Influence of Laboratory Ware Related Changes in Conformational and Mechanical Properties of Collagen

R. Sripriya,[1] Md. Rafiuddin Ahmed,[2] P. K. Sehgal,[1] R. Jayakumar[2]

[1] *Bioproducts, Central Leather Research Institute, Adyar, Chennai 600 020, India*
[2] *Bioorganic Laboratories, Central Leather Research Institute, Adyar, Chennai 600 020, India*

ABSTRACT: Enzyme-treated acid-solubilized bovine collagen (ESBC Type I) prepared in laboratory-grade glass and polypropylene ware under identical conditions have shown different conformational and mechanical properties. Circular dichroism (CD) studies on ESBC have shown higher triple-helical contents in polypropylene laboratory-grade ware compared to laboratory ware of glass. Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetric (DSC) studies have supported the conformational changes performed on both preparations. Both preparations, in membrane form, were evaluated for their mechanical properties—viz., shrinkage temperature (St), dissolution temperature (Dt), and tensile strength (Ts). The collagen membrane of ESBC prepared in polypropylene showed significantly higher values compared to glass ones. No difference was seen in shrinkage temperature data of the two membranes. Reduced viscosity of ESBC was also measured for both preparations, and it had a significantly higher value in polypropylene ware compared to ESBC prepared in glassware. We postulate that the presence of silicate ions (SiO_3^{-2}) in glassware has a role in all these changes. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 87: 2186–2192, 2003

Key words: Achilles tendon; bovine collagen; polypropylene ware; glassware; conformational properties; mechanical properties

INTRODUCTION

Collagen constitutes the major structural protein in the extracellular matrix, providing mechanical strength and structural integrity to the various tissues in the body. The stabilization of collagen fibers during development and through growth to maturation is fairly well understood.[1] Variation in physical properties of fish and invertebrates collagen has been reported.[2] In spite of different raw materials (tissues) available to obtain pure collagen, the processing techniques of apparatus used were also found to play a major role in physical properties of the collagen. Though it is a chief structural protein and distributed in different parts of the vertebrates, the term *collagen* usually implies the collagen present in skin, tendon, and bone. Tendon and bone contain about 90% collagen and skin contains more than 50% collagen.[3] The most abundant among the different types of collagen is the Type I collagen. Because of its abundance in nature and its unique physicochemical and biological properties, Type I collagen has been used extensively to formulate medical materials.[4] Presently, pure type I collagen has been marketed in different forms like *Lyostypt* of bovine origin, *Osteovit* of bovine origin,[5] and *Bio-Gide B* and *Bio Gide,* both of porcine origin.[6] *Kollagen,* from the serosal layer of bovine, and *NeuSkin*, from bovine Achilles tendon, have been marketed.[7] Bovine tendons are rich in pure Type I collagen and found abundantly and easily available.[8,9] Porcine skin was also used in the extraction of Type I collagen.[10]

Collagen is a unique protein possessing different levels of primary, secondary, tertiary, and quaternary structural orders. The structure of collagen was investigated extensively by Ramachandran.[11] The individual molecules of collagen are made of three polypeptide chains, each containing over 1200 residues. In Type I collagen, the triple-helical structure contains two chains that are identical and are termed as $\alpha_1(I)$, and the third chain with a somewhat different primary structure is called $\alpha_2(I)$.[12] Each of these chains occurs in a left-handed helix related to polyproline and polyglycine, and these three chains are supercoiled in a right-handed fashion to form a triple helix. In contrast to other ordered conformations such as the α-helix and β-sheet structures, the triple helix of collagen (Type I) is a compact conformation. The conformation of collagen is related to the polyproline II helix. The covalent intermolecular crosslinks between collagen molecules in macromolecular fibrils are essential for its stability and various physicochemical properties. After solubilization of collagen, some of these

crosslinks were also found, allowing the preparation to have monomers, dimers, and oligomers. Ramachandran et al.[13] pointed out that a water molecule forming an interchain bridge was in a position to form an H-bond to the hydroxyl group of hydroxyproline and further stabilize the triple helix. Materials suited for medical systems have always been a product of interdisciplinary collaboration among polymer chemists, biochemists, and clinicians. Collagen, along with other polymers, conceivable qualifies as a biomaterial based on its physicochemical, biochemical, mechanical, and biological properties. But most of the polymers other than collagen lack the capacity to direct or regulate biological response. Collagen is unique in a way, being capable of resorption and integration with a body in a controlled manner through crosslinking. The massive research effects expended on collagen make it one of the best polymers today, and this research adds to new knowledge in the field of polymer engineering and applied polymer science. Even though there are many isolation and purification methods available,[14] the main difficulty encountered depends on the different conditions and containers used for solubilization of collagen. While preparing soluble collagen from these tissues, we have noticed changes in the secondary and tertiary behavior of collagen when we switched over from glass to polypropylene ware. Changes were also seen in mechanical properties and viscosity. These changes were quite interesting and we wanted to investigate them systematically. In this study, we have compared the enzyme-treated acid-solubilized bovine (ESBC) collagen from tendon in both glass and polypropylene ware by their characterization techniques, such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE), differential scanning calorimetry (DSC), Fourier transform infrared (FTIR), circular dichroism (CD), and shrinkage temperature.

Results of the study reported in this article could contribute to new knowledge in the field of applied polymer science and technology.

MATERIALS AND METHODS

Preparation of collagen membrane

The Achilles tendons, collected fresh from a local slaughterhouse, were manually dissected out from surrounding fascia, followed by washing in tap water. They were cut into small bits of 3–4 mm each with a sharp knife and were solubilized by a patented procedure developed at CLRI.[15] To explain in brief: The bits were minced in a mincer (Model 4612, Hobart, USA). The minced tissues were divided into two equal parts, and one was taken in a glass and another in a plastic container; subsequent processes were carried out separately in the respective containers. The minced tissues were washed using a nonionic surfactant. The washed tissues were suspended in sodium peroxide solution for swelling. The tissue was washed with distilled water (qs) after the coagulation of the swollen mass. The coagulated collagen was then suspended in phosphate buffer solution of pH 8.5 and treated overnight with trypsin (0.5% w/w). The tissue was again washed in distilled water to deactivate the enzyme and the dissolved salts were removed. The coagulated tissue was swollen again in distilled water after adjusting the pH of water to 2.5 with HCl, and treated with pepsin (0.3% w/w) overnight. After the second enzyme treatment, tissues were washed repeatedly in water to deactivate the enzyme. The coagulated collagen was dissolved in Millipore water (0.06 μs purity) acidified to pH 3.5 using HCl to get pure collagen solution. The undissolved proteins were removed by centrifugation at 10,000 rpm for 30 min using standard polypropylene centrifuge tubes. All the above operations were performed at a temperature of 15 ± 2°C. Purity of collagen was checked by physicochemical measurements as described here.

The pure solubilized collagen solution was degassed. The clear solution was allowed to dry in Teflon troughs kept in a dust-free chamber and maintained at a temperature of 22 ± 2°C. Collagen membranes were formed in 30–34 h. The membranes were washed with Millipore water (0.06 μs purity) and sealed in polyethylene sachets.

Hydroxyproline estimation

The purity of soluble collagen was estimated by the standard method of Neuman and Logan.[16] Hydroxyproline present in collagen was estimated by hydrolyzing the lyophilized sample of the material in 6N HCl in a sealed tube at 110°C for 16–24 h. This ensures complete availability of hydroxyproline present in the material. The hydroxyproline obtained is oxidized using copper sulfate and hydrogen per oxide to get pyrrole carboxylic acid. Pyrrole carboxylic acid was condensed with paradimethylamino benzaldehyde to give a purple color, which was read visible range at 540 nm and the values are compared with hydroxyproline standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The lyophilized collagen was dissolved in a sample buffer containing β-mercaptoethanol and was incubated at 65°C for 30 min. These will separate α-, β-, γ-, and other isomers of collagen. These samples were loaded in 8% separating gel. The protein–SDS complex were negative in charge and moved toward anode; components were separated based on the molecular weight. The gels were fixed in 50% methanol and 10%

acetic acid, and were stained using Coomassie blue (CBB) and destained with 10% methanol acetic acid mixture.

PAGE of the soluble collagen was determined by the Laemmli method[17] using a 5% stacking gel and 8% separating gel. The gels were stained with CBB, and destained with acetic acid and methanol.

CD studies

The CD studies on soluble collagen solution were carried out using a spectropolarimeter (JASCO J-715 model). Before conducting the experiments, the instrument was purged with nitrogen gas at a constant pressure of 3–5 mm Hg for a period of 30 min. A cell with a 1 mm path length was employed. The solvent (HCl pH 3.5 in Millipore water 0.06 μs purity) was taken in a cuvette for carrying out baseline adjustment. CD measurements of each of the samples were made from 190 to 300 nm. The CD was measured as molar ellipticity ($[\theta]$ deg cm^2 dmol^1), using the formula $[\theta] = (\theta_{obs} \times 10)/1 \times c'$; θ_{obs} is observed ellipticity in degrees; 1 the path length in decimeter, and c' the concentration in moles residue/1.

DSC studies

DSC studies of the soluble collagen membrane, as described in method of preparation, was recorded using a calorimeter (DSC 204). Samples of collagen membrane were shielded in aluminum containers. The heating rate 1 K (Kelvin) per minute and temperature range between −5 and 200°C in an N$_2$ atmosphere, were maintained.

FTIR spectroscopy studies

FTIR studies of the material (collagen membranes) were recorded using a FTIR instrument (Nicolet 2DDKB FTIR spectrometer).

Dissolution temperature

The dissolution temperature of the collagen membranes was measured as described by Bell and Bello.[18] To describe in brief: Collagen membranes were incubated at neutral pH (7.4) at 37°C. Temperature of the water bath was raised at a rate of about 10°C/h and the disappearance of the opaque membrane was observed. The dissolution temperature was taken as that temperature at which almost all the collagen was dispersed.

Tensile strength measurements

Collagen was tested in Instron Series II Automated Materials Testing System. The collagen membranes were cut into 16 mm dumbbells with 5.15 mm inner width and immersed in distilled water (1–2 min) before testing the samples. A tensile force was applied at an extension rate of 10 mm/min. The ends of the sample were held by pneumatic grips (40 psi grip pressure). The ability of the collagen biomaterial to bear loads can be studied by its stress–strain behavior. The slope of the plot in the stress–strain curve is termed *modulus*.

Shrinkage temperature

The shrinkage temperature of the collagen membranes was measured in a microshrinkage meter fitted with a field microscope. Wet Collagen membrane (1 sq cm, in distilled water) was placed on the wet surface (distilled water) of a microslide and the temperature of the slide was raised slowly (1°C/min). The shrinkage of the membrane can be visualized through the microscope. The temperature at which the membrane shrinks approximately half to its original size is taken as the shrinkage temperature.

Reduced viscosity

The relative viscosity of collagen solution was measured using a Ubblehode capillary viscometer from Messrs Schott and Genosser, Meizz, Germany.[19,20] Reduced viscosity was calculated from relative viscosity as follows:

Relative viscosity = Flow rate of solution (collagen)/Flow rate of solvent (HCl pH 3.5)
Specific viscosity = 1 − Relative viscosity
Reduced viscosity = Specific viscosity/Concentration of the solution (g/100 mL)

RESULTS AND DISCUSSION

Purity of collagen

The electrophoretogram of ESBC is given in Figure 1. The α, β, and γ are clearly shown in the PAGE run. No other major bands were observed, indicating purity of the collagen preparations processed separately in polypropylene and glass ware. The pattern of α-, β-, and γ-isomers of collagen compares favorably with the standard values reported earlier.[10] No change in PAGE pattern is observed of ESBC preparations in polypropylene and glass ware.

Hypro estimations have shown more than 89% of collagen (on moisture basis) present in both preparations. This clearly shows no contamination is offered to the collagen solution during its preparation in both the systems.
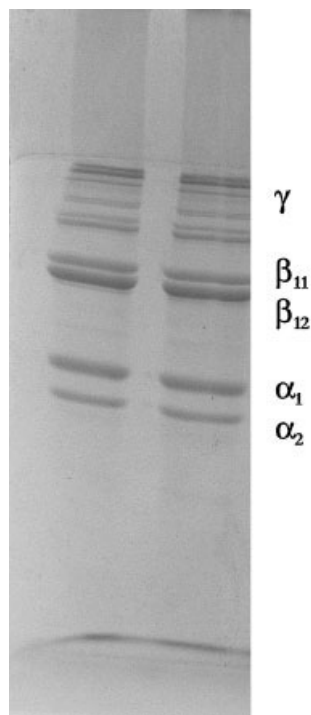
Figure 1 Comparative SDS-PAGE analysis of pure ESBC in (a) glassware and (b) polypropylene ware.

CD studies

Comparative CD spectra for both the preparations are given in Figure 2, and values of $\pi-\pi^*$ given in Table I. The CD spectra of ESBC shown in Figure 2 are dominated by $\pi-\pi^*$ amide transitions at ~200 nm and positive $n-\pi^*$ transitions at ~222 nm. The intensity of two bands is a measure of the triple-helical content of a given sample.[21,22] ESBC in polypropylene has higher value of $\pi-\pi^*$ transition (Fig. 2, curve b) compared to ESBC in glass (Fig. 2, curve a). thus indicating more triple-helical content in polypropylene preparation. The $n-\pi^*$ transition remains same for both the preparations. A positive peak at 225 nm is characteristic of the collagen molecule as determined by the CD spectrum.[23] Brahmachari *et al.*, related the relative band strength, i.e., ratio of rotational strengths of positive and negative CD band to the conformation in aqueous solution and in films cast from aqueous solutions.[24] The ratio of ESBC in polypropylene showed a lower value compared to the ESBC of glass, indicating increased solvation of the collagen carbonyls in polypropylene. Since the major stabilizing forces in the triple helix are intra- and interchain van der Waals forces and hydrogen bonds, perhaps the silicate (SiO_3^{-2}) ions in glassware participate in interchain hydrogen bonds directly through water bridges, which has a destabilizing effect and ultimately results in decreased solvation of ESBC in glassware's and lower triple-helical content.[25] In collagen, because of the close association of the three chains, there are no interior spaces or cavities in the triple helix. In this assembly, all residues are located on the surface. The polypeptide backbones of the three chains reside near the surface and all peptide —C≡O and NH groups are available for interaction with solvent and surface of glass or polypropylene. Polypropylene, perhaps, does not offer any resistance to the presence of more of these moieties, which serve as hosts for tightly bound structural H_2O.

Our results of CD studies have clearly indicated that even high-quality glass material can play an important role in altering the secondary and tertiary structure (triple-helical content) of collagen.

DSC studies

Figure 3 shows thermograms of both the membranes prepared in polypropylene and glass ware. The differences of the two-denaturation temperatures are clearly seen between the two-collagen membranes in the thermograms.

Curve a in the figure shows the thermogram of ESBC membrane prepared in glassware. The thermogram shows two thermal transitions (endothermal peaks) (ΔH 445 J/g collagen dry matter) at 81.0 and 116.7°C corresponding respectively to collagen denaturation temperature (Td) and the loss of water presence in the material. The first peak (81.0°C) initiated at 77°C, with one shoulder at 83°C and extending up to 84°C. This transition has a narrower range and occurs at a higher temperature than the membrane prepared in polypropylene ware. It indicates comparatively less water content in membranes prepared in glassware. Similarly, the second denaturation temperature, which is due to the loss of water, occurs at a lower temperature (116.7°C) in membranes prepared in glassware compared with the membrane prepared in polypropylene ware (132.2°C), thus indicating loss of
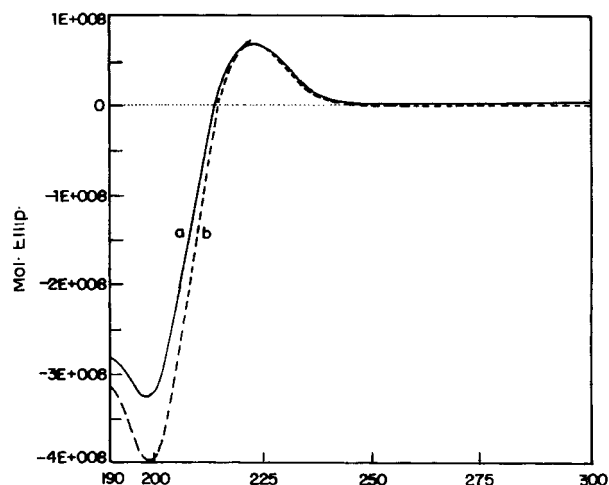

Figure 2 Comparative CD spectrum of ESBC in (a) glassware and (b) polypropylene ware.

TABLE I
DSC Studies, CD Studies, and FTIR Studies for Collagen Membranes Prepared in Polypropylene and Glass Ware

Property	ESBC membrane in polypropylene ware[a]	ESBC membrane in glassware[a]
DSC	Denaturation temperature is 77.6°C and loss of water at 132.2°C	Denaturation temperature is 81°C and loss of water at 116°C
FTIR	Ratio 1238.35/1454.83	Ratio 1239.14/1452.22
CD	π–π* Transition intensity 3.9859E	π–π* transition intensity 3.2747E

[a] Mean value of three determinations.

water for polypropylene ware membranes having higher retention of water content compared to glass ones. This supports higher triple-helical contents as shown in CD studies in collagen membranes prepared in polypropylene ware.

Curve b in the figure shows the thermogram of the ESBC membrane prepared in polypropylene ware. The thermogram shows two thermal transitions (endothermal peaks) (ΔH 390.8J/g collagen dry matter) at 77.6 and 132.2°C respectively corresponding to collagen denaturation temperature (Td) and the loss of water presence in the material. The first peak (77.6°C) initiated at 70°C, with two shoulders at 79 and 83°C and extending up to 88°C. This transition has a broader range and occurs at a higher temperature than the normal aqueous collagen dispersions, which usually range between 35 and 50°C. This is because the denaturation of the dried collagen matrix occurs at a much higher temperature than the aqueous dispersions of collagen. Indeed, it is known that reduced water content leads to higher denaturation temperature of collagenous tissue.[26] The values of denatur-

ation temperature and loss of water temperature are also given in Table I.

DSC experiments explain the significant role of hydrogen-bonding, hydrophobic, and electrostatic interactions in the stability of collagen, as reported recently.[27]

FTIR spectroscopy studies

Figure 4 shows the FTIR spectra of ESBC membranes prepared in glassware (curve a) and polypropylene ware (curve b). The amide I band, with characteristic frequencies in the range from 1600 to 1660 cm^{-1}, is associated with the stretching vibrations of the peptide carbonyl groups. The exact frequencies of these stretching vibrations depend both upon the strength of the hydrogen bond to the carbonyl oxygen and upon the environment dictated by the local peptide conformation. The amide II band (~1550 cm^{-1}) is related to NH bending and CN stretching, and amide III (1320–1220 cm^{-1}) is due to CN stretching and NH
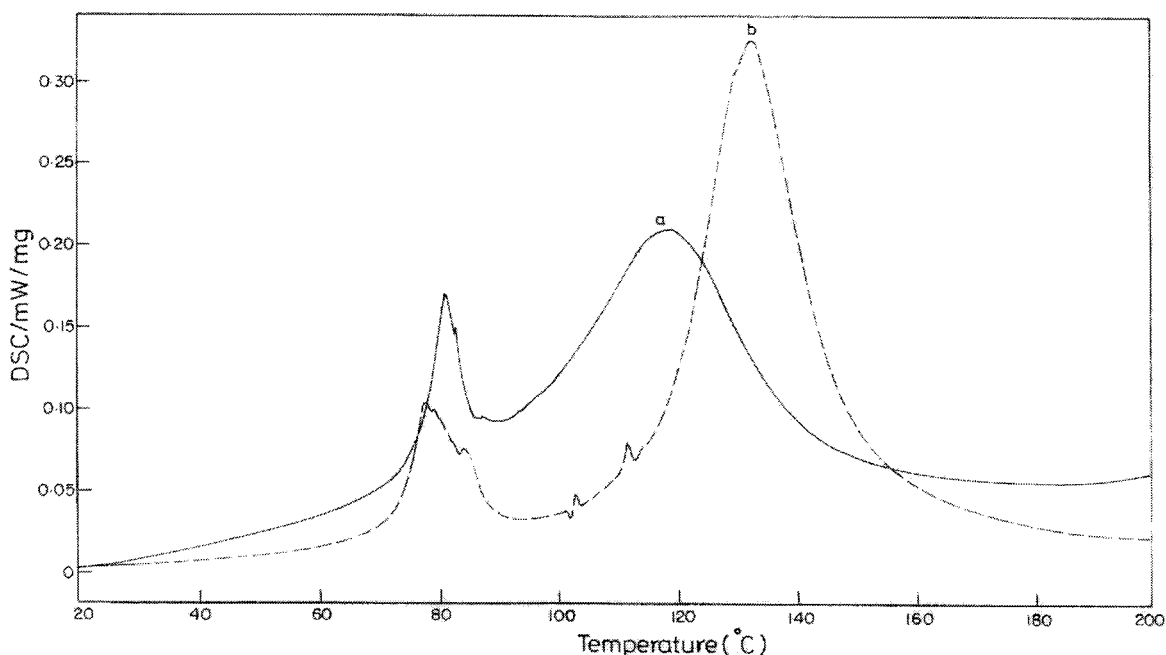

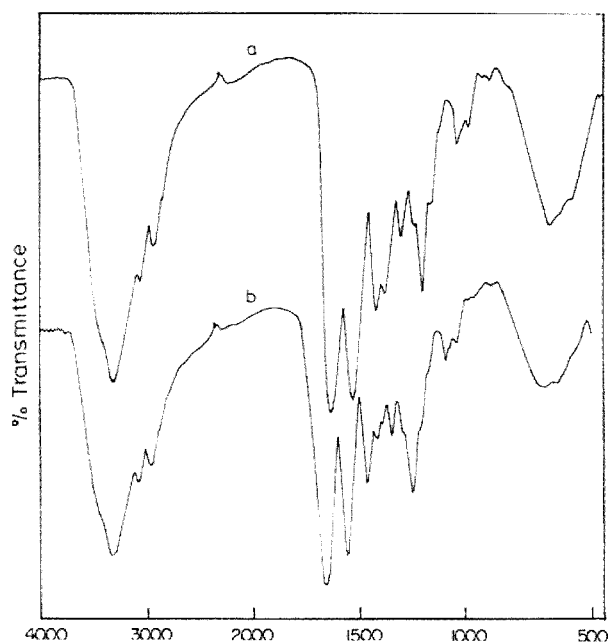**Figure 3** Comparative DSC Studies of ESBC in (a) glassware and (b) polypropylene ware.

Figure 4 Comparative FTIR spectrum of ESBC in (a) glassware and (b) polypropylene ware.

bending modes. The band close to 1450 cm^{-1} is probably associated with CH bending modes.[28–31]

The amide–A band (NH stretching), as observed at 3323 cm^{-1}, is almost symmetric, suggesting that the amount of water must be low. If any detectable amount of bound water is present under these conditions, its absorption band must practically overlap with the amide A band at 3323 cm^{-1}, implying an unlikely low frequency shift of ~100 cm^{-1} for the H$_2$O absorption band.

The IR absorption ratio (1238.35/1454.83) for ESBC membrane prepared in polypropylene ware is more than one and higher than compared to IR absorption ratio (1239.14/1452.22) for ESBC prepared in glassware. As a measure of protein denaturation, we used the absorption maximum of the CH$_2$ stretch vibration band at ~2973 cm^{-1}. This band is present in both preparations, showing no denaturation occurred in collagen during its preparation.[32] Absorption ratios of the above values are given in Table I.

Mechanical properties and reduced viscosity measurement

Mechanical properties viz., dissolution temperature, shrinkage temperature, and tensile strength of collagen membranes prepared in polypropylene and glass ware are given in Table II. Values of dissolution temperature and tensile strength are significantly higher in collagen membranes prepared in polypropylene ware. No significant change is observed in shrinkage temperature value for both preparations. Reduced viscosity measurement of ESBC solution showed a significantly higher value for collagen prepared in polypropylene ware. This indicates increased solvation of ESBC in polypropylene ware, which is directly linked to higher values of triple-helical contents. Mechanical data gave further evidence of properties linked with the conformation of collagen; shrinkage temperature data more or less indicate no change for both preparations, showing little effect in conformational changes on this property. Water plays a significant part in the matrix stability of collagenous structures. Secondary and quaternary structure of collagen implicates a hydrogen-bond network involving intermolecular and intramolecular interaction in collagen. Recently effect of urea on the dimensional stability of rat tail tendon (RTT) collagen has been reported.[33]

Laboratory ware glass (code 7280) contains, by wt %, 71% SiO$_2$, 1% LiO$_2$, 12% Na$_2$O, and 16% ZrO$_2$.[34] Presence of silicate ions of Na, Li, or Zr in the glass might interfere in the preparation of collagen. It ultimately moves into collagen, with different conformational and mechanical properties. Recently, the importance of the conditioned bioactive glass 45S5 as a substrate for nucleus pulposus cells has been reported.[35] Polypropylene resists strong acids (except nitric acid) and alkali up to 60°C. Perhaps it offers a better surface to process collagen tissues.

To know if the silicate ions play any role in collagen conformation and its mechanical behaviors, we added sodium silicate to a collagen solution (pH 3.5) and studied the reduced viscosity changes. Here, we have observed a drop in reduced viscosity of collagen solution in the presence of silicate ions. We are carrying out more experiments. Once we complete these exper-

TABLE II
Hydroxyproline, SDS-PAGE, Dissolution Temperature, Shrinkage Temperature, Tensile Strength, and Reduced Viscosity for Collagen Membranes Prepared in Polypropylene and Glass Ware

Property	ESBC membrane in polypropylene ware[a]	ESBC membrane in glass ware[a]
Hydroxyproline	89 ± 0.5%	89 ± 0.5%
SDS-PAGE	α, β, and γ bands present	α, β, and γ bands present
Dissolution temperature (°C)	88 ± 5	79 ± 5
Shrinkage temperature (°C)	54 ± 2	53 ± 2
Tensile strength (kg/cm^2)	112 ± 3	58 ± 3
Reduced viscosity	136 ± 4	39 ± 4

[a] Mean value of five determinations.

iments, we will know for sure the role of silicate ions in collagen conformational changes. We are conducting detailed studies using the presence of silicate ions in collagen preparation. These studies will be published separately.

CONCLUSION

Collagen is well recognized as a useful and an important biopolymer in the field of biomaterial engineering. In order to gain satisfactory results from collagen while being used as a biomaterial, it becomes necessary to improve its physical, chemical, and biological properties. Researchers[36] are investigating the effects of functionalization and sequence variation on triple-helical stabilities and molecular properties to design superior biomaterials based on collagen. Earlier, we were of the opinion that good-quality laboratory glassware (grade 7280) is the best material for handling collagenous tissues, and they can be used for preparing pure collagen. Our studies reported in this paper do not support this view, and we found polypropylene ware are comparatively better and perhaps among the best that can be used for handling and preparing collagenous tissues. The conformation of collagen is related to the conformation of imino acids present in collagen monomer. These conformational changes have been systematically studied using CD, DSC, and FTIR. The conformational changes are well supported by the mechanical and physicochemical properties of collagen studied in both preparations. This study has been reported for the first time, and we feel it would be useful for scientists and technologists to design their experiments better using collagen.

References

1. Bailey, A. J.; Gordon, P. Proc Indian Acad Sci (Chem Sci) 1999, 111, 57–69.
2. Miles, A. C.; Bailey, A. J. Proc Indian Acad Sci (Chem Sci) 1999, 111, 71–81.
3. Friess, W. Eur J Pharm Biopharm 1998, 45, 113–136.
4. Vijaya, R. D.; Sehgal, P. K. Indian Drugs 2000, 37(3), 119–128.
5. Wachol-Drewek, Z.; Preiffer, M.; Scholl, E. Biomaterials 1996, 17, 1733–1738.
6. Schlegel, A. K; Mohler, H.; Busch, F.; Mehl, A. Biomaterials 1997, 18, 535–538.
7. Eucare Pharmaceuticals (P), Ltd. Product monograph of Kollagen, Chennai, India, 2000.
8. Radhika, M.; Babu, M.; Sehgal, P. K. Comp Biochem Physiol 1999, 124, 131–139.
9. Manimalha, B.; Sehgal, P. K. In Vitro Cell Dev Biol Animal 1997, 33, 223–227.
10. Hsiu-O, H.; Lun-Huei, L.; Ming-Thau, S. J Cont Release 1997, 44, 103–112.
11. Ramachandran, G. N. Int J Peptide Protein Res 1988, 31, 1–16.
12. Miller, E. J. In Collagen Vol. I—Biochemistry; Nimni, M. H., Ed.; CRC Press: Boca Raton, FL, 1988; pp 139–157.
13. Ramachandran, G. N.; Bansal, M.; Bhatnagar, R. S. Biochim Biophys Acta 1973, 322, 166–171.
14. Piez, K. A. In Extracellular Matrix Biochemistry; Piez, K. A., Reddi, A. H., Eds.; Elsevier: New York, 1984; pp 1–40.
15. Sehgal, P. K.; Manimalha, B.; Ramesh, D. V. Indian Pat. Appl. No. 1351 Del-99, 1999.
16. Neuman, R. E.; Logan, M. A. J Biol Chem 1950, 186, 549–556.
17. Leammli, U. K. Nature 1970, 227, 680–681.
18. Bell, J.; Bello, H. R. Biochim Biophys Acta 1967, 147, 272–279.
19. Sehgal, P. K; Thomas Joseph, K; Leather Sci 1984, 31, 229–235.
20. Radhika, M.; Sehgal, P. K. J Biomed Mater Res 1997, 35, 497–503.
21. Jenness, D. D.; Sprecher, C.; Johnson, W. C., Jr. Biopolymers 1976, 15, 513–521.
22. Caldwell, J. W.; Applequist, J. Biopolymers 1984, 23, 1891–1904.
23. Kersteen, A. E.; Raines, T. R. Biopolymers 2001, 59, 24–28.
24. Brahmachari, S. K.; Bansal, M.; Ananthanarayanan, V. S.; Sasisekharan, V. Macromolecules 1979, 12, 23–28.
25. Ramachandran, G. N.; Bansal, M.; Bhatnagar, R. S. Biochim Biophys Acta 1973, 322, 166–174.
26. Kopp, J.; Bonnet, M.; Renou, J. P. Matrix 1989, 9, 443–450.
27. Usha, R.; Ramasami, T. Termochim Acta 1999, 338, 17–25.
28. Susi, H.; Ard, J. S.; Carroll, R. J. Biopolymers 1972, 10, 1597–1604.
29. Payne, K. J.; Veis, A. Biopolymers 1988, 27, 1749–1760.
30. Gordon, P. L.; Huang, C.; Lord, R. C.; Yannas, I. V. Macromolecules 1974, 7, 954–956.
31. Silvester, M. F.; Yannas, I. V.; Forbes, M. J. Thromb Res 1989, 55, 35–48.
32. Friess, W.; Lee. G. Biomaterials 1996, 17, 2289–2294.
33. Usha, R.; Ramasami, T. J Appl Polym Sci 2002, 84, 975–982.
34. Boyd, D. C.; Thompson, D. A. Encyclopedia of Chemical Technology, 3rd ed.;, John Wiley: New York, 1980; Vol. 2, pp 807–880.
35. Gan, J. C.; Ducheyne, P.; Vresilovic, E.; Shapiro, I. M. J Biomed Mater Res 2000, 51, 596–604.
36. Goodman, M.; Kwak, J. Proc Indian Acad Sci (Chem Sci) 1999, 111, 35–49.