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Dielectric properties of the collagen–glycosaminoglycans scaffolds in the temperature range of thermal decomposition

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Abstract

Dielectric spectroscopy has been applied to study the decomposition process of unmodified collagen and chondroitin sulfate (CS)- and hyaluronic acid (HA)-modified collagen. Measurements were performed over the frequency range from 10 Hz to 100 kHz and at temperatures from 22 to 260 °C. According to the Kramers–Kronig relationship a dispersion is apparent in both ε' and ε'' for the three materials below 140 °C and at higher temperatures as a broad peak around 220–230 °C, respectively. The values of ε' and ε'' at the same temperature for constant frequency are higher in HA-modified collagen than in the unmodified collagen. However, small differences are shown in these parameters between CS-modified collagen and unmodified collagen. The observed dispersion around 220–230 °C corresponds to the decomposition of unmodified and CS- and HA-modified collagen. Power-low responses are observed for the frequency dependence of ac conductivity for unmodified and modified collagen. The behaviour observed for temperature dependencies of the exponent n for the three materials is considered to be related to the proton polarization and conduction processes.
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Keywords: Collagen; Thermal decomposition; Dielectric properties; Activation energy; Cross-linking

1. Introduction

An important role in creation of functional scaffolds for tissue engineering play both collagen and glycosaminoglycans (GAG) such as hyaluronic acid (HA) and chondroitin sulfate (CS) [1,2]. Tissue engineering offers the potential to create functional and viable tissue constructs for patients requiring organ or tissue replacement [3]. The main approaches towards tissue engineering involves the in vitro culturing of cells on biodegradable polymeric scaffolds/matrices to form neo-organs that are then implanted into the body at a relevant anatomical site [4]. For a biologically active scaffold to promote cell adhesion and growth, it must satisfy a number of requirements. Among others the scaffold must be non-immunogenic, non-toxic, biocompatible, biodegradable and structurally stable.

Collagen is the most abundant protein in mammalian tissues and is the major component of the natural extracellular matrix (ECM) [5]. Collagen has found use in the biomedical field due to its hemostatic properties, low antigenicity, appropriate mechanical characteristics and cell-binding properties for use in tissue engineering applications [6]. Meanwhile, CS and HA belong to the connective-tissue mucopolysaccharide group of substances that are present mainly in articular cartilage, vitreous humour and synovial fluid but they are widely distributed in other tissues and most body liquids [7]. They have unique physicochemical properties and distinctive biological functions. They are preliminary located on the surface of cells or in the ECM.

Attachment of HA or CS to reconstituted collagen may offer an opportunity to exploit the biocharacteristics of these polysaccharides and valorize collagen as a biomaterial. Composites collagen–GAG have been used for a wide variety of in vitro studies of cellular migration, contraction, and tissue growth [1,2,8]. The cellular events in these scaffolds must be controlled. Without such regulations, the regenerated tissue

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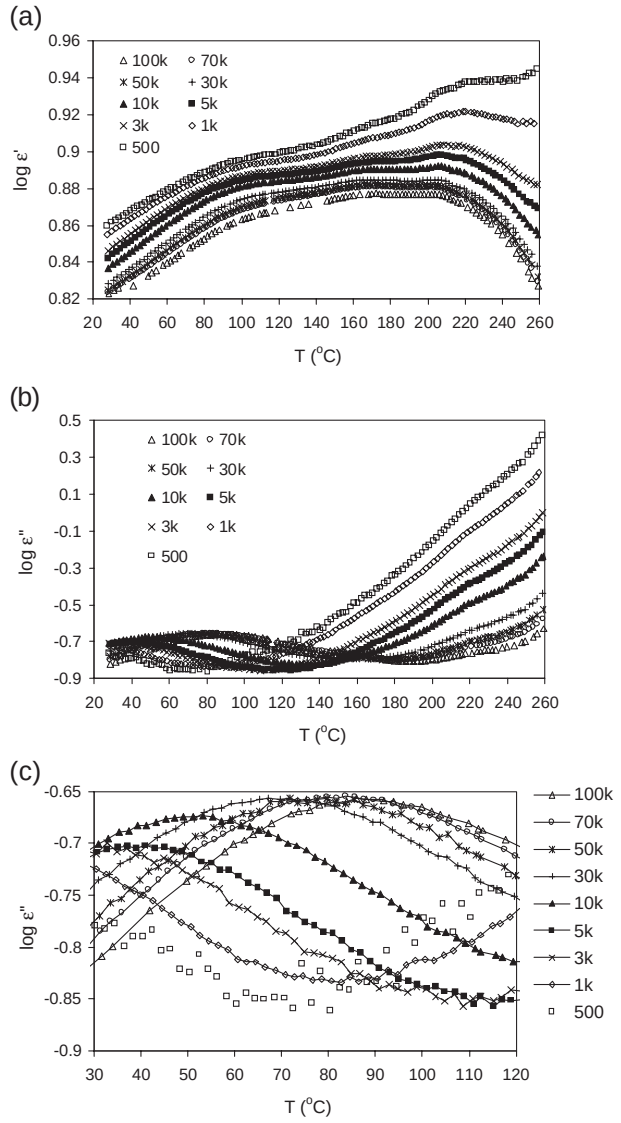

Fig. 1. Temperature dependencies of (a) ε′ and (b) ε″ up to 260 °C, and (c) ε″ up to 120 °C for unmodified collagen.

will not acquire a normal function. Electric phenomena are intrinsic in biological systems and their role in regulating cellular events no doubt contribute to tissue and organ regeneration. The electrically conductive and biodegradable polymers are of interest because certain characteristics of the collagen–GAG analogues of ECM can influence the density and distribution of cells within the matrix, and thus affect the regeneration process. Although numerous papers have been devoted to collagen–GAG [9–15], there are no literature reports on the electric behaviour of such valuable composite materials. Since collagen is constituted by polar repeating units –CO–CR–NH– and GAG contains polar groups (COO^-, OSO_3^-, OH^-), the dielectric techniques are particularly sensitive to analyze relaxation phenomena. These techniques [16–19] used to study the effects of water and electric field frequencies on the dielectric properties of constituent phases of unmodified collagen. In view of the above, in this study we are interested in the dielectric properties of collagen–HA and collagen–CS scaffolds. To prepare collagen modified by CS or HA two complementary cross-linking methods, dehydrothermal (DHT) pre-treated and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) have been introduced. The cross-linking is an effective method to modify the stability towards biodegradation and to optimize the mechanical and thermal properties of collagen or collagen–GAG scaffolds [9,20]. Besides, DHT cross-linking owing formation of peptide bonds between adjacent chains of collagen macromolecule stabilizes it and prevents matrix collapse [21].

2. Experimental procedures

2.1. Materials

Collagen type I was derived from purified porcine Achilles tendons by pepsin digestion and acetic acid

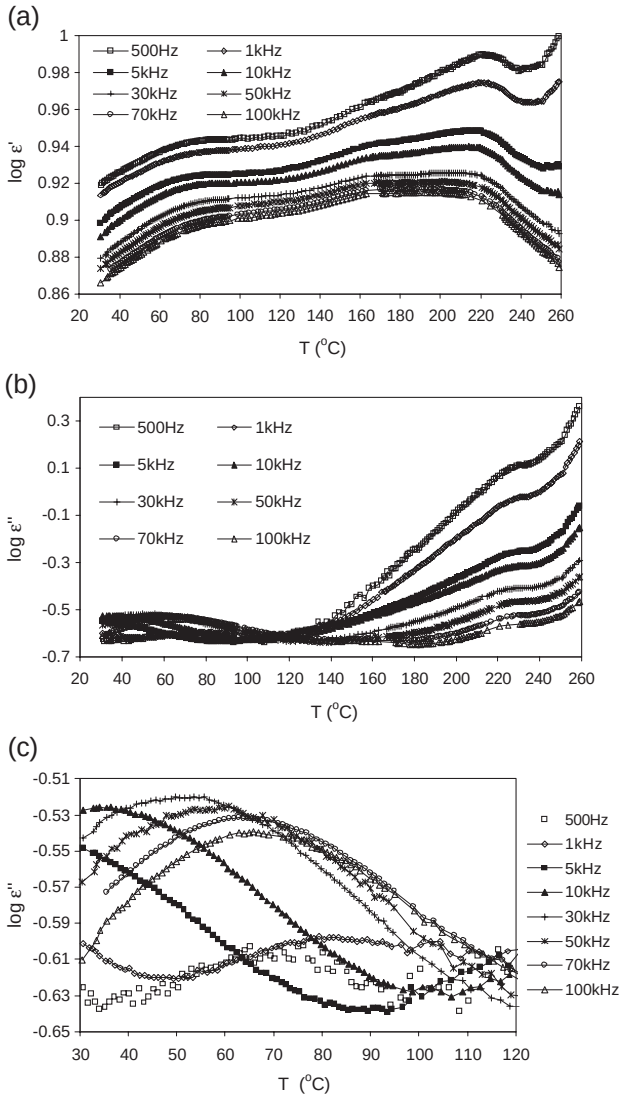

Fig. 2. Temperature dependencies of (a) ε′ and (b) ε″ up to 260 °C, and (c) ε″ up to 120 °C for CS-collagen.

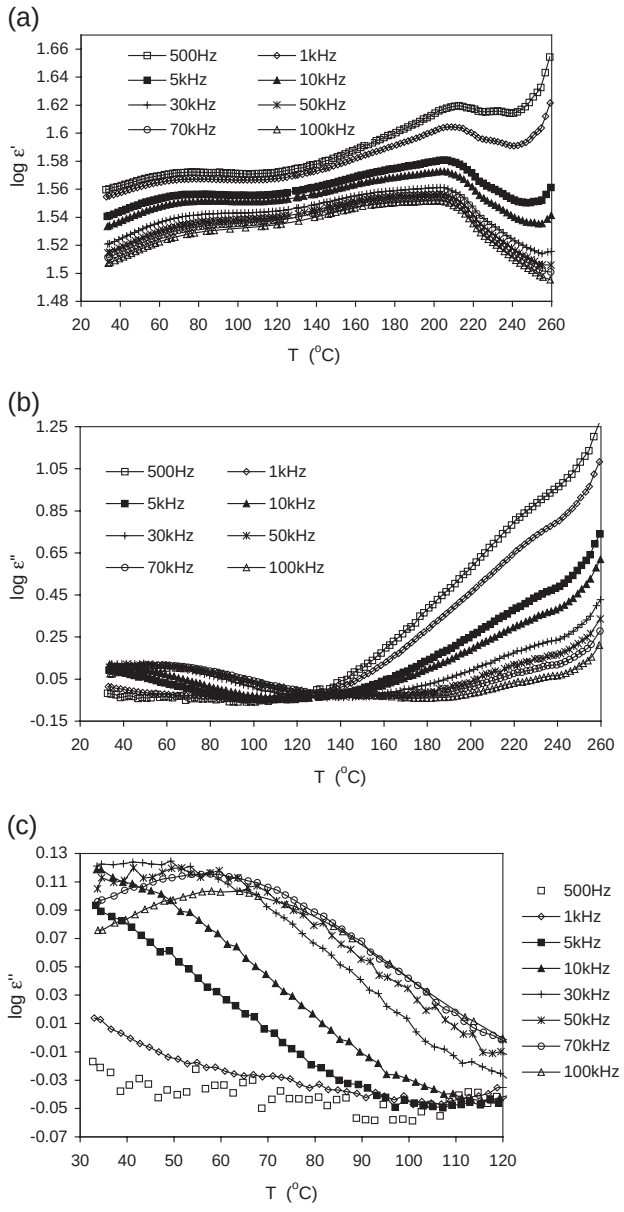

Fig. 3. Temperature dependencies of (a) ε′ and (b) ε″ up to 260 °C, and (c) ε″ up to 120 °C for HA-collagen.

dissolution to prepare 0.5% w/w dispersion. Further details of this procedure and methods of collagen investigation are given elsewhere [22]. The collagen solution was characterized by molecular weight, $M_v = 3.9 \times 10^5$, denaturation temperature, $T_d = 38$ °C and pH = 3.5. Analysis of amino acid composition (analyzer Jeol ILC-3 BC2) showed a high content of glycine, proline and hydroxyproline (almost 60% of all amino acid residues) but a very low amount of tyrosine (0.1/1000 amino acid residues). Its antigenicity is very low owing to the removal of telopeptides by pepsin treatment.

Hyaluronic acid (HA), chondroitin-4-sulfate (CS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and 2-morpholino-ethane (MES) were purchased from BioChemika Fluka. All other chemicals of analytical grade used in this work were purchased from POCh-Giwice, Poland.

2.2. Preparation of scaffolds

All experiments were carried out using collagen films as a model substrate. For film preparation, the collagen dispersion after deaeration was cast on the dish and then dehydrated by slow drying under a laminar airflow to obtain film of about 0.1 mm thickness. Some collagen films without any further treatment were taken as controls. Other films were dehydrothermally cross-linked by heating them at 80°C for 48 h under a vacuum of 50 mTorr. Thereafter, collagen films were cross-linked using EDC with NHS in the presence of 2.75% (w/v) chondroitin-4-sulfate or 0.5% (w/v) hyaluronic acid. The preparation of the collagen–CS/HA scaffolds was similar to the method given by Pieper et al. [9]. For the cross-linking reaction, 1.73 g EDC and 0.42 g NHS in 200 ml 50 mM MES buffer (pH 5.5) was used per gram of collagen. The addition NHS increases the rate and degree of cross-linking [23]. In order to minimize hydrolysis of EDC, cross-linking was carried out in buffer of 2-morpholino-ethane sulfonic acid (MES) [24]. After the cross-linking reaction, the samples were washed in 0.1 M Na_2HPO_4 (pH 9.1) and with 1 M NaCl, respectively. Finally, the matrices were rinsed with distilled water and dehydrated again by slow drying under a laminar flow hood.

2.3. Dielectric measurement

Measurements of the complex permittivity ε* ($\varepsilon^* = \varepsilon' - j\varepsilon''$) and conductivity σ ($\sigma = 2\pi f \varepsilon_o \varepsilon''$) were carried out using an impedance analyzer HIOKI 3522-50 LCR over the frequency range of 10 Hz–100 kHz and temperatures from 22 to 260 °C. The temperature of the collagen sample was measured by a constantan-copper thermocouple (Th). The electromotive force of the thermocouple was indicated by a digital voltmeter. The temperature controller (type 650, UNIPAN, Poland) was connected to an electric heater in the sample cell. The results were recorded by a computer.

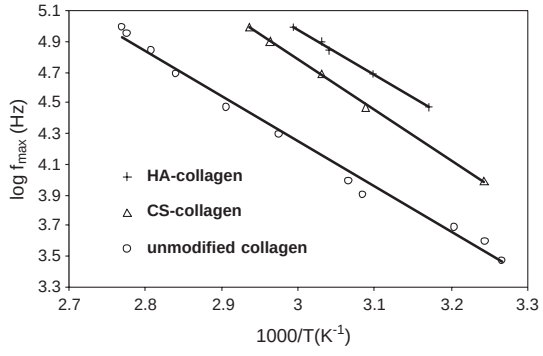

Fig. 4. Plots of $\log f_{max}$ vs. $1/T$ for unmodified collagen, CS-collagen and HA-collagen.

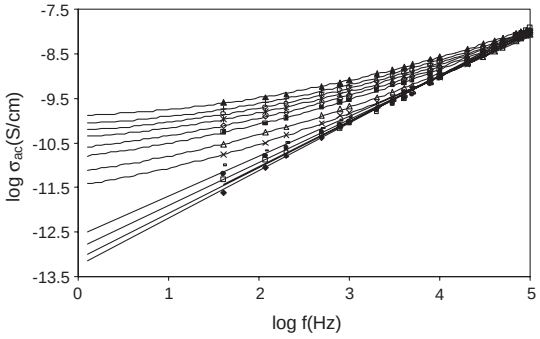

Fig. 5. The variation of σ_{ac} vs. frequency for unmodified collagen at selected temperatures of 50 (♦), 100 (□), 120 (●), 140 (−), 160 (×), 180 (△), 200 (■), 210 (◊), 220 (*), 230 (○) and 240 °C (+).

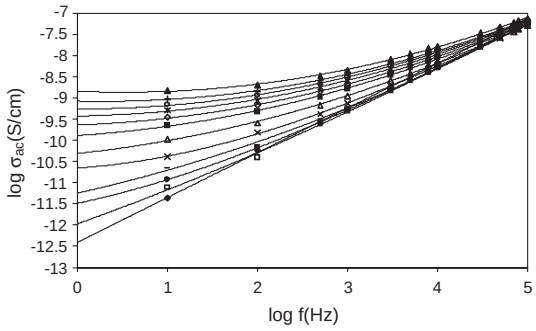

Fig. 7. The variation of σ_{ac} vs. frequency for HA-collagen at selected temperatures of 50 (♦), 100 (□), 120 (●), 140 (−), 160 (×), 180 (△), 200 (■), 210 (◊), 220 (*), 230 (○), 240 (+) and 250 °C (▲).

Prior to the dielectric measurement the collagen sample was covered with silver paste electrodes and subjected to elimination of loosely bound water by keeping it in the measuring cell at a constant temperature of 120 °C for 2 h. After this time the sample was cooled to room temperature and was subjected to dielectric measurements in the cycle of heating from room temperature to 260 °C at a rate of about 1 °C/min.

In our experiments we assumed, that electrode polarization effects are negligible. These effects has been taken into account in such materials as food products [25], apples [26] and hydrogels [27] with very high water contents, whose conductivity at low frequencies is greater than 10^{-8} $(\Omega\ \mathrm{cm})^{-1}$ [28]. In this study, the conductivities in the low frequencies and in the whole temperature range are lower than 10^{-8} $(\Omega\ \mathrm{cm})^{-1}$ for unmodified collagen, and CS- and HA-modified collagen, so the electrode polarization effects from the water in these materials should not be significant.

3. Results and discussion

Figs. 1–3 present the temperature dependencies of the relative permittivity ε′ and dielectric loss ε″ for unmodified collagen and CS- and HA-modified collagen for various frequencies, respectively. The values of ε′ and ε″ at the same temperature are higher in HA-modified collagen than in the unmodified collagen. However, small differences are shown in these parameters between CS-modified collagen and unmodified collagen. As seen in each figure, according to the Kramers–Kronig relationship a dispersion is apparent in both ε′ and ε″ below 140 °C and at higher temperatures as a broad peak around 220–230 °C, respectively. For a better representation of the low T dispersion, the plots of ε″ in Figs. 1–3(b) are shown only to 140 °C in Figs. 1–3(c). For this dispersion the variation in ε′ occurs near the peaks in ε″ which shift to lower temperatures as the frequency decreases. Fig. 4 presents the Arrhenius plots of the frequency maximum, whose slopes yield the activation energy ΔH for the relaxation of all the samples. The dispersion curves for CS- and HA-modified collagen appear at lower temperatures than for unmodified collagen. The ΔH takes the values 56 kJ/mol for unmodified and HA-modified collagen, and 62 kJ/mol for CS-modified collagen. These values of ΔH are similar for three materials, which would indicate on the same polarization and conduction mechanisms for the low T dispersion.

The second relaxation process shows no frequency dependence and is manifested as peaks for ε′ and ε″. These peaks are much clearer in the ε′ dependence for all three materials. However, determination of a well-defined peak width or peak temperature is difficult without a complete

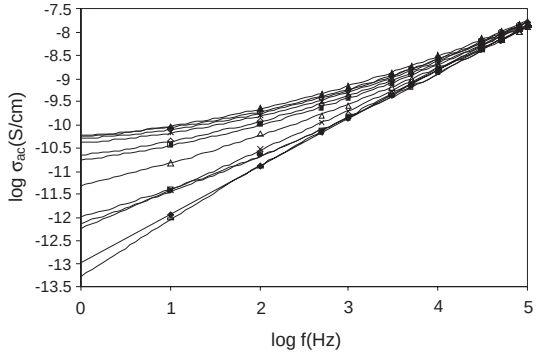

Fig. 6. The variation of σ_{ac} vs. frequency for CS-collagen at selected temperatures of 50 (♦), 100 (□), 120 (●), 140 (−), 160 (×), 180 (△), 200 (■), 210 (◊), 220 (*), 230 (○), 240 (+) and 250 °C (▲).

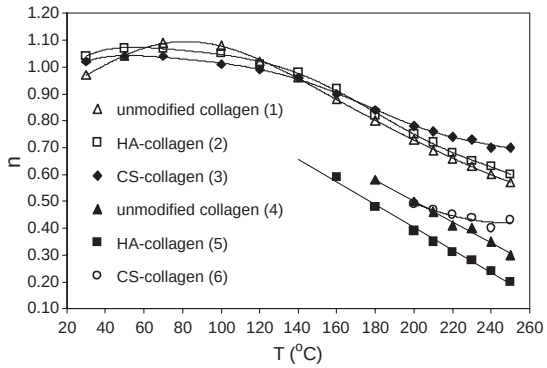

Fig. 8. The variation of the exponent n as a function of temperature for unmodified collagen, CS-collagen and HA-collagen.

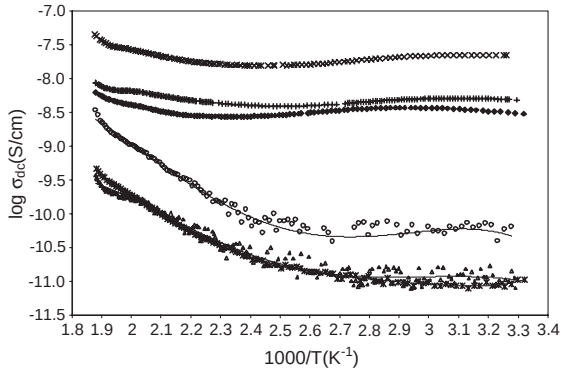

Fig. 9. Plots of log σ_{ac} vs. $1/T$ for unmodified collagen (*, 100 Hz; ♦, 30 kHz), CS-collagen (▲, 100 Hz; +, 30 kHz) and HA-collagen (○, 100 Hz; ×, 30 kHz).

modelling of all dispersions. As seen in Figs. 1–3(a–b), there appears to be another dispersion which occurs at higher T than those used in the experiments. The observed dispersion around 220–230 °C corresponds to the decomposition of unmodified and CS- and HA-modified collagen. The occurrence of decomposition process of these materials at the same temperature range has been confirmed by differential scanning calorimetry (DSC) and thermogravimetric (TG) analysis [19].

Figs. 5–7 show the frequency dependence of ac conductivity σ_{ac} for unmodified collagen, and CS- and HA-modified collagen at different temperatures, respectively. The curves present the typical conductivity power-law variation with frequency $\sigma_{ac}=\sigma_{dc}+Af^n$, where σ_{dc} is dc conductivity, A is a constant and n is the power law exponent. Fig. 8 compares the temperature dependencies of the exponent n for the three materials, obtained from the linear fit of the curves presented in Figs. 5–7. These fits were obtained for each material in the three ranges of frequency and appropriate temperature range. Curves (1), (2) and (3) were obtained in the range 10 Hz–100 kHz and 500 Hz–100 kHz up to 100 °C, 120 °C and 160 °C, and above these temperatures up to 250 °C, respectively. The coefficient of determination, r^2, was greater than 0.996 for each fit. The curves (4) ($r^2>0.981$), (5) ($r^2>0.975$) and (6) ($r^2\sim 1$) correspond to the low-frequency range 10–500 Hz and temperatures above 180 °C. Up to 180 °C the n values lay between 0.8 and 1, which indicates that in this temperature range, the hopping conduction of protons is the dominant charge transport process. The observed low T relaxation in Figs. 1–3 (c) can be attributed to the proton polarization and conduction processes. In an earlier paper [18] we discussed the mechanism of proton transport for collagen and other proteins in the same temperature range. Above 180 °C for curves (1–3) the n values decrease up to about 0.5–0.6. For low frequencies in the case of curves (4–6) the n values lay below 0.4. The changes in the parameter n are accompanied by an increase in ε' and ε'' for each frequency and the appearance of the high T relaxation as shown in Figs. 1–3(a, b). Moreover, above 200 °C the conductivity σ_{ac} (Figs. 5–7) flattens out sufficiently at low frequencies to get dc conductivity.

In order to compare the dynamics of the high T relaxation for unmodified and modified collagen we presented in Fig. 9 the Arrhenius plots of the ac conductivity against T^{-1} for 100 Hz and 30 kHz, so for the frequencies from the low- and high-frequency range. In the temperature range 3.3 K^{-1} (30 °C)–1.87 K^{-1} (260 °C), the values of σ_{ac} are significantly higher for HA-modified collagen than unmodified collagen at the same temperature. However, small differences in the conductivity appear for low frequencies between CS-modified collagen and unmodified collagen. Only for higher frequencies, the values of σ_{ac} are a little bit higher in CS-modified collagen than in unmodified collagen. Most probably, the conductivity in collagen–CS and collagen–HA is due to a large density of charge carriers and the formation of additional hydrogen bonds for charge transport as a result of the cross-linking reactions. The most important reactions occurring in collagen–CS/HA are two forms of cross-linking: collagen–collagen and collagen–CS/HA (Fig. 10). As it was shown [10,11] in both reactions, EDC and NHS activates carboxylic groups of glutamic and aspartic acid residues in collagen or carboxylic group of glucuronic acid residues in CS or HA. The reaction between NHS-activated carboxylic group of collagen, CS and HA

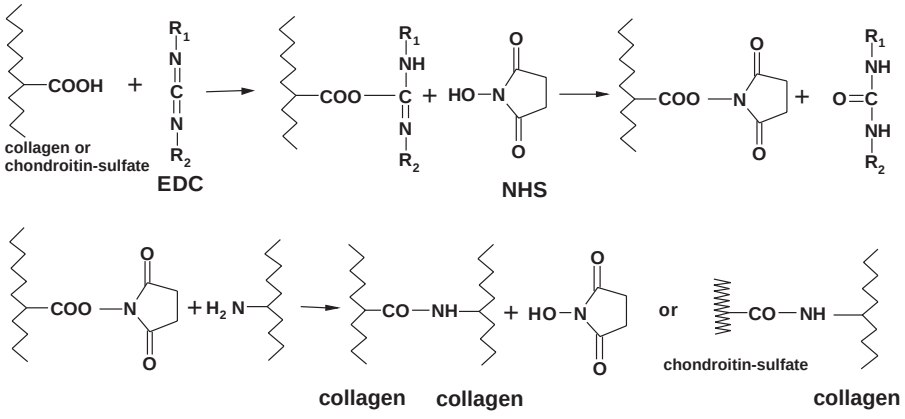

Fig. 10. Reaction of collagen or CS with EDC and NHS. $R_1=-CH_2-CH_3$; $R_2=-(CH_2)_3-NH^+-(CH_3)_2Cl^-$.

with ε-amino groups from lysine and hydroxylysine of collagen induces the formation of amide bonds –CO–NH–.

4. Conclusions

The temperature and frequency dependencies of the dielectric parameters for unmodified and modified collagen show low and high *T* relaxation. The influence of CS and HA on dielectric behaviour of collagen is manifested by a shift of the dielectric loss maximum associated with low *T* dispersion towards lower temperatures. The activation energy for this dispersion is similar for the three materials of about 60 kJ/mol. The high *T* relaxation is accompanied by an increase in ε' and ε'' for each frequency and small changes in σ_{ac} in the low-frequency region.

The result of this study indicate that the dielectric spectroscopy is useful in observing the differences in the low and high temperature behaviour between unmodified and modified collagen.

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References

[1] C.R. .Lee, H.A. Breinan, S. Nehrer, et al., Articular cartilage chondrocytes in type I and type II collagen–GAG matrices exhibit contractile behaviour in vitro, Tissue Eng. 6 (2000) 555–565.
[2] J.M. Zaleskas, B. Kinner, T.M. Frejman, et al., Contractile forces generated by articular chondrocytes in collagen–glycosaminoglycan matrices, Biomaterials 25 (2004) 1299–1308.
[3] Q. Lu, K. Ganensan, D.T. Simionescu, N.R. Vyavahare, Novel porous aortic elastin and collagen scaffolds for tissue engineering, Biomaterials 25 (2004) 5227–5237.
[4] M.H. Fittkau, P. Zilla, D. Bezuidenhout, et al., The selective modulation of endothelial cell mobility on RGD peptide containing surfaces by YIGSR peptides, Biomaterials 26 (2005) 167–174.
[5] F.H. Silver, D.L. Christiansen, Biomaterials Science and Biocompatibility, Springer-Verlag, New York, 1999.
[6] F.J. O'Brien, B.A. Harley, I.V. Yannas, L.J. Gibson, The effect of pore size on cell adhesion in collagen–GAG scaffolds, Biomaterials 26 (2005) 433–441.
[7] H. Bayraktar, E. Akal, O. Sarper, et al., Modeling glycosaminoglycans-hyaluronan, chondroitin, chondroitin sulphate A, chondroitin sulphate C and keratin sulphate, J. Mol. Struct., Tcheochem. 683 (2004) 121–132.
[8] C.R. Lee, A.J. Grodzinsky, M. Spector, Structural investigations of cross-linked hyaluronan, Biomaterials 22 (2001) 3145–3154.
[9] J.S. Pieper, A. Oosterhol, P.J. Dijakstra, J.H. Veerkamp, T.H. van Kuppevelt, Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern, Biomaterials 20 (1999) 847–858.
[10] J.S. Pieper, T. Hafmans, J.H. Veerkamp, T.H. van Kuppevelt, Development of tailor-made collagen–glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects, Biomaterials 21 (2000) 581–593.
[11] M.J.B. Wissink, R. Beernink, A.A. Poot, et al., Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices, J. Control. Release 64 (2000) 103–114.
[12] R. Servaty, J. Schiller, H. Binder, et al., Hydration of polymeric components of cartilage—an infrared spectroscopic study on hyaluronic acid and chondroitin sulfate, Int. J. Biol. Macromol. 28 (2001) 121–127.
[13] S.N. Park, J.C. Park, H.O. Kim, M.J. Song, H. Suh, Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide cross-linking, Biomaterials 23 (2002) 1205–1212.
[14] Y.S. Pek, M. Spector, I.V. Yannas, et al., Degradation of a collagen–chondroitin-6-sulfate matrix by collagenase and by chondroitinase, Biomaterials 25 (2004) 473–482.
[15] D.L. Ellis, I.V. Yannas, Recent advantages in tissue synthesis in vivo by use of collagen–glycosaminoglycan copolymers, Biomaterials 17 (1996) 291–299.
[16] V. Samouillan, A. Lamure, E. Maurel, J. Dandurand, C. Lacabanne, F. Ballarin, M. Spina, Characterisation of elastin and collagen in aortic bioprotheses, Med. Biol. Eng. Comput. 38 (2000) 226–231.
[17] V. Samouillan, A. Lamure, C. Lacabanne, Dielectric relaxations of collagen and elastin in the dehydrated state, Chem. Phys. 255 (2000) 259–271.
[18] E. Marzec, W. Warchol, Dielectric properties of a protein–water system in selected animal tissues, Bioelectrochemistry 65 (2005) 89–94.
[19] W. Friess, G. Lee, Basic thermoanalytical studies of insoluble collagen matrices, Biomaterials 17 (1996) 2289–2294.
[20] K. Pietrucha, M. Zaborski, Thermal and mechanical properties of collagen–chondroitin sulphate sponges as scaffold for tissue engineering, Ann. Pol. Chem. Soc. 3 (2004) 715–718.
[21] K.S. Weadock, E.J. Miller, E.L. Keuffel, M.G. Dunn, Effect of physical cross-linking methods on collagen fiber durability in proteolytic solutions, J. Biomed. Mater. Res. 32 (1996) 221–226.
[22] K. Pietrucha, Effect of irradiation on collagen solutions in relation to biomedical applications, Polym. Med. 19 (1989) 3–18.
[23] L.H.H. Olde Damink, P.J. Dijkstra, J. Feijen, M.J.A. van Luyn, P.B. van Wachem, P. Nieuwenhuis, Cross-linking of dermal sheep collagen using a water-soluble carbodiimide, Biomaterials 17 (1996) 765–773.
[24] M.A. Gilles, A.Q. Hudson, C.L. Borders, Stability of water-soluble carbodiimides in aqueous solutions, Anal. Biochem. 184 (1990) 244–248.
[25] B. Bodakian, F.X. Hart, The dielectric properties of meat, IEEE Trans. Dielectr. Electr. Insul. 1 (1994) 181–187.
[26] F.X. Hart, W.H. Cole, The dielectric properties of apples in the range 0.1–100 kHz, J. Mater. Sci. 28 (1993) 621–631.
[27] P. Pissis, A. Kyritsis, Electrical conductivity studies in hydrogels, Solid State Ionics 97 (1997) 105–113.
[28] M. Davies, Practical correction for electrode polarization effects, in: N.E. Hill, W.E. Vaugham, A.H. Price, M. Davies (Eds.), Dielectric Properties and Molecular Behaviour, Van Nostrand-Reinhold, London, 1969, pp. 285–287.