as shown in Fig. 3. The K_T value for any temperature may then be calculated by obtaining the intercept on the ln K_T axis and the slope and inserting the values in Eq. (2), for each particular temperature. With known K_T values, a prediction of shelf-stability may then be obtained by inserting the necessary data into Eqs. (3) and (4), depending if an absolute activity or a percentage deactivation model is used.

In order to test this model, a temperature of 37°C was chosen as the storage temperature. Using the slope and intercept obtained from Fig. 3, and substituting into Eq. (2), a K_T value of 0.0024 was obtained, which gave a predicted shelf stability (T_{50}) of 20.81 days at this temperature.

The experimental evaluation of the deactivation of glucose oxidase in the unstabilised dry form was carried out using enzyme dehydrated as described and stored at 37°C for 166 days. The remaining activity was tested periodically. From a plot of ln (% Response) vs. Time (see Fig. 4) a K_T value of 0.00234 was calculated giving a predicted shelf-life of 21.37 days. This is a difference of 2.62% from the estimated value using the prediction model and is therefore a fairly good estimation. This was not found to be the case for the stabilised preparation as the predicted values differed somewhat from those derived experimentally. This indicated that the mechanism of deactivation of the stabilised enzyme was not first order and may even be different in nature to the unstabilised

Table 1
Comparison of experimentally derived and predicted values of K_T and T_{50} values for dehydrated preparations of unstabilised and stabilised glucose oxidase at 37°C

Dehydrated glucose oxidase	K_T experimental values at 37°C (days)	T_{50} at 37°C calculated experimentally (days)	K_T calculated values from Fig. 3 (days)	T_{50} at 37°C calculated from Fig. 3 (days)	Difference
Unstabilised	0.0234	20.81	0.0240	21.37	2.62%
Stabilised	0.00365	136.99	0.00426	117.37	16.7%

Table 2
Comparison of experimentally derived and predicted values of K_T for solutions of goat anti-human IgA–alkaline phosphatase conjugates at 55°C

Goat anti-human IgA alkaline phosphatase conjugate	K_T experimental values at 55°C (days)	T_{50} at 55°C calculated experimentally (h)	K_T calculated values from Fig. 5 (days)	T_{50} at 55°C calculated from Fig. 5 (h)	Difference
Unstabilised	2.316	5.18	2.60	4.62	11%
Stabilised	1.598	7.51	1.855	6.47	14%

preparation. However there is clearly a stabilisation effect observed, the results are summarised in Table 1.

5.2. Solution stabilised goat anti-human IgA–alkaline phosphatase conjugate

Two samples of the goat anti-human IgA–alkaline phosphatase conjugate, one unstabilised and one stabilised, were heated to 55°C and tested periodically for activity of the enzyme label. The calculated K_T values are given below in Table 2. This form of accelerated degradation is very widespread in the diagnostics industry as it gives a rapid estimate of the probable shelf-life of reagents in a very short time. Plotting Ln K_T vs. the reciprocal of the temperature gave good linearity, with an r^2

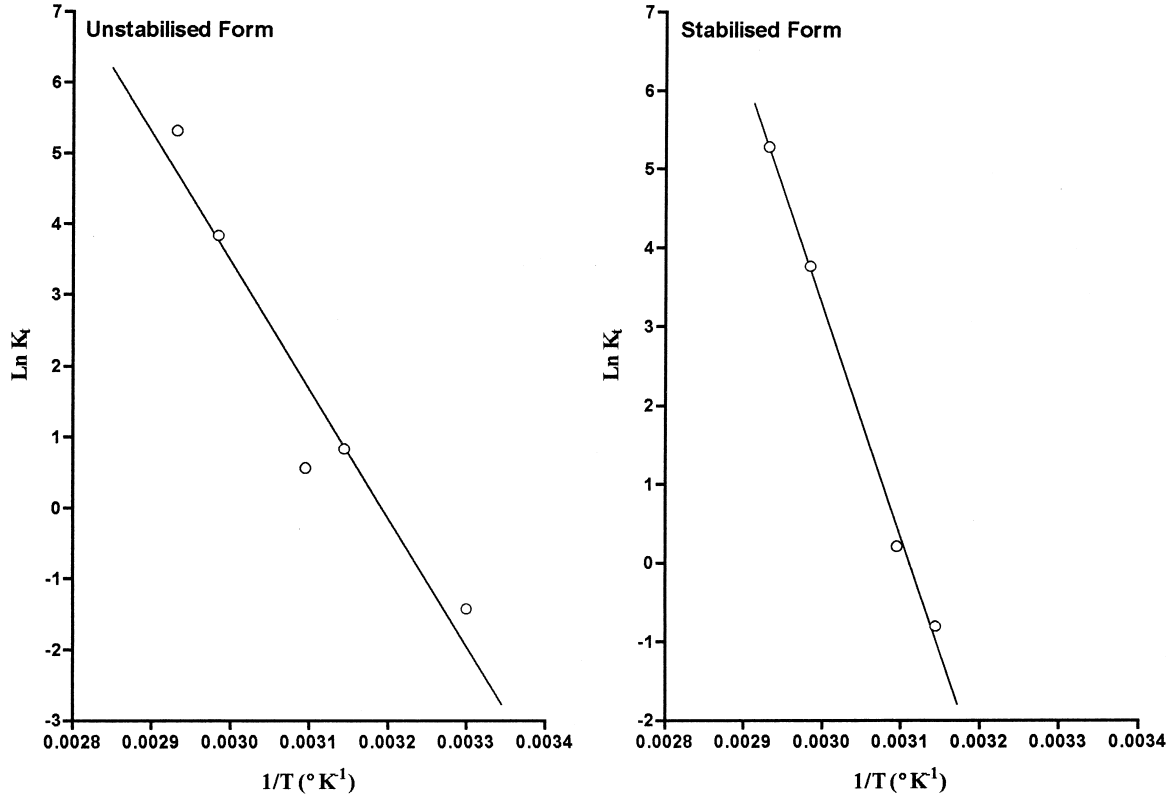


Fig. 5. Plot of ln K_T vs. $1/T$ for control (unstabilised) and solution stabilised goat anti-human IgA–alkaline phosphatase conjugate. Solutions of goat anti-human IgA–alkaline phosphatase conjugate were stressed at 55°C in the presence and absence of stabilisers and the activity of the enzyme label was measured. It can be seen that the stabilisers have a significant effect on the slope and intercept of the graph of ln K_T vs. $1/T$. The r^2 values are 0.9299 for the unstabilised conjugate and 0.9963 for the stabilised conjugate.

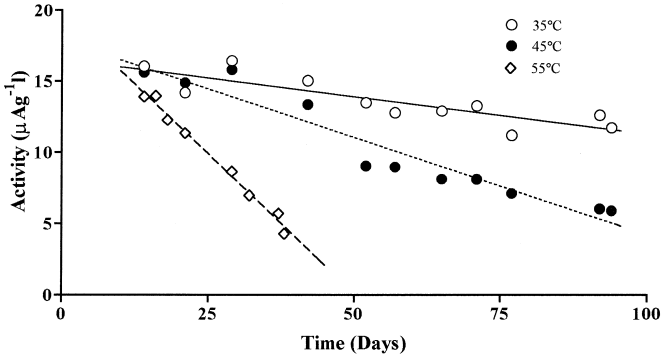


Fig. 6. Activity of glucose oxidase modified carbon paste electrodes stored at three different temperatures. The experimental activity of the glucose oxidase biosensors is shown at three different temperatures. The r^2 values are 0.728 at 35°C, 0.922 at 45°C and 0.988 at 55°C, indicating that on elevating the temperature the deactivation rate tends to cancel out errors in measurement due to variable paste composition. The main error here is likely to be inefficient mixing during paste preparation, leading to variable enzyme loading in the biosensor cartridge and this can be corrected by very thorough mixing of the components of the paste (usually mechanical devices have to be used to produce very accurate results). The results above were derived from pastes that were mixed by hand.

value of 0.9299 for the unstabilised form and 0.9963 for the stabilised form, Fig. 5. This indicates that the method should work well for prediction of shelf-life, even though the estimated deactivation coefficients for both the unstabilised and stabilised forms of the enzyme show some variation of approximately 11–15%. If we use the data to predict the shelf-life of the conjugate at temperatures of 25°C, we get a figure of 9.67 days for the unstabilised solution and one of 650 days for the stabilised one.

5.3. Glucose oxidase carbon paste sensor

Fig. 6 shows a plot of enzyme activity versus time for the three storage temperatures chosen for the carbon paste sensors. As can be seen, a reasonable degree of linearity was obtained with each temperature (r^2 values are 0.728, 0.922 and 0.988 at 35, 45 and 55°C respectively). The experimentally derived deactivation constants obtained were plotted against each of the temperatures as shown in Fig. 7. Using the data in Fig. 7, the values of K_T were calculated for temperatures of 4°C and 20°C, respectively and the predicted shelf-life of the GOx carbon-based sensors was calculated, using Eq. (3).

The initial enzyme activity, A°, recorded at $t = 0$ for these sensors was measured and values of 10% and 20% drop in the enzyme activity A^d were chosen as representative values of sensor

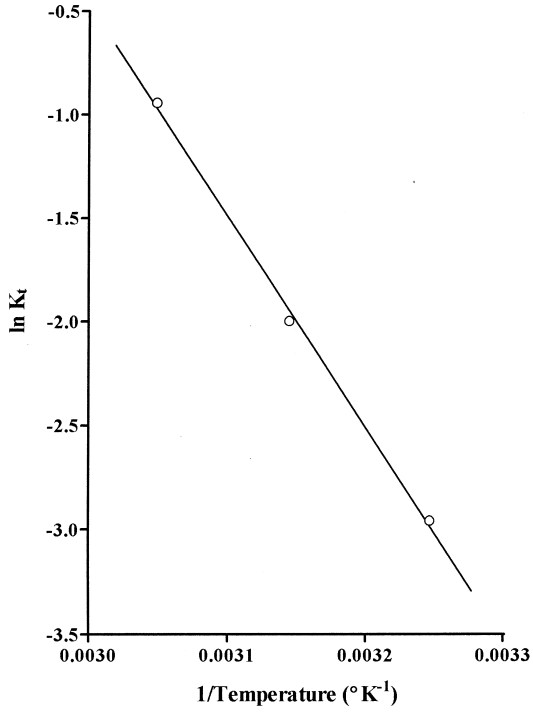


Fig. 7. Plot of ln K_T vs. $1/T$ for glucose oxidase modified carbon paste electrodes. The plot of ln K_T vs. $1/T$ for the glucose oxidase biosensors gives a linear relationship, with an r^2 value of 0.998. This indicates an excellent agreement for the predictive model and has been used to give predicted values of shelf-life which differ by only 2% from the experimental data derived from long term stability trials.

Table 3
Predicted enzyme activity loss for glucose oxidase carbon paste sensors

Initial activity A° (μA g^{-1} l)	Calculated K_T values	Temperature (°C)	Activity loss. A^d (%)	Predicted shelf-life (years)
15.355	0.00126	4	10	3.3
	0.00126	4	20	6.6
	0.00937	20	10	0.46
	0.00937	20	20	0.88

viability, results in Table 3. The validity of the model was then tested experimentally using a number of the GOx carbon paste sensors stored at 20°C and independently tested for a similar period of time.

From the predicted model, it was estimated that when stored at 20°C it would take approximately 10.5 months to lose 20% enzyme activity. When analysed experimentally, it was found that the GOx sensors that were stored at 20°C, showed a 22% drop in activity after 10 months. This is in good agreement with the predicted value.

6. Conclusion

A predictive model for use in the estimation of shelf-life of enzyme based diagnostics and biosensors has been demonstrated. Use of the model in the estimation of shelf-life of unstabilised and stabilised dry enzyme preparations, a solution stabilised antibody–enzyme conjugate and an enzyme modified carbon paste experiments has been carried out and in most cases the predicted results are in good agreement with experimentally derived data. However, some cases revealed a certain variation in the accuracy of the results and in particular, where stabilisers are added to the dehydrated or solution matrix.

One reason for the variability is almost certainly due to the inter-sample variability that is present within all test systems to a certain degree. With the glucose oxidase carbon paste sensor, this variability is possibly due to a lack of consistency within the packed cartridge itself and is possibly related to the paste manufacturing process. The experimental process for the dry preparations of glucose oxidase is rather simpler than for the biosensors, giving less variability from batch to batch, which is reflected in the difference between the estimated and calculated K_T values for the unstabilised preparation, which is only 2.62%. However when the stabilised preparations are examined a large difference in the values is seen, which is unlikely to be due to experimental process variation. This is believed to be due to modified deactivation processes due to the addition of the stabilisers.

One of the problems associated with the prediction model is the necessity for initial studies of stability and the assumption that the degradation seen in the early stages, reflects the later stages of deactivation. The accuracy of the predictive model, depends how accurate these short term studies are, as the longer the continuation of the experimental stability study, the more accurate will be the r^2 values obtained for the linear regression and therefore the more accurate the values of K_T. In all cases where shelf stability is an issue, the prediction of shelf-life should be backed up by the correct long term experimental studies. This method may be useful to give an indication of the real shelf-life of a protein formulation, analytical test or biosensor, but the real test comes under ‘live’ conditions.

The overall conclusion is that the predictive model, though not necessarily absolutely accurate, may be used as a rough estimation of shelf-life of complex analytical systems and biosensors, allowing a rapid take up of new

formulations to be put into more accurate trials for stability estimation.

Acknowledgements

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