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INVESTIGATION INTO THE STRUCTURAL INTEGRITY OF COLLAGEN BASED DRUG DELIVERY SYSTEMS AFTER EXPOSURE TO ETHYLENE OXIDE AND GAMMA STERILISATION TECHNIQUES

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Abstract

For wound healing, collagen demonstrates the ability to easily absorb large quantities of tissue exudates, as well as shielding against mechanical harm. This study investigates the effect of two established sterilisation techniques, ethylene oxide (EtO) and gamma (γ) irradiation on collagen sponges. Thermal analysis studies found two peaks on each of the sponges tested: the first one, located around 100-106°C is a result of water bonded to molecules and unfolding of the triple helical structure, and the second peak 125-145°C is due to the decline of cross-links. Hydration studies have shown that, the EtO sterilised collagen sponge absorbed the greatest amount of deionised water after a period of 3 days, while the gamma sterilised gentamicin sulphate sponge completely dissolved after 24 hours. This is a good indication that gamma sterilisation breaks down cross-links or larger chains of the material, thus reducing the mechanical integrity of the sponge.

Keywords and phrases: collagen, sterilisation, ethylene oxide, gamma, thermal analysis.
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1. Introduction

Structural collagen proteins are obviously highly represented in extracellular matrices, and their inherent high biocompatibility and amenability to cellular remodelling has resulted in extensive adaptation, to tissue engineering and device applications [3, 6]. In an ideal situation, sterilisation of such products should free collagen based biomaterials from all viable micro organisms without inducing changes in the protein chemistry, the mechanical properties and the degradation behaviour. However, all currently used methods will inherently damage and alter collagen in a way that might affect its *in vivo* absorption rate, mechanical strength or performance in combination with drugs [9, 10, 13, 32].

Biomedical devices incorporating biodegradable polyesters are traditionally sterilised by ethylene oxide (EtO), because other sterilisation methods, include irradiation, heat, steam or acid can cause significant physical deformation, and accelerated polymer degradation. However, EtO exposure is still associated with some of the latter effects as well as the risk of retention of residual gas within the polymer matrix, and there are greater restrictions on its use in Europe [7]. EtO exposure occurs under conditions of high relative humidity and raised temperature, which results in negligible degradation, but the release of lysine and hydroxylysine residues suggests that, there is chemical reactivity between EtO and amino groups on the protein. This interaction results in reduced helix stability and greater resistance to enzymatic degradation due to restricted matrix access on the part of collagenase, at least in non-porous preparations [9, 23]. According to Gorham [12], γ-irradiation from a ^{60}Co source has become very commonly applied to collagen preparations. A dose of 2.5mRad (25kGy) is reported to cause effective sterilisation. The effect of γ-irradiation on collagen biochemistry has been particularly evaluated for collagen extracted from tendons [2, 5, 28].

Studies have shown that γ-irradiation brings about chain scission causing polymer fragmentation. Typically, some end users compensate for this increased fragmentation by deliberately cross-linking the protein and *in vivo*, covalent cross-linking of collagen molecules in connective tissues

contributes to normal functionality. The majority of gamma irradiation damage is induced by free radicals resulting from the radiolysis of water molecules [1, 30]. However, γ- irradiation leads to significant collagen sponge degradation (whether cross-linked or not) and to a decrease in denaturation temperature and tensile strength [15, 21, 28, 31]. This study focuses on sterilisation techniques, ethylene oxide (EtO), and gamma (γ) irradiation, and how the thermal and mechanical properties of collagen sponges (with or without drugs) are affected by such process treatments. Cross-linking has been shown to induce dehydration of collagen fibres and improve associated increased temperature stability and ionizing irradiation increases cross-linking and resultant viscosity [18, 19].

2. Experimental

2.1. Materials

The collagen sponges used in this study were:

- Non-sterile collagen sponges;
- γ- irradiation collagen sponges;
- EtO sterilised collagen sponges;
- Non-sterile gentamicin sulphate collagen sponges;
- γ- irradiation gentamicin sulphate collagen sponges;
- EtO sterilised gentamicin sulphate collagen sponges.

2.2. Differential scanning calorimetry (DSC)

The collagen samples were tested by using a TA 2010 differential scanning calorimeter with sample sizes between 5.4 and 6.2mg. The samples were placed in sealed pans, and tested over a temperature range of 20 to 300°C at a heating rate of 10°C per minute. All DSC tests were carried out under a 20mL per minute flow of nitrogen to prevent oxidation. Calibration of the instrument was performed by using indium as standard (156.1°C +/–0.5°C).

2.3. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Fourier transform infrared spectroscopy was carried out by using the attenuated total reflectance (ATR) mode on a Nicolet Avator 360 FTIR, with a 32 scan per sample cycle and a resolution of 8cm^{-1}. The samples were scanned between, 400 to 4000cm^{-1}.

2.4. Dynamic mechanical thermal analysis (DMTA)

DMTA scans were carried out by using a Rheometric Scientific Mark 3 DMTA instrument in tensile mode. The temperature profile ranged from 30 to 200°C at a 2°C per minute heating rate with a frequency of 1Hz. The specimens for DMTA testing ($12\text{mm} \times 4\text{mm} \times 1\text{mm}$) were cut from the selected samples.

2.5. Hydration studies

The samples used were weighed and measured ($\text{L} \times \text{B} \times \text{H}$). The samples were placed in glass Petri dishes containing deionised water. These Petri dishes were then stored in an incubator at 37°C. After a period of 24 hours, the Petri dishes were removed from the oven, and the weights and dimensions of each sample were recorded. This procedure was repeated every 24 hours for two weeks and each batch of membranes were measured in triplicate.

2.6. Viscometry

0.56g of the collagen material and 0.2ml of acetic acid was place in 99.2ml of deionised water; the solution was then heated to 37°C. The pH value was monitored, 4.5 +/–0.2, and when applicable 0.1M NaOH was added to bring the solution into the required pH for homogenisation. The samples were then homogenised by using a Cookworks blender. This procedure was repeated for each of the collagen sponges, and the homogenised solutions were then analysed by using an Ostwald viscometer. The average time values obtained for the solution with the collagen dispersion was denoted as *"tsol",* and the average time for the solution containing no collagen dispersion (deionised water and the acetic acid) was "*to*".

Using the values obtained, *to* and *tsol*, the relative and specific viscosity were calculated for each sponge, where

$$\text{Relative viscosity} \quad \eta_r = tsol / to, \tag{1}$$

$$\text{Specific viscosity} \quad \eta_{sp} = tsol - to / to. \tag{2}$$

3. Discussion of Results

3.1. Thermal analysis of collagen sponges

Collagen denaturation is defined as a transition from the triple helix to a randomly coiled form, taking place in the domains between the cross-links. Hydrogen bonds, hydrophobic interactions, van der Waal's forces, and electrostatic interactions between oppositely charged residues on side chains all contribute to stabilisation of the superhelical tertiary structure. The non-random distribution of ionisable and hydrophobic side chains along with the repeating unit results in the occurrence of charged, and hydrophobic patches that contribute to stabilisation of the structures through electrostatic and hydrophobic interactions, whereas hydrogen bonded water plays a big part in the stabilisation of the molecule. All of the aforementioned non covalent bond methods break down upon heating, where the breakdown occurs at the weakest points of the helix, between the stabilising clusters [4].

As outlined in Figure 1, the DSC thermograph illustrated two peaks on each of the sponges tested: the first one, located around 100-106°C is a result of the water bonded to molecules and unfolding of triple helical structure, and the second peak 125-145°C is due to the melting of cross-linked part of collagen. Looking towards the melting temperature of the sterilised sponges, there was much more energy required to melt the EtO sterilised collagen sample, which suggests cross linking occurred as a result of the sterilisation process, in accordance with prior evidence [19].

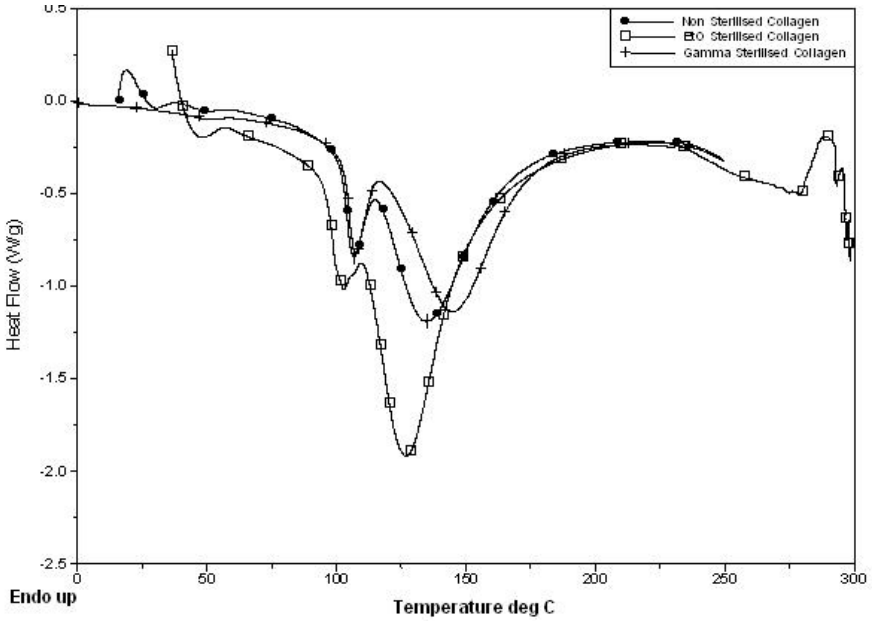

Figure 1. DSC thermograph of non-sterilised, EtO sterilized, and gamma sterilised collagen sponges.

With the incorporation of gentamicin sulphate as illustrated in Figure 2, the non-sterilised gentamicin sulphate collagen sample follows the same melting profile as the gamma sterilised gentamicin sulphate collagen sample in comparison to the EtO sterilised gentamicin sulphate collagen sample, which has a lower melting temperature. This would suggest that, the gentamicin is stabilising the cross-links between the structures for the non-sterilised and gamma sterilised samples.

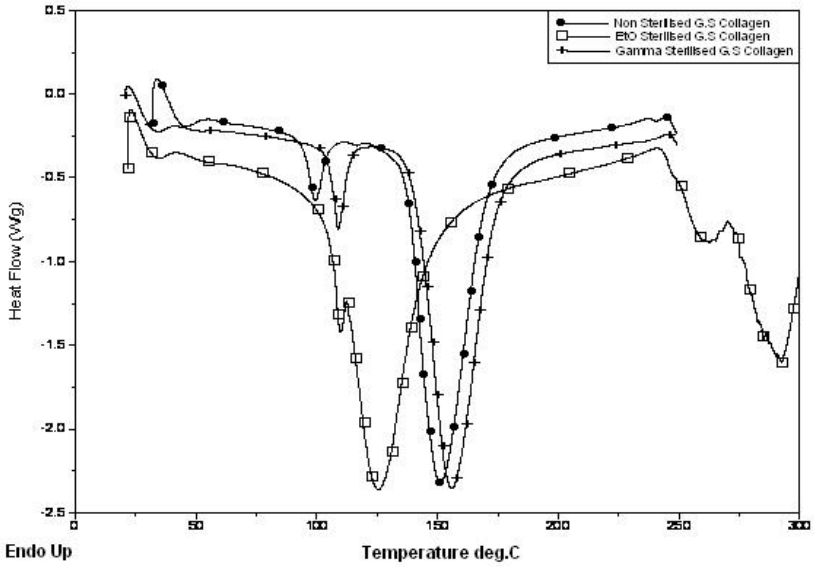

Figure 2. DSC thermograph of non-sterilised, EtO sterilized, and gamma sterilised collagen sponges, which contain gentamicin sulphate.

The endothermic peaks due to vaporisation of bound water at the temperature range 100-115ºC are also affected by the presence of gentamicin. Siapi et al. [29] found that the reabsorbance of water is more significant in the samples containing gentamicin after 24 hours equilibrium time indicating that the antibiotic makes a more stable complex with collagen molecules aiding this process. Siapi et al. [29] also suggested that collagen denaturation occurs at higher temperatures, when gentamicin is incorporated in fibrous membranes, thus the gentamicin stabilises the crosslinking between the structural units (covalent, hydrophobic links) due to its interactions with collagen sponge and water.

3.2. Infrared spectroscopy analysis of the collagen sponges

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) has been used to study changes in the secondary structure of collagen. It has been used to study collagen cross-linking [24], denaturation [8] as well as thermal self assembly [11, 14]. The spectral

changes, which are indicative of changes in collagen secondary structure have been shown to include changes in the amide A [17], amide I (1636-1661cm^{-1}), amide II (1549-1558cm^{-1}) [27], and the amide III (1200-1300cm^{-1}) regions [8].

Fibrillogenesis (self assembly) of collagen has been found to be associated with (a) broadening and a slight shift to a lower wave number of the amide A peak [17], (b) increase in intensity and slight shift to lower wave number of amide III peak [14], (c) band broadening and shift of amide I peak to lower wave number [11, 14, 26], and (d) a shift of the amide II peak to a lower wave number [11, 14]. Shifts of amide I, II, and III peaks to lower wave numbers, and the increase in intensity of the amide III and the broadening of amide I are therefore associated with increased intermolecular interactions such as hydrogen bonding, Figure 3.

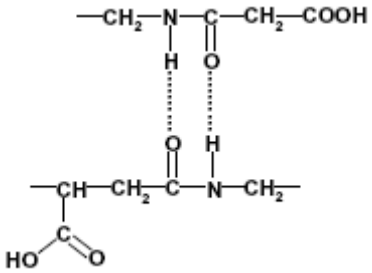

Figure 3. Hydrogen bonding in collagen.

Denaturation of collagen, on the other hand, has been found to lead to reduction in the intensity of amide A, I, II, and III peaks [8], narrowing of amide I band [26], an increase in amide I component located at around 1630cm^{-1}, and reduction in the intensity of amide I component, found at around 1660cm^{-1} [11, 25, 27].

The sterilised and non-sterilised collagen exhibits a series of absorptions from 1240 to 1650cm^{-1}, as illustrated in Figure 4. Bands at 1239 ± 3cm^{-1} are assigned to amide III. Bands of C–H twist vibration appear at 1454 ± 2cm^{-1}. It has been reported that, three components of carbonyl vibration are at 1631cm^{-1}, 1643cm^{-1}, and 1660cm^{-1}. There

are bands present at $1645 \pm 10\text{cm}^{-1}$ in the spectra, which originate from C=O stretching vibrations coupled to N–H bending vibration. These bands are assigned to amide I, which may be the combination of the three carbonyl bands referred to above. Bands at $1550 \pm 2\text{cm}^{-1}$ are assigned to the amide II, which arises from the N–H bending vibrations coupled to C–N stretching vibrations. Bands at $2977 \pm 55\text{cm}^{-1}$ are discrete and inconspicuous and are assigned to a C–H stretch. The band at $1402 \pm 2\text{cm}^{-1}$ and $2362 \pm 3\text{cm}^{-1}$ are related to carbonate ($-CO_3$) and atmospheric carbon dioxide, respectively. Weak water bands are present at $3292 \pm 45\text{cm}^{-1}$. When comparisons are made between the gentamicin sulphate collagen samples, also illustrated in Figure 4, to the collagen samples the only difference is a large peak at $1060 \pm 2\text{cm}^{-1}$. This is due to the presence of the drug. The process of sterilisation has shown no chemical damage towards the collagen sponges.

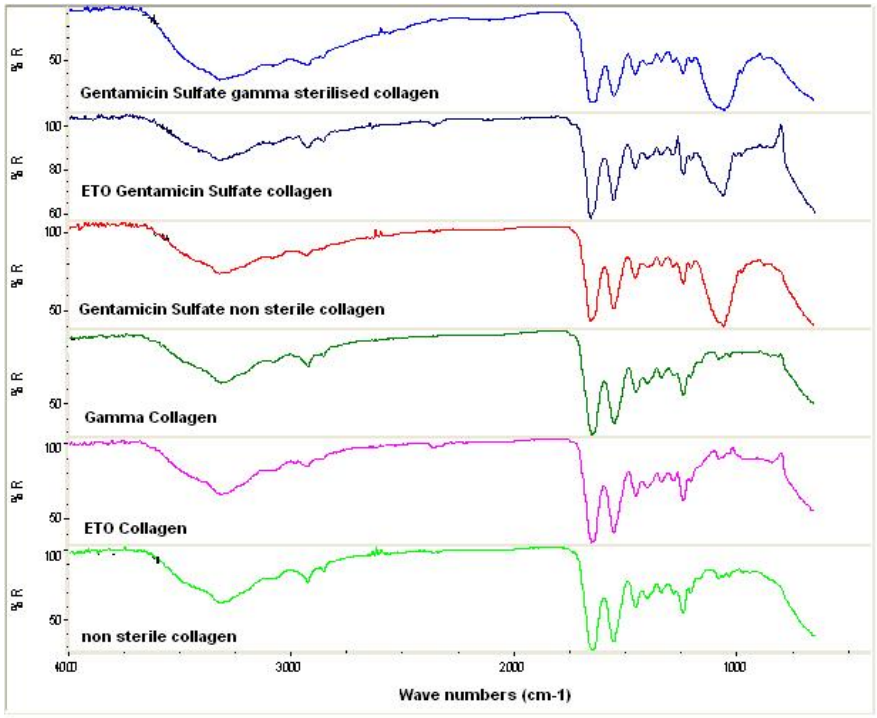

Figure 4. Comparison between all the different collagen samples ATR-FTIR spectra.

3.3. Thermal mechanical analysis of the collagen sponges

From the DMTA findings as presented in Figure 5, the gamma sterilised gentamicin sulphate collagen sponge sample has a similar DMTA profile to that of the non-sterile gentamicin sulphate collagen sponge sample. However, in relation to the EtO sterilised gentamicin sulphate collagen sponge a peak at 50ºC appeared. This peak may be attributed to the collapse of the triple-helical domain of the collagen, which corresponds with the DSC findings of Siapi et al. [29]. Therefore, it may be suggested that EtO sterilisation reduces the helix stability with the presence of gentamicin, which would reflect the differences between it and the gamma sterilised gentamicin sponge sample. When comparing the collagen sponges without drug, there is no apparent difference between either of the sterilisation techniques used. The peaks between 40-50ºC may be the collapse of the triple-helical domain.

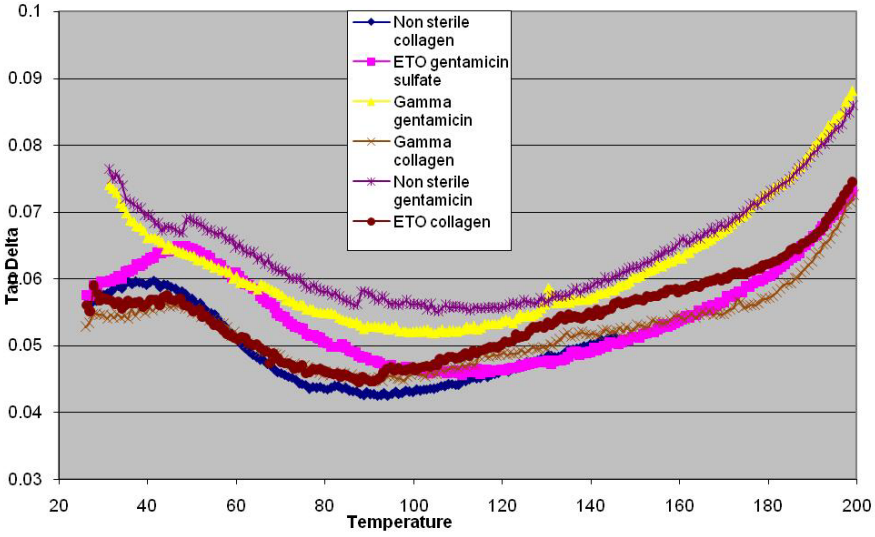

Figure 5. Comparisons between all of the collagen DMTA scans.

3.4. Hydration analysis of collagen sponges

Both intra-and inter-chain hydrogen bonding within the triple helices has long been thought to play an important role in forming the structure of collagen molecules, along with a highly ordered inner hydration layer of water molecules that forms hydrogen bonds along the peptide chains. Additionally, water has been shown to form hydrogen-bonded "bridges", which further contribute to the structure of collagen by forming intra-and inter-chain links within molecules, along with intermolecular bridges between neighbouring triple helices that presumably contribute to the structure and properties of the collagen fibrils [20].

As would be expected, there was an increase in the overall weight of each sample, when swollen with one exception, the gamma sterilised gentamicin sulphate collagen sponge, Figure 6. This sample dissolved after one day, which suggests that, the gamma process on the collagen structure initiated chain scission resulting in a lower molecular weight material with fewer cross-links, which corresponds with the findings of Noah et al. [21]. Thus, with the addition of the drug, the intermolecular distance between cross-links increased, thus reducing the overall strength of the material in an aqueous environment. The break down in the collagen structure by gamma sterilisation was also evident in the swelling behaviour of the gamma sterilised collagen sponge, where the sample only slightly absorbed deionised water and after a period of two weeks, the sample lost much of its structural integrity.

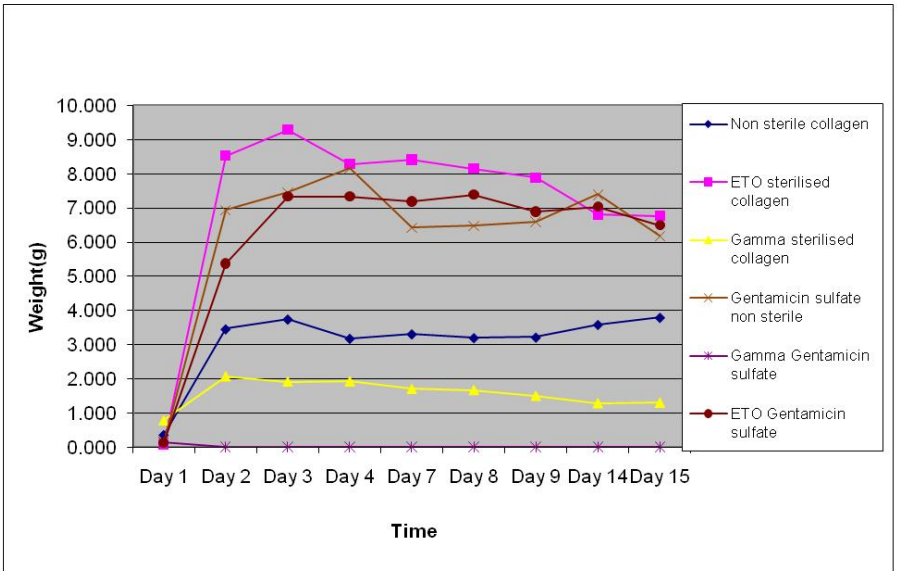

Figure 6. Graphical representations illustrating the increase in swelling behaviour over time by using collagen sponges in an aqueous medium.

One factor which may have caused this accelerated breakdown was the presence of moisture in the sample pre-sterilisation. Friess [9] suggested that, the effect of gamma sterilisation on collagen structure indicated chain scission resulting in a fraction of lower molecular weight components. This fragmentation may be compensated by the formation of additional cross-links, which depends on the moisture content of the product. Thus, the initial amount of water present in the sample before sterilisation alters the strength of the sponge. However, the rate at which the sample degrades could be beneficial to the end user, for example, if the intended sponge was used as a haemostat; the collagen sponge has achieved its goal once the blood clot has formed. The largest weight occurred in the samples containing the EtO sterilised gentamicin sulphate collagen, EtO sterilised collagen, and the non-sterile gentamicin sulphate collagen samples. It would suggest that the EtO sterilised sponges have an increased cross-linked density or chain lengths, when compared to the gamma sterilised sponges. Therefore, as the sponge is

immersed in an aqueous environment, the drug is released out of the matrix as the water penetrates, thus increasing the overall weight of the sponge. It is worth noting that the EtO sterilised sponges have a similar swelling profile to the non-sterilised gentamicin sulphate sponges.

3.5. Viscosity analysis

Intrinsic viscosity can be determined from a single point viscosity measurement of a dilute solution [16]. This method has the advantage of being more rapid and adequate, when a large number of samples have to be analysed in short periods of time. Moreover, it has the advantage of being independent of a constant value, in contrast to graphical extrapolation determinations. The formula used to calculate the intrinsic viscosity (η) is presented in Equation 3, where the concentration C, equals 1 and the η_r and the η_{sp} are calculated as described in Subsection 2.6. Thus, the results obtained from this study are presented in Table 1.

$$[\eta] = (3 \ln \eta_r + 3/2\, \eta_{sp}^2 - 3\, \eta_{sp})^{1/3} / c\,. \tag{3}$$

Table 1. Viscosity results obtained for each of the collagen sponges tested at 37°C

Samples	η_r	η_{sp}	η
EtO sterilised gentamicin sulphate collagen sponge	1.31	0.31	0.286
Gamma sterilised gentamicin sulphate collagen sponge	1.25	0.25	0.232
Non-sterilised gentamicin sulphate collagen sponge	1.12	0.12	0.113
EtO sterilised collagen sponge	4.47	0.47	2.296
Gamma sterilised collagen sponge	1.28	0.28	0.263
Non-sterile collagen	1.6	0.6	0.534

As presented in Table 1, the intrinsic viscosity results are relatively low, when compared to the result obtained for the EtO sterilised collagen sponge sample. This would seem to suggest that the EtO sterilised collagen sponge is much more cross-linked, when compared to the other sponges, and this is backed up by the large endothermic melting point as shown in Figure 1. The remaining sterilised samples are consistent with

each other, but it is worth remembering that the gamma sterilised gentamicin sulphate collagen sponge, when placed in deionised water at 37°C, Figure 6, completely dissolved within 24 hours. Ogawa et al. [22] has found that one of the physicochemical characteristics of collagen is its high viscosity at room temperature. The results found by Ogawa are presented in Figure 7. As illustrated by the graph, the viscosity of the samples decrease as the temperature is increased. Based on this assumption, each of the collagen sponges had relatively low viscosity at 37°C, which corresponds with the findings of Ogawa et al. [22].

It is worth mentioning that an extensive literature search has not yielded any actual intrinsic viscosity data on collagen based sponges. Thus, it was difficult to truly characterise the viscosity results. It is for this reason, the results were based on the collagen obtained from seabream as found by Ogawa et al. [22]. Also, the homogenisation process used is a shear dependant system, which alters the molecular structure of the collagen sponges, which dictates the actual viscosity of the material. Care was taken in trying to keep the homogenisation process as consistent as possible.

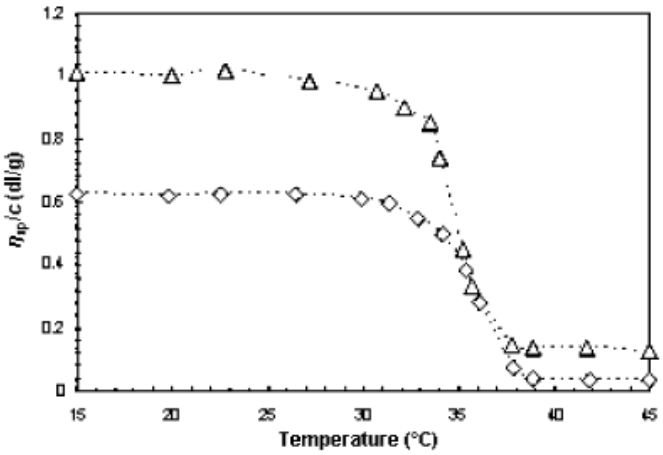

Figure 7. Thermal denaturation curves of bone, pepsin-solubilized collagen, measured by viscosity. Each value was the mean of three determinations. Unfilled diamond with broken line, black drum. Unfilled triangle with brokenline, sheepshead, where Black drum and Sheepshead are forms of seabream [27].

4. Conclusion

In conclusion, thermal analysis has shown that two peaks exists for all of the collagen samples tested: the first one, located around 100ºC is due to the water bonded to molecules and unfolding of triple helical structure and the second one, which varied between 125 and 145ºC is due to the melting of cross-linked part of collagen. However, from the DSC thermographs, there was much more energy required to melt the EtO sterilised samples, which suggest a much more cross-linked material. The swelling studies has shown that, the EtO sterilised collagen sponge absorbed the most amount of deionised water after a period of 3 days, while the gamma sterilised gentamicin sulphate sponge completely dissolved within 24 hours. This is a good indication that, the gamma sterilisation process breaks down the cross-links or larger chains of the material, thus reducing the mechanical integrity of the sponge. The intrinsic viscosity results are relatively low, when compared to the result obtained for the EtO sterilised collagen sponge sample indicating that, the EtO sterilised collagen sponge was much more cross-linked or had larger chains present within its structure than that of the remaining sponges; this trend was also evident in the swelling studies. It is well established that γ-irradiation increases collagen fragmentation, which implies that EtO sterilisation is the best method for retaining structural integrity. However, the level of difference observed, the preservation of gentamicin profile and the literature consensus, would suggest that overall, γ-irradiation offers the most advantages, provided the intended application is not compromised by the known deficits.

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