

**NPL REPORT
DEPC-MPR-049**

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SCAFFOLD DEGRADATION**

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MARCH 2006



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ELECTROCHEMICAL ASSESSMENT OF TISSUE SCAFFOLD DEGRADATION

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ABSTRACT

Degradation of a commercially available collagen-glycosaminoglycan dermal equivalent material accelerated by exposure to gamma radiation followed by storage at elevated temperatures or exposure to enzymes, i.e. collagenase significantly increases its permeability to a small molecule probe, potassium ferrocyanide.

The time dependent diffusion of the molecular probe through the dermal equivalent material was monitored via changes in the oxidation peak current of cyclic voltammograms. Experimentally such measurements can be made using a simple diffusion cell with the membrane positioned well away from the electrodes or in a specially designed Rank cell where the sample is in direct contact with the working electrode. Both techniques have their advantages and disadvantages.

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ISSN 1744-0270

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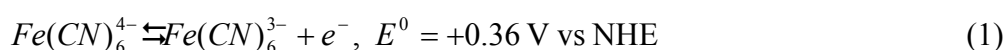
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1. INTRODUCTION

Tissue scaffolds are temporary matrices for supporting cells to grow new, functional living tissue. The materials from which the scaffolds are manufactured range from ‘stiff’ materials such as coral to highly hydrated gel-like materials depending on the intended application. Scaffold materials can be either synthetic or naturally occurring. Synthetic materials can be manufactured with predictable and reproducible chemical, mechanical and physical characteristics, but they tend to elicit an inflammatory response in the host. Therefore, naturally occurring materials such as collagen and glycosaminoglycans have been widely investigated as alternatives tissue scaffold materials^(1, 2). To date there is no consensus as to which are the ‘best’ materials to use for a particular application, which structures provide the optimal environment for cell culture or what are the most appropriate mechanical and chemical conditions. Lack of understanding of the structure of tissue scaffolds and how they change with time is key to the development of optimal designs and materials.

Various techniques have been utilised in an attempt to quantify these often very complex structures including x-ray micro-computed tomography, mercury porosimetry, capillary flow porometry and the analysis of electron micrographs, all with limited success. These limitations are due to a combination of the constraints of the underlying physics of the investigative methods, the difficulty of representing complex structures as simple numerical parameters and the limited length-scale over which the structures are studied. Degradation or remodelling of the scaffold over time is an integral aspect of scaffold performance that needs to be managed such that it happens over an appropriate timescale: too soon and the scaffold/cell complex may lose its mechanical integrity, too long can cause problems with cell integration. Rapidly degrading materials *in vivo* may induce an inflammatory response or have an adverse effect on the stability of the local pH, both *in vitro* and *in vivo*.

Data obtained from experimental methods, such as mercury porosimetry or image analysis that are designed to investigate the structure of tissue scaffolds can be difficult to interpret in terms of how the scaffold will actually perform in service. Measures of pore sizes, porosity and the pore size distribution are useful quantities for developing scaffolds and ensuring consistency between batches but measures of the rate at which different molecular species diffuse through the scaffold may actually be more valuable. In a previous paper we reported the use of a small molecule redox couple, ferri/ferrocyanide, which is a classical, reversible molecular probe used in cyclic voltammetry, a technique that is able to monitor reactions that occur at electrode surfaces³. Such measurements can be used to determine an effective diffusion coefficient for the molecular probe through a material. There are many other well-documented redox systems that can be considered as a standard, but the ferri/ferrocyanide used in this investigation⁴ is readily monitored using platinum electrodes;



Here the NHE is the normal hydrogen electrode against which all standard potentials (E^0) are related. Under conventional solution conditions this redox couple has fast electron transfer kinetics and is a simple single electron reaction without any additional side reactions, giving rise to well defined single oxidation and reduction peaks⁵. This

redox couple is useful for investigating different electrode systems as the solution response is already well characterised. Potassium ferrocyanide (MW = 422.41), although not physiologically relevant can be used as a probe to assess the permeability of porous membranes as a probe compound will be discussed below.

In this report we have extended this work to investigate different approaches to accelerating the rate of scaffold degradation in a collagen-based material used commercially as a dermal substitute. Collagen-based matrices are commonly used materials for skin, connective tissue and peripheral nerve tissue engineering applications^{1,2}. Such materials have an obvious physiological advantage over synthetic polymers since collagen is a natural component of extracellular matrix. Its microstructure and stability also play a crucial role in cell-cell and cell-matrix interactions.

Exposure to γ -radiation is a commonly used method in medicine for sterilising materials. Indeed this is often the only route for sterilising materials that are thermally sensitive and contain significant amounts of water. This approach has a downside in that radiation exposure can lead to chain scission or cross-linking of polymers. The former effect has been well documented for medically relevant materials such as poly(methyl methacrylate) and polyethylene and less well so for natural materials such as collagen. Radiation can potentially induce structural changes in materials that may impact on their performance; which will be enhanced through cross-linking or reduced by chain scission. A technique such as cyclic voltammetry, can be used to detect small changes in structure by monitoring the permeability of the material to a small molecule probe. Other approaches have been used to accelerate degradation of naturally occurring polymers; Pek et al, reported the use of enzymes such as collagenase and chondroitinase to degrade a collagen-glycosaminoglycan (C-GAG) skin substitute⁶. Holly et al, have used pH as a means of accelerating the rate of degradation in PLGA (poly(lactic-co-glycolic acid) scaffolds found that the rate of degradation of PLGA increased at lower pH⁷.

In this report we have used cyclic voltammetry to study changes in the diffusion behaviour of a simple redox couple through collagen-based scaffolds that have been subjected to different doses of γ -radiation followed by accelerated ageing at elevated temperatures or in the presence of enzymes or at different pHs.

2 MATERIALS AND METHODS

A commercially available collagen-glycosaminoglycan (C-GAG) dermal equivalent scaffold nominal (thickness = 1.5 mm) was removed from storage in 70% isopropanol (IPA) and cut into approximately 10 x 10 mm squares prior to being irradiated. The protective silicone cover that is attached to one side of the hydrogel matrix was carefully removed prior to radiation exposure.

2.1 γ -Irradiation

Two groups of C-GAG samples were sealed in small glass containers in the presence of 70 – 30 IPA/water mixture and irradiated at room temperature using a source with an

average homogeneous dose power of 1 kGy/hr. The exposure levels for each sample were 25 and 50 kGy respectively. Non-irradiated samples were used as controls.

2.2 Degradation Studies

γ -Irradiated C-GAG samples (γ -C-GAG) were removed from the IPA/water solution and thoroughly rinsed for one minute in normal saline prior to application. All chemicals used were analytical grade or equivalent and were used as received.

- *Temperature Effect*

γ -C-GAG samples were immersed in PBS (phosphate buffer saline) at physiological pH (pH = 7.4) and incubated for periods of 12, 24 and 48 hours at two different temperatures: i) T = 37°C (physiological conditions); and ii) T = 44°C (inflammatory conditions).

- *Enzymatic Degradation*

γ -C-GAG samples were immersed in PBS containing two different collagenase (Aldrich) concentrations: i) 5 μ g/ml; and ii) 10 μ g/ml. The samples were incubated for periods of 12, 24 and 48 hours under physiological conditions (37°C and pH 7.4). After the incubation period, the samples were removed from the solutions, rinsed thoroughly with deionised water and preserved in a 70/30 IPA/water mixture until required.

- *pH Effect*

γ -C-GAG samples were immersed in phosphate buffer solution (made using PBS tablets, Aldrich) and stored at two different pH's; pH = 7.4 (physiological conditions) and pH = 5 (inflammatory conditions). Samples were incubated at 37°C for periods of 12, 24 and 48 hours.

2.3 Scanning Electron Microscopy

Scanning electron microscopy (SEM) of the reference G-GAG and hydrogels treated by γ -radiation and by collagenase were performed. Specimens of G-GAG were dehydrated by a series of acetone dilutions followed by critical point drying. Finally specimens were gold sputtered and analysed using a 6310 Joel microscope.

2.4 Cyclic Voltammetry

Two experimental approaches, shown in Figures 1 and 2, were used to obtain cyclic voltammograms (CVs). In figure 1, the sample, after rinsing in distilled water and soaking in PBS buffer, was placed over a platinum (Pt) working electrode with a Pt wire as counter-electrode and an Ag/AgCl reference both immersed in the bulk solution above the membrane. The sample in the Rank cell (Rank Brothers, Cambridge, UK) is thus in direct contact with the 12 mm² working electrode. Excess buffer was removed before adding an aqueous solution of 0.1 M potassium ferrocyanide (K₄Fe(CN)₆·3H₂) in PBS at pH 7.4. A software controlled μ Autolab II (Ecochemie, the Netherlands) potentiostat was used to record all voltammetric experiments. All potentials were recorded with respect to the Ag/AgCl electrode. Sequential recordings of the CVs were made taking the time at which the sample was exposed to the redox couple as time zero.

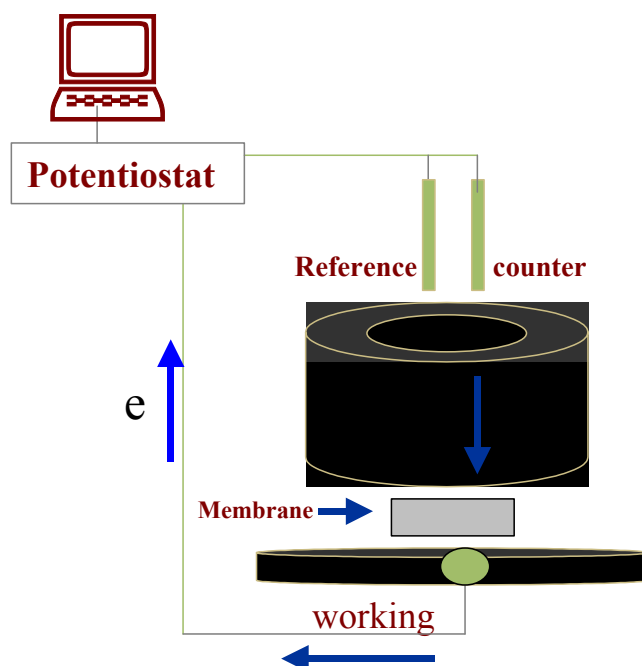


Figure 1. The Rank cell contains a sheet of collagen-GAG matrix that is in direct contact with the Pt working electrode.

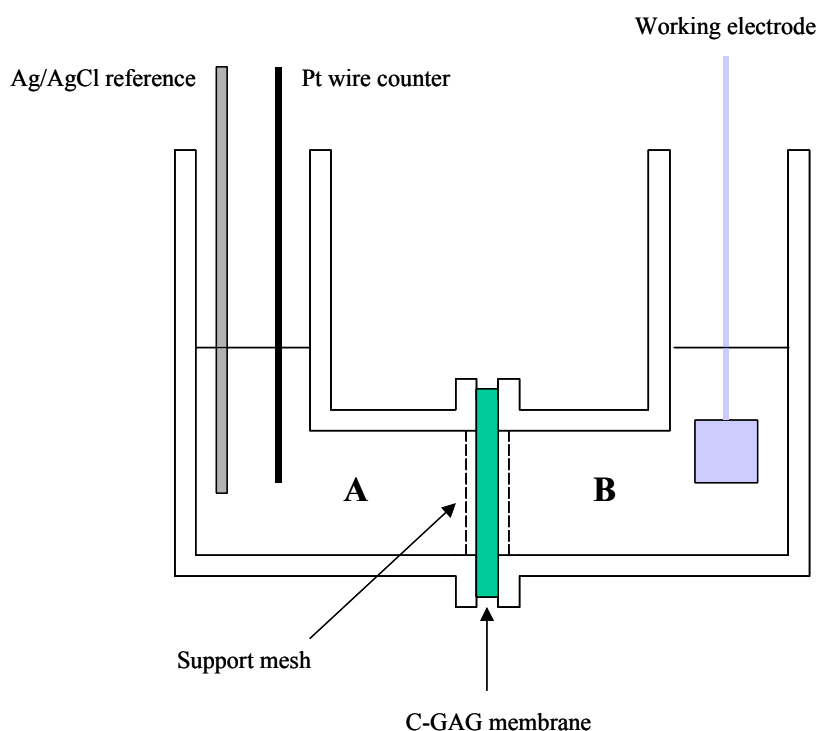


Figure 2. A sheet of collagen-GAG matrix is sandwiched between two sheets of a coarse mesh in a simple diffusion cell. Chamber A contains the redox couple in PBS and chamber B contains only buffer.

A sample with a diameter of 10 mm was used in the simple diffusion cell shown in Figure 2. The sample was sandwiched between two sheets of a coarse mesh used for containing gel-like materials in size exclusion chromatography columns. The mesh served to support the sample and helped to maintain a uniform thickness. The Pt working electrode was sited in chamber B that initially contained PBS buffer. The Pt wire counter-electrode and a Ag/AgCl reference electrode were placed in chamber A which contained the redox couple, 0.1 M dissolved in PBS. Care was taken to ensure that chambers A and B were filled at the same rate with buffer and buffer-redox couple respectively and up to the same level. Care was also taken to ensure that the diffusion cell itself was level to avoid any undesirable bulk movement of fluid through the sample. Small magnetic stirrers placed in each of the chambers were used to gently agitate the fluid between measurements.

3 RESULTS

3.1 Sensitivity of CVs to the Electrolyte Solution

In conventional electrochemical experiments the redox couple is dissolved in an electrolyte solution, typically potassium chloride, KCl. The presence of the electrolyte ensures that mass transport due to migration is minimised and the effects of the electrode's electric field is confined to a small distance from the surface. The electrolyte should be inert in the potential range of the experiment and carries the current of the electrochemical cell and thus minimises any problems due to solution resistance. The electrolyte concentration in PBS buffer is lower than in the KCl experiments and therefore there may be an effect visible from a difference in the solution resistance.

Figure 3 shows CVs obtained using the Rank cell shown in Figure 1 in the absence of a member sample for ferrocyanide in PBS and KCl respectively. The results are typical of a single electron transfer process. The locations of the oxidation and reduction peaks are the same ($\pm 0.325\text{V}$ for oxidation peak and $\pm 0.181\text{V}$ for the reduction peak) between the two solutions, although there is a slight increase in solution resistance when using PBS. However, the voltammogram has good shape and current amplitude and since the PBS system is closer to the buffer systems used elsewhere in this work, it has been used for these electrochemical experiments.

3.2 Scan Rate Dependence of Cvs in the Rank Cell

Figure 4 and 5 show the sensitivity of the CV's to different scan rates for a non-irradiated C-GAG covered electrode and a control. For a reversible reaction, such as that for the redox couple used here, the peak potential should be independent of the scan rate. The peak current should also be proportional to the square root of the scan rate.

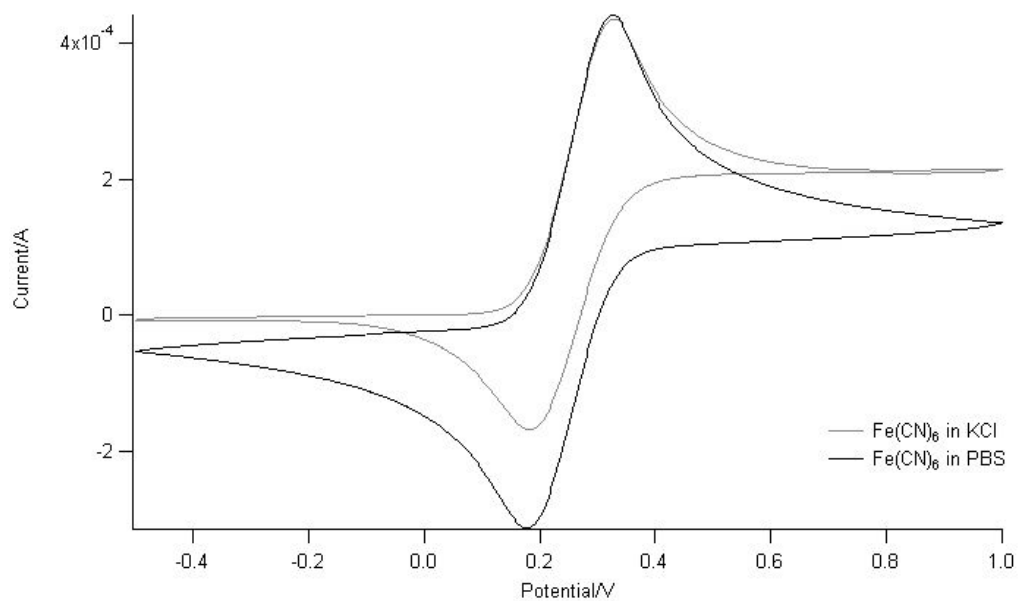


Figure 3. Comparison of CVs measured in KCl and PBS in the absence of a sample measured using a scan rate of 100mVs^{-1} .

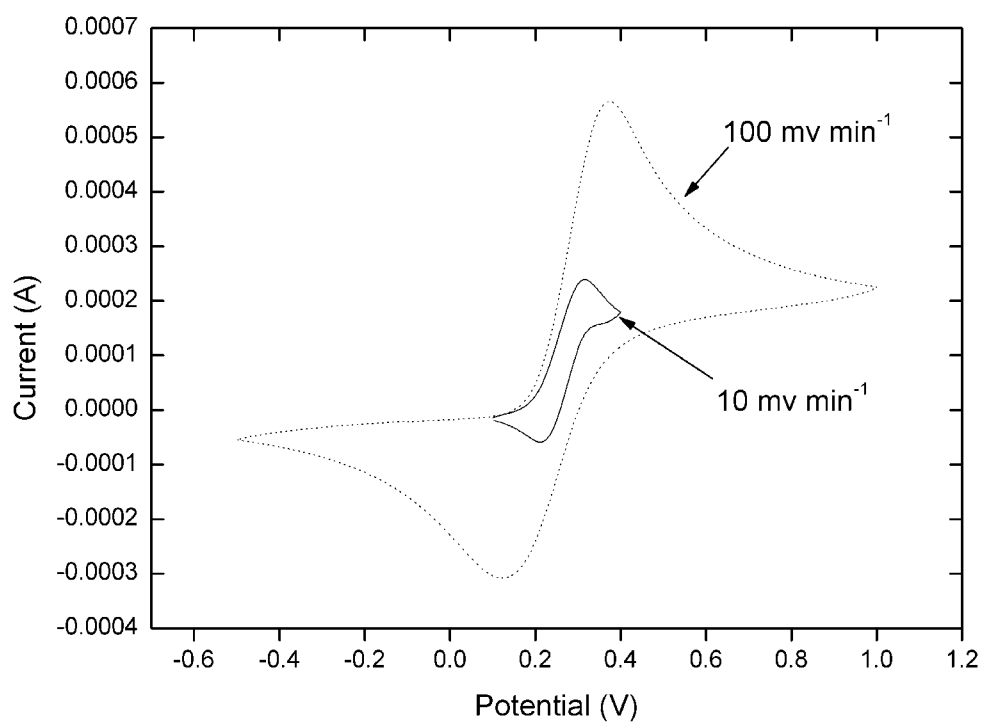


Figure 4. The dependence of CVs on scan rate for a bare electrode Rank cell.

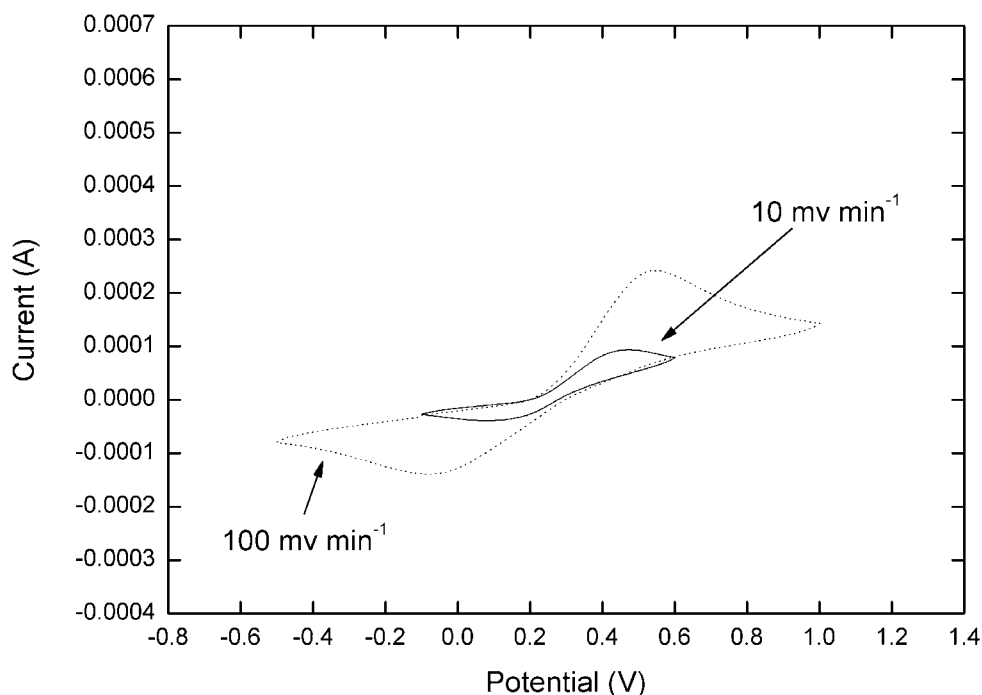


Figure 5. The dependence of CVs for a C-GAG covered electrode on scan rate for the Rank cell. These measurements were made at 20 and 24 minutes respectively after introducing the ferrocyanide/PBS solution into the chamber.

Both figures show that the oxidation and reduction peak potentials do change with scan rate so the system retains has deviated from its reversible characteristic. Given that an essentially aqueous gel phase is in contact with the electrode, alteration to the redox chemistry is highly unlikely. This implies that the differences between the CVs are due to changes in the diffusivity of the electroactive species. Moreover both the oxidation and reduction the peak heights increase with increasing scan rate and if peak height is proportional to the square root of the scan rate we would expect a threefold increase in peak height with a change in scan rate from 10 to 100 mVs^{-1} , in fact there is a loss of this relationship. Also, the peak height for the control is approximately twice that of the C-GAG covered electrode. This also indicates that the sample covering the electrode is likely to be affecting the diffusion of ferrocyanide to the electrode surface.

Subsequent measurements were made using a scan rate of 100 mV^{-1}

3.3 Comparison of Experimental Methods for Determining CVs

The amplitude of the oxidation peak, i_p (A) in a cyclic voltammogram is proportion to the bulk concentration of the redox couple, C (mol cm^{-3}) according to the Randles-Sevcik equation;

$$i_p = (2.686 \times 10^5) n^{3/2} \nu^{1/2} D^{1/2} A C \text{ at } 25^\circ\text{C} \quad (2)$$

where n is the number of electrons involved in the half reaction, D is the diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$), ν is the scan rate (V s^{-1}) and A is the area of the electrode (cm^2)⁶. Equation (2) can be applied to reversible electrode reactions and used to obtain estimates of D , the diffusion coefficient for solute migration to the electrode surface.

The oxidation-reduction processes at the electrode face resulting from changes in the potential between the working and counter electrode are diffusion limited. In practical terms these reactions and ion movements during repeat scans take place within a boundary layer that extends some 200 nm away from the surface of the working electrode. For most of the experimental arrangements described here we can therefore assume that the diffusion coefficient for the redox couple will be the same as that for an aqueous solution i.e. $7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Bard and Faulkner⁵).

Figures 6 and 7 show the time dependent evolution of CVs obtained from both the Rank cell and diffusion cell during the first few minutes of exposing the C-GAG membrane to the redox couple. The amplitude of the current is much less in the diffusion cell system than in the Rank cell system, in fact by an order of magnitude. This difference is due to the dilution of the redox couple by the buffer in chamber B, a practical consequence of these very weak currents is that data obtained at times below approximately 250 s are prone to noise. The dilution problem could be overcome by doubling the concentration of ferrocyanide in the diffusion cell: but this would complicate comparisons of the two approaches, i.e. the buffer concentration would arguably need to be increased. Using a diffusion cell that contains smaller volumes of solution will also not solve the problem. For this reason the Rank cell method would be more suitable for measuring solute diffusion through the C-GAG membrane as lower probe solute concentrations could be utilised.

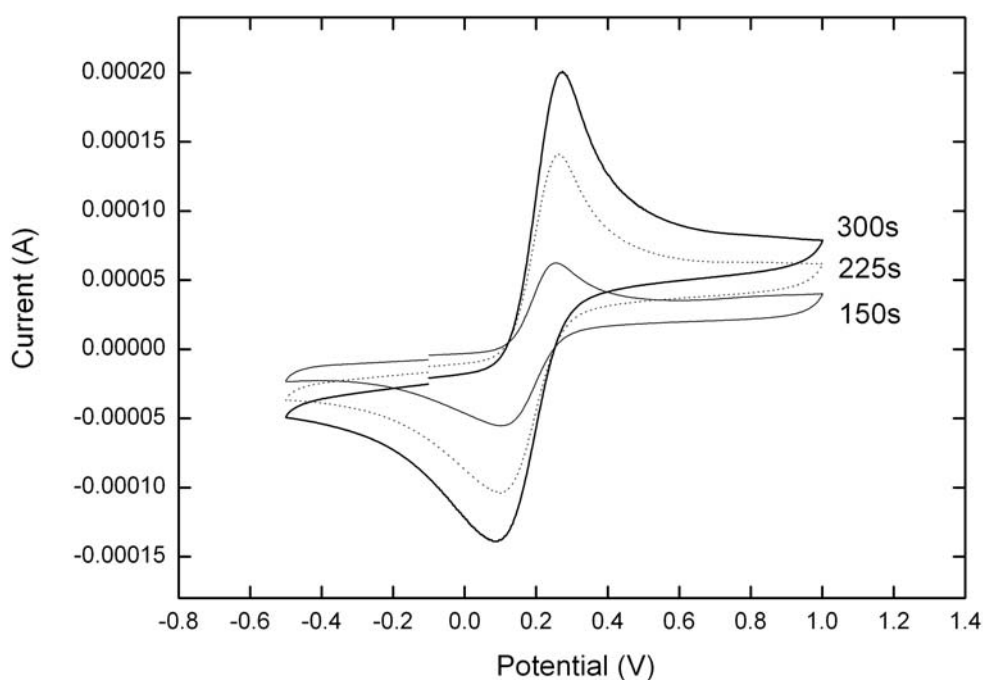


Figure 6. The short time increase in the amplitude of CVs obtained at the times indicated using the Rank cell.

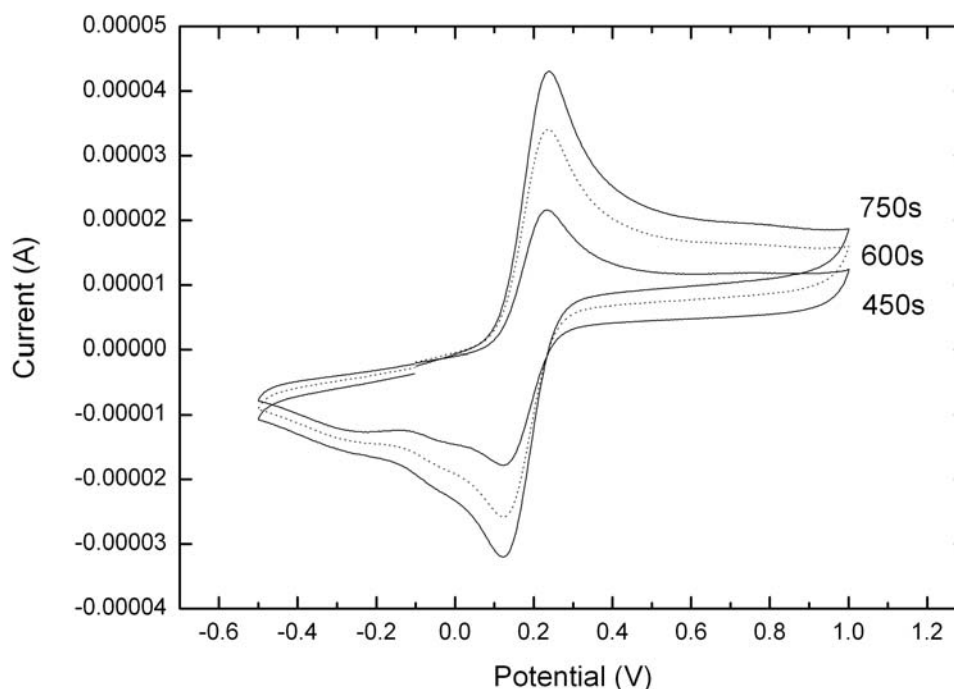


Figure 7. The short-time increase in the amplitude of CVs obtained at the times indicated using the diffusion cell. The peak currents are less than those measured in the Rank cell.

The time dependent increase in the amplitude of the oxidation peak can be used to measure the change in concentration of the redox couple in the immediate vicinity of the working electrode following equation (2). Figure 8 shows a comparison of measurements obtained from the Rank cell and the diffusion cell. From this figure it is clear that there is a distinct difference in the results obtained using the Rank and diffusion cells. The former shows a distinct peak in the time dependence of the oxidative peak current, this is evident in the CVs where the increase in amplitude at short times decreases with increasing measurement time, Figure 9. Corresponding measurements made using the diffusion cell show an almost linear increase in intensity. Although as previously mentioned the oxidative peak current in the diffusion cell is lower than that measured in the Rank cell.

According to the Randles-Sevcik function, equation (2), any changes in the peak current, i_p for a given experimental system are proportional to changes in the concentration of the redox couple, the area of the working electrode and the diffusion coefficient of oxidizable molecules in the immediate vicinity of the working electrode. We would expect a progressive increase in i_p with time for both experimental arrangements due to diffusion of the redox couple through the C-GAG samples, whilst this is the case for the diffusion cell data it is not so for the Rank cell. The decrease in the oxidation peak intensity is most likely to be due to collapse of the unsupported C-GAG on to the working electrode that reduces its available area for solute transport.

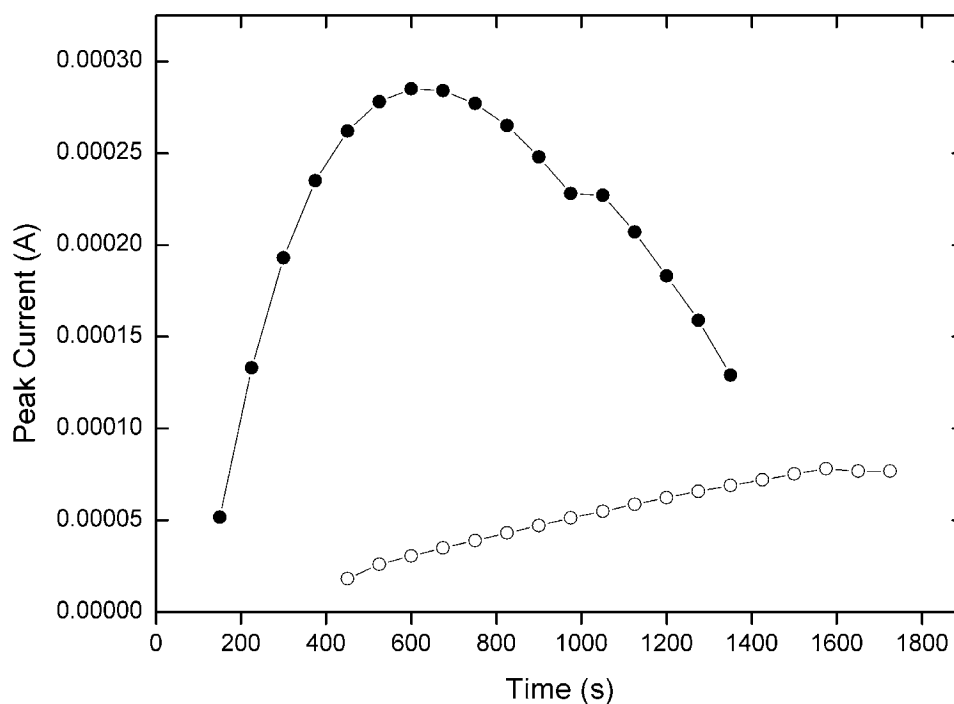


Figure 8: A comparison of the time dependent oxidation peak current in a Rank cell (●) and the diffusion cell (○).

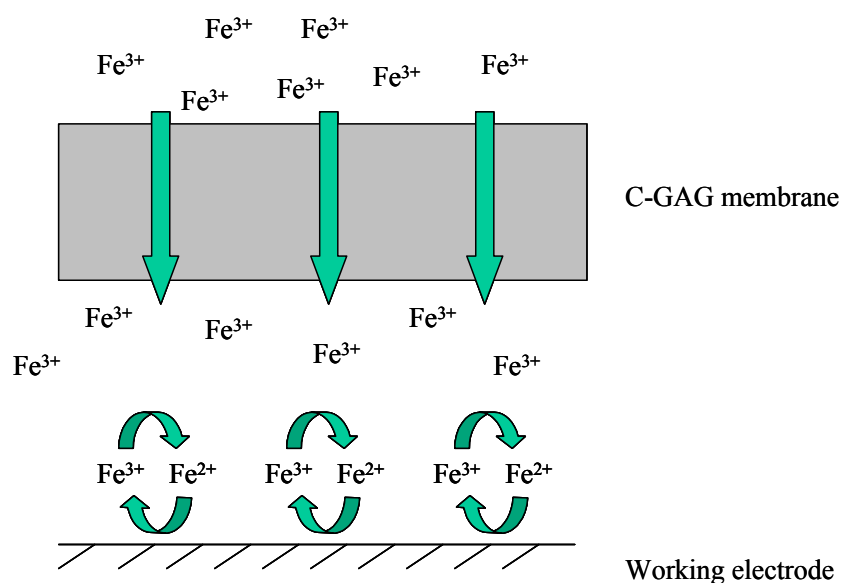


Figure 9. The concentration of redox couple in initial 200 nm range of the working electrode is determined by the rate at which it diffuses through the C-GAG membrane.

Figure 9 shows a schematic representation of the diffusion of the redox couple through the C-GAG membrane and activity at the working electrode. The concentration of redox couple in the PBS buffer will increase with time due to diffusion across the C-GAG membrane. This will be detected by a change in the concentration of redox couple very close to the electrode surface, potentially in a thin film between the electrode and

the C-GAG layer. The oxidation/reduction processes that give rise to the CVs occur within this narrow distance range and therefore the diffusion coefficient of the redox couple is independent of the presence of a sample, i.e. changes in i_{pp} are simply proportional to changes in the concentration of redox couple in this interfacial zone. Following this assumption this experimental arrangement can be used as a means to monitor the flux of redox couple through the membrane.

It is important to note that the concentration of electroactive species in chamber A of the diffusion cell changes significantly with time since an equivalent volume of buffer is contained in chamber B. This limitation can be overcome by only considering short-time data where the concentration of redox couple in chamber A will essentially be that of the original solution. This dilution issue is not a concern for the Rank cell since the volume between the sample and the working electrode is much less than the volume of solution above it as shown in Figure 10.

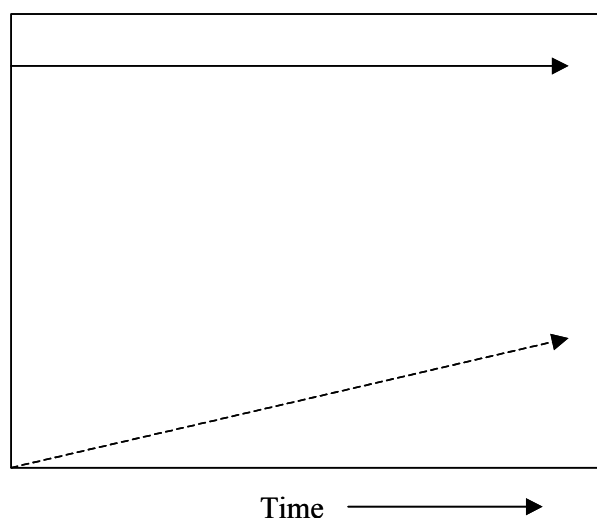


Figure 10. The concentration of redox couple between the working electrode and the C-GAG membrane will increase with time (dashed line) in the Rank cell although the concentration of redox couple in the excess bulk solution above the membrane remains effectively constant.

3.4 The C-GAG Matrix and Degradation

The micrographs shown in Figure 11 are of the natural C-GAG matrix. These images show a complex porous structure that appears to have gossamer-like sheets of C-GAG copolymer. The pores visible in the micrographs are relatively large – on a scale of 50 μm – 100 μm , although the structure suggests that passage from one side of the membrane to the other may be tortuous. The electron micrograph shown in Figure 12 from an irradiated sample shows a comparable structure i.e. any changes that may result from irradiation do not alter the appearance of the microstructure. In contrast, the micrographs in Figure 13 of an irradiated C-GAG matrix that has been incubated with collagenase show a fibrous structure with little evidence of the C-GAG sheets. This fibrous structure appears is much more ‘open’ than the native or irradiated material

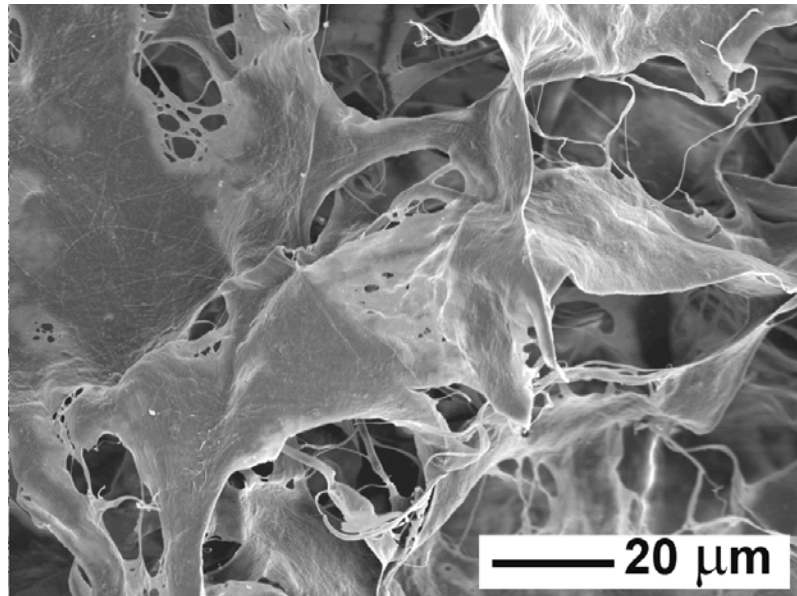


Figure 11. A scanning electron micrograph of non-degraded collagen/GAG matrix reveals a highly porous structure composed of sheet-like walls.

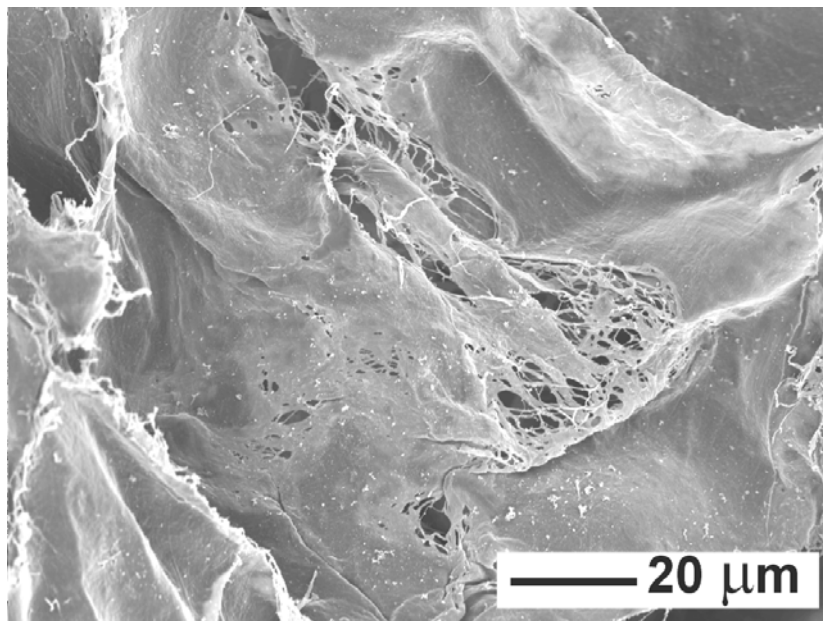


Figure 12. The structure of irradiated C-GAG sample (Dose = 50 kGy) incubated at 37 °C, pH 7.4 is very similar to the non-irradiated material.

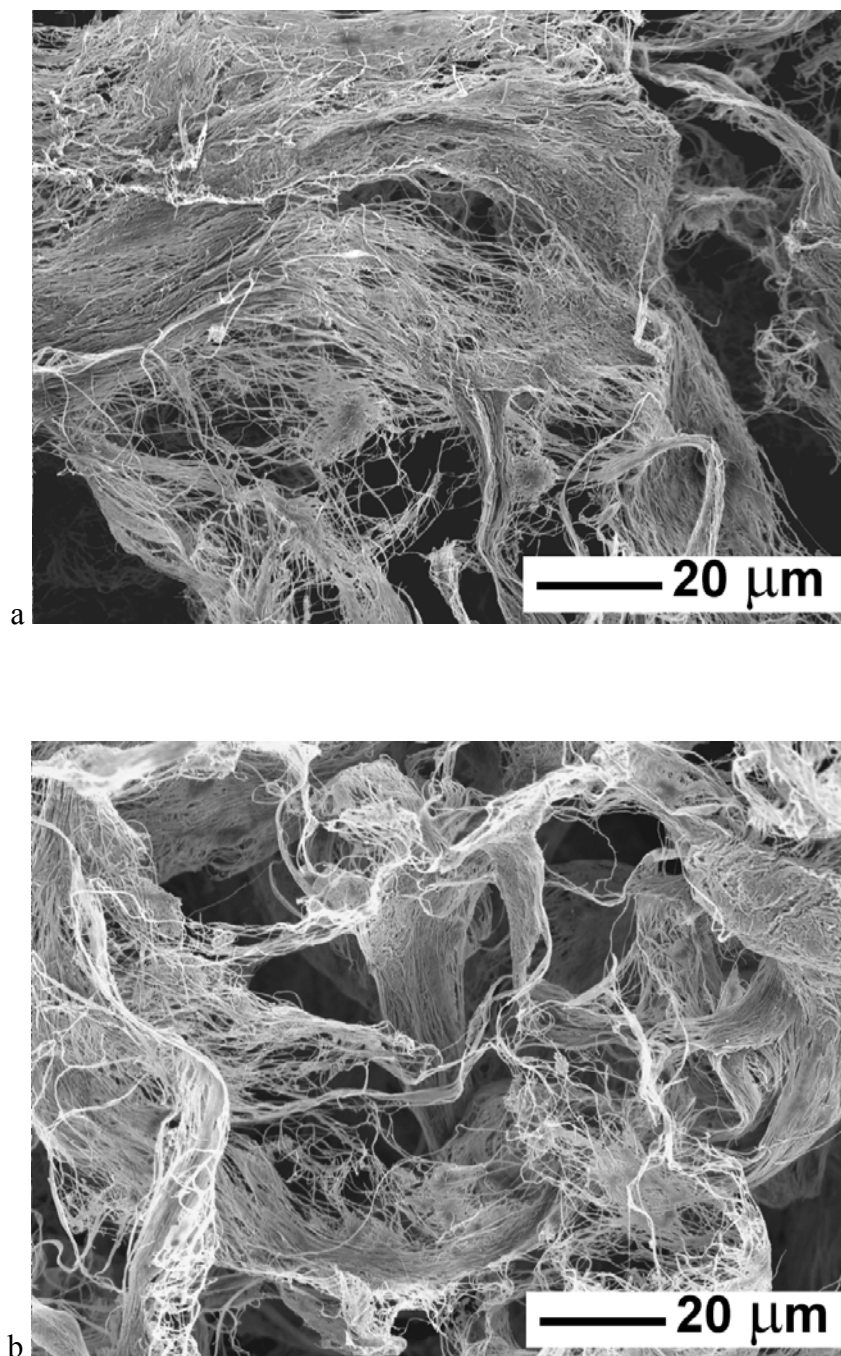


Figure 13. SEM images of irradiated C-GAG sample ((Dose = 50 kGy) incubated at 37 °C, pH 7.4 in the presence of collagenase (10 μg/ml in PBS) is an open structure composed of sheaths of fibres.

3.5 Effect of Degradation on the CVs of Degraded C-GAG

The following sections show the effect of temperature conditioning, exposure to enzymes, storage at different pHs and protein adsorption have in CVs measured using the Rank cell (Figure 2). This apparatus was chosen in preference to the diffusion cell as the measured currents were larger.

3.5.1 Effect of Temperature

Figure 14 shows a comparison of the peak oxidation current, i_p , measured in C-GAG samples that were exposed to different levels of radiation and stored for a period of 48 h at 37 °C and 44 °C respectively, with that of the control. The results from the non-irradiated control sample stored at 4 °C show a well-defined peak in oxidative current, Figure 16, quite different behaviour to that of the degraded materials. These show a slight increase in i_p over a period of about 30 minutes which then essentially remains independent of time. The magnitude of this current does not appear to correlate with the radiation exposure level or the incubation temperature, Figure 15.

The magnitude of i_p for the degraded material is about 1/3 that of the non-irradiated control. This behaviour suggests that the radiation/thermal treatment of the C-GAG matrix increases its permeability to the redox couple compared with the control. This is presumably due to dilution of the ferrocyanide by buffer trapped in the C-GAG membrane itself and between the membrane and the working electrode. This volume of buffer is not controlled and not easy to control.

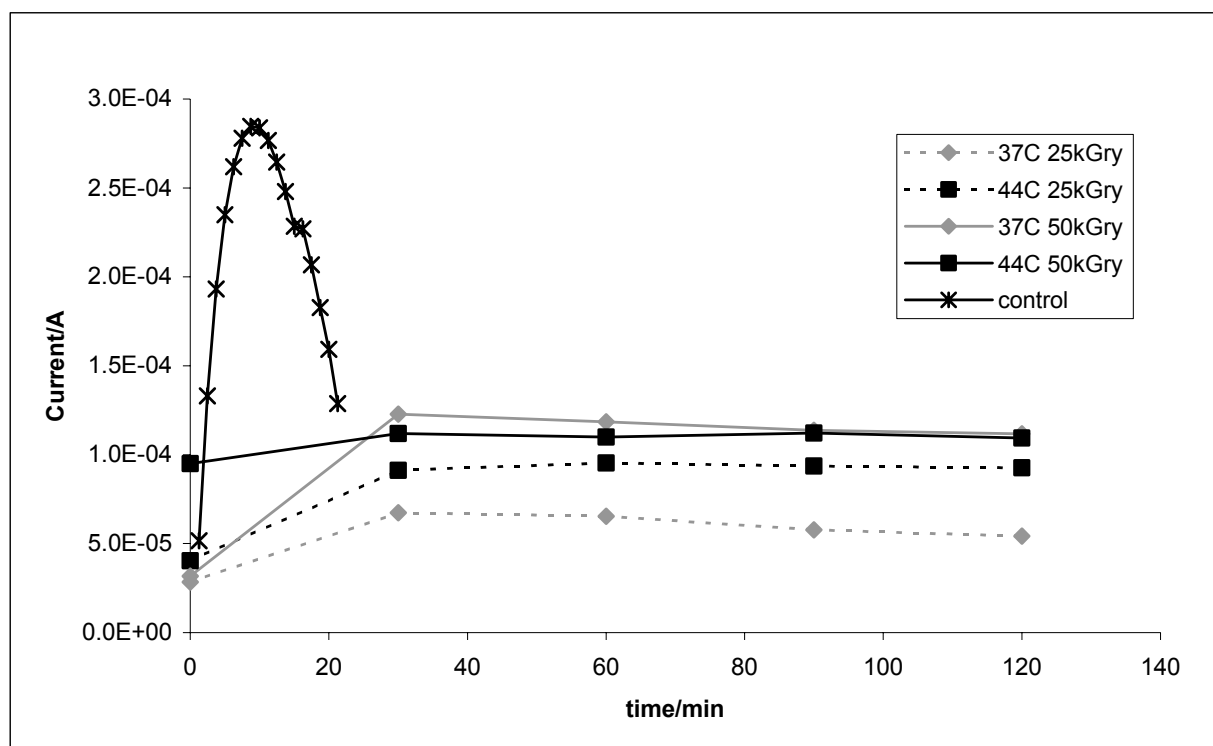


Figure 14. A comparison of the time dependence of the oxidation peak current, i_p for a non-irradiated C-GAG control stored at 4°C with samples exposed to different levels of radiation i.e. 25 or 50 kGry and incubated at different temperatures for a period of 48h.

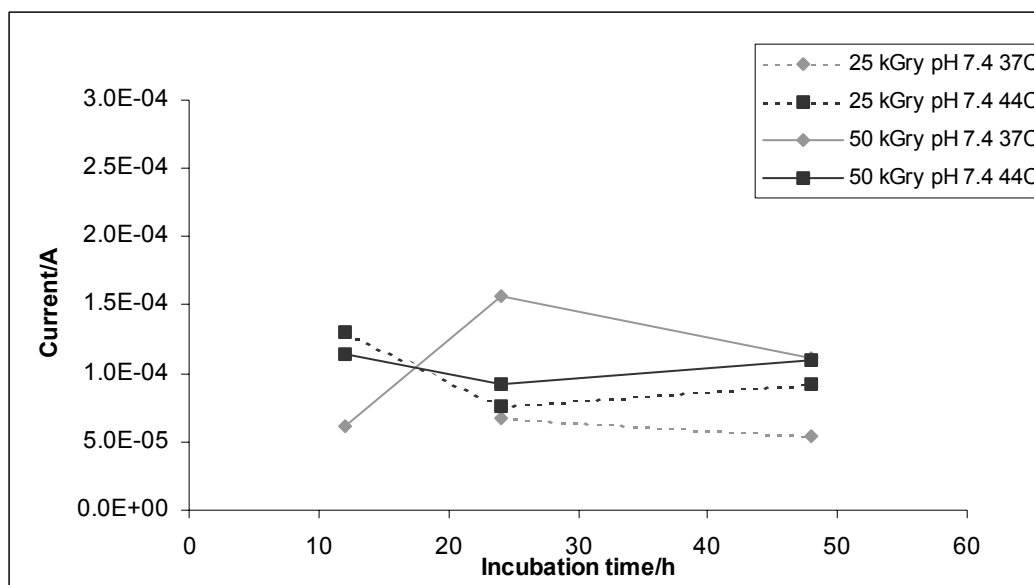


Figure 15. The oxidation peak current, i_p as a function of incubation time at different temperatures for samples that have been exposed to different levels of radiation.

3.5.2 Effect of Collagenase

Figure 16 shows the change in the magnitude of the oxidation peak as a function of time for irradiated samples that have been incubated with different amounts of collagenase for a period of 48h. These results show that the measured current is essentially independent of time which indicates that the samples remain permeable to ferrocyanide. Correlating differences in i_p with differences in radiation exposure or collagenase concentration is difficult to probable variations in the way different individual samples behaved either with measurement time or incubation time (Figure 17).

3.5.3 Effect of pH

Figure 18 suggests that the permeability of the C-GAG membrane is sensitive to the pH of the storage solution. Irradiated samples stored at pH 7 show comparable behaviour or samples stored at elevated temperatures or exposed to collagenase. However, samples stored at pH 5 have very low oxidative peak currents. There are two possible explanations for this behaviour; either the sample structure has been altered such that its permeability to ferrocyanide has significantly changed or the sample has collapsed on to the working electrode, effectively reducing its effective area. Further work is being carried out to identify which of these hypotheses is true.

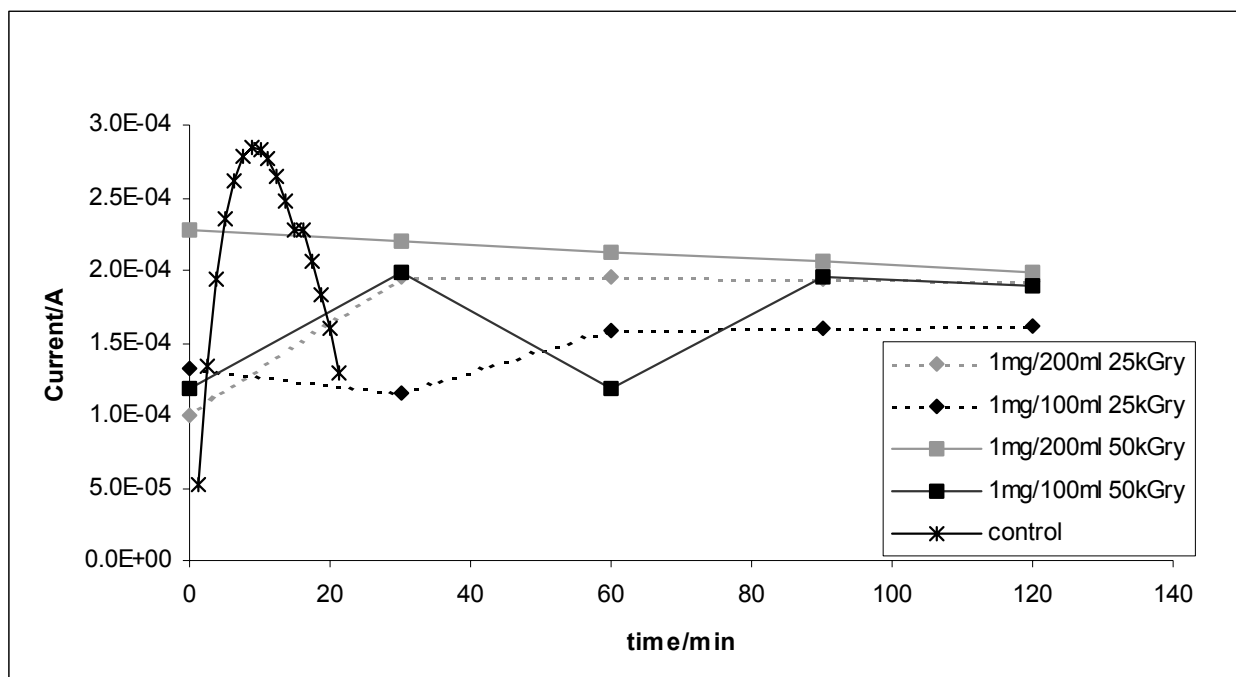


Figure 16: Effect of collagenase concentration versus time for irradiated samples after conditioning at 48h.

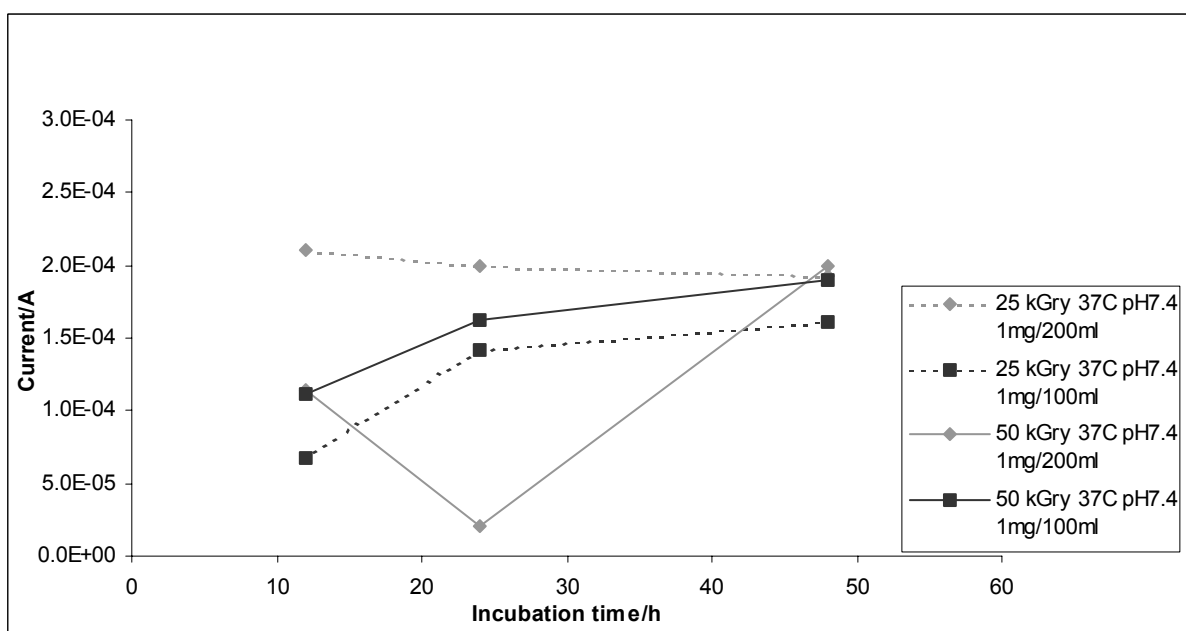


Figure 17. The effect that different conditioning times (12, 24 and 48h) has on the CVs of irradiated samples incubated with different concentrations of collagenase.

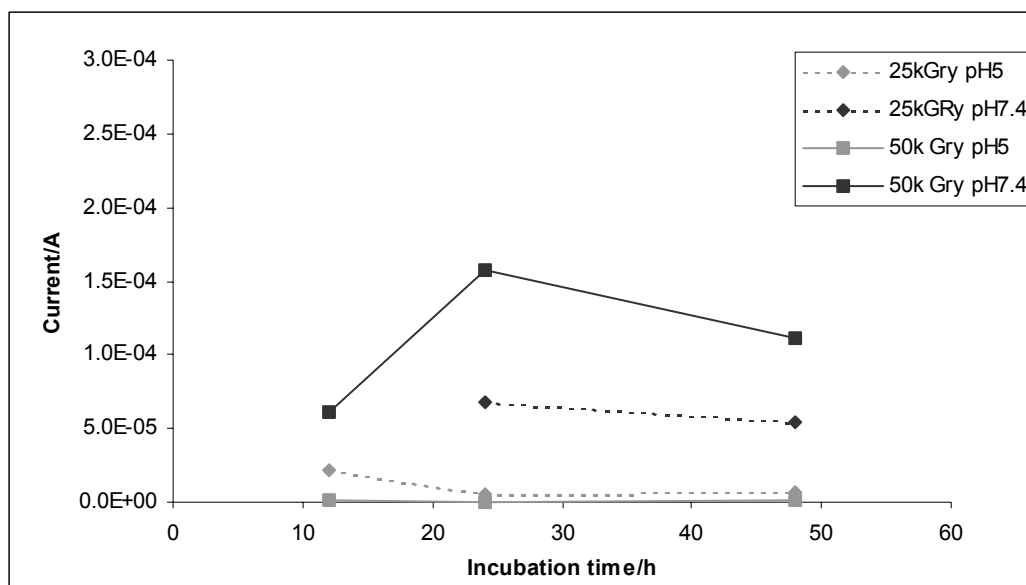


Figure 18. The effect of pH on the permeability of irradiated C-GAG samples to ferrocyanide.

4 DISCUSSION

In this investigation we have compared two experimental approaches for measuring the permeability of a hydrogel-like membrane to small molecule probes. The diffusion cell in which the sample is located well away from the electrodes, at first sight has a significant advantage over the Rank cell as it avoids any complications due to the sample collapsing on to the working electrode effectively reducing its effective area. The measured currents in the diffusion cell are much less than those measured in the Rank cell due to dilution effects. Practically, ensuring that the diffusion cell is level and filled with the same volume of fluid at the same rate is challenging but necessary in order to avoid complicating pressure effects. Constant dilution of the stock ferrocyanide solution in chamber A (Figure 1) into the matched volume of chamber B reduces the magnitude of the diffusion gradient between the two chambers.

In the Rank cell the sample is sited over the working electrode thus minimizing the volume of buffer contained between it and the working electrode. The volume of solution containing electroactive species above the sample is typically contained at about 1 cm³ to minimize the pressure acting on the sample. However, this relatively small volume of solution becomes diluted with buffer contained within the sample and the space between it and the working electrode. This ‘unknown’ film of buffer is difficult to control and may distort quantitative measurements based on CVs.

Currents measured in the Rank cell arrangement are much higher than those in the diffusion cell for a given concentration of electroactive species as the dilution effect is reduced. However, the system may be susceptible to electrode surface blocking, i.e. the functional area of the working electrode may be reduced through intimate contact by the sample. This behaviour may account for the CVs of non-degraded C-GAG samples that show an initial increase in the peak current, i_p followed by a significant decline.

Exposure to radiation and storage under different environments that are designed to accelerate the rate of degradation of the C-GAG matrix can produce a significant change in the structure of the material. Exposure of native material to different doses of radiation has no observable effect on the sheet-like porous structure of the material. Similarly incubation of irradiated material at different temperatures for different times has no discernible effect on the structure of the material. However, incubation of the hydrogel matrix with collagenase has a dramatic change in the hydrogel structure reducing the sheet-like appearance to a fibrous mesh. In terms of the measured CVs, irradiated samples, thermally conditioned irradiated samples and irradiated samples treated with collagenase, all significantly increase the permeability of C-GAG scaffolds to ferrocyanide. This increased permeability is independent of the measurement time and incubation time suggesting that the treated hydrogels are much more permeable than the native material.

Storing irradiated samples in an acidic environment (pH 5) or in a solution containing fibrinogen has a significant effect on measured CVs reducing the oxidation peak current to very low levels. This behaviour might be explained by collapse of the sample on to the working electrode surface (pH 5 storage).

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6 ACKNOWLEDGEMENTS

This work was funded by the United Kingdom Department of Trade and Industry as part of the Measurements for Processability and Performance of Materials (MPP) programme.