XXXI IULTCS Congress
Valencia (Spain) - September 27th-30th 2011

Paper A8

Collagen stabilization using functionalized nanoparticles

Kalarical Janardhanan Sreeram, Marimuthu Nidhin, Selvam Sangeetha, Nishtar Nishad Fathima, Usha Ramamurthy and Balachandran Unni Nair
Chemical Laboratory, Central Leather Research Institute (CSIR), Adyar, Chennai, 600020, India
Corresponding Author: Tel. +91 44 2441 1630, Fax. +91 44 2491 1589, Email. kjsreeram@clri.res.in

1. Introduction

The leather making protein collagen is the most abundant protein and a major component of connective tissue. It is also one of the most common biomaterials with broad applications ranging from drug delivery to tissue-engineering, scaffolds and materials for wound dressing (1). Stabilization of collagen, thus confering mechanical stability and retardation of biodegradation is an important requisite for industrial application. Crosslinking procedures include reactions with glutaraldehyde, polyphenols, isocyanates, epoxides, bis imidates, metal ions such as Cr, Al, Zr, Fe etc as well as thermal treatment, UV or gamma-ray irradiation, and photo-oxidation. Many of the chemical crosslinking methods incorporate a "bridge" molecule as a part of the crosslink. Another way to chemically crosslink collagen is to use so called "zero-length" linkers; coupling agents capable of forming peptide bonds between collagen units such as EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) (2, 3). Irrespective of the coupling agent employed, a two-point connection between collagen units is created, while multiple-way crosslinkers are known to result in development of collagen materials exhibiting novel properties (4). It is in this regard that the Cr(III) ions are effective in bringing about inter-molecular and intra-molecular crosslinking, thus enhancing the stability of the collagen units. In biomedical applications, multiple-way crosslinks allow the incorporation of multiple functionalities within the collagen matrix in one step (5). It has been found that hydrogen bonds can be formed between amine as well as amide groups from collagen and hydroxyl groups from the crosslinking agent (tanning agent in the case of leather) (6).

In biomedical applications, an agent like glutaraldehyde is replaced, owing to toxicity. Replacements include L-DOPA (3,4-dihydroxyphenylalanine), a neurotransmitter, which is a naturally present compound in the living system (7). In leather making also, several of the chemicals, when employed in large quantities contribute to a significant pollution of soil and ground water, thereby questioning the very existence of the tanning industry. Tanning industry can perhaps draw similarities and clues from the biomedical applications of collagen in developing new tanning agents that are benign and provide higher atom economy through better diffusion and fixation.

Collagen stabilization strategies for biomedical applications: Nanosilver without any functional groups was reported to alter the positions of amide I and amide II bands of collagen (8). Cross linking with gold nanoparticles or silicon carbide nanowires has been shown to improve the mechanical properties of collagenous tissues and increase their resistance to degradation (9). Nanoparticles functionalized using appropriate reagents such as tiopronin (N-(2-mercaptopropionyl)glycine) (4), gelatin (10), amine groups (11) has been linked to collagen using EDC, resulting in the formation of an amide bond. However, the

EDC has lower crosslinking because of two point fixation (12). Polyamidoamine (PAMAM) dendrimer with surface amine groups in association with EDC provided substantial stability (12). This is attributable to the multiple crosslinks that the dendrimer can provide as against the two-way crosslinks. Substantiating this observation, poly(allylamine) based collagen-mimic compound, bearing more collagen model peptides than the dendrimer, exhibited a thermally stable higher order structure (13).

Taking clue from the previous studies, current work reports the synthesis of nanoparticles of chromium(III) oxide, functionalizing it with polymers capable of interacting with the collagen side chains and bringing about a higher degree of stability to the collagen. The novelty of our work is from the background knowledge that nanoparticles can be functionalized to provide for effective crosslinking with proteins, leading to a greener approach to the stabilization of protein without use of harmful chemicals such as chromium ions, aldehydes, etc. The nanoparticles can be effectively functionalized such that penetration of the nanoparticles through three dimensional, multipores collagen scaffolds can be effectively achieved prior to tunable fixation through electrostatic or hydrophobic interactions.

2. Materials and Methods

All chemicals used in this work were purchased from M/s. Sigma Aldrich and used as received. Collagen fibers were teased out from tails of six-month-old male albino rats (Wistar strain) and thoroughly washed and stored at -20°C until needed. Collagen extracted from rat-tail tendon (RTT) by known procedures (by acetic acid extraction and salting out with NaCl) was used for circular dichroism, viscosity and fluorescence studies. The collagen content of the solution was estimated by the standard procedures. Purity of collagen was checked by SDS-PAGE.

Synthesis of Cr_2O_3 nanoparticles: 0.025 moles of $Cr(NO_3)_3.9H_2O$ and 0.075 moles of urea were milled together at the rate of 600 rpm for 60 minutes, resulting in a dark green complex of urea with chromium ions. This complex was then treated in an autoclave for 20 minutes at a temperature of about ca.120ºC and pressure of 15 lbs. The resulting product was then dried in an air oven at 90ºC for 10 hours. The greed solid obtained was calcined in furnace at 800ºC for 60 minutes at the rate of 5ºC/min. After calcination, Cr_2O_3 formed as light green dense-less powder.

Synthesis of chromium oxide copolymer nanostructures: A stock solution of chromium oxide nanoparticles was prepared in THF (1mg/mL) and poly(styrene)-block-poly(acrylic acid) (PS-b-PAA) was prepared in DMSO (10mg/mL). To encapsulate the chromium oxide nanoparticles, 50µL of PS-b-PAA stock solution was diluted with a mixture of 2450µL of DMSO and 2000µL of THF and sonicated for 30 minutes. To this 500µL of chromium oxide nanoparticle stock solution was added gradually under sonication. After 1 hour, 20mL of water was added to it, and left in sonication for further 90 minutes. During the addition of water, the solution turned as milky suspension indicating the encapsulation of polymer on chromium(III) oxide (Cr-PS-b-PAA).

Characterization: Cr_2O_3 nanoparticles were characterized using FTIR, UV-Vis and Scanning Electron Microscopy (SEM). The nature of interaction between collagen and PS-b-PAA/Cr-PS-b-PAA was

studied using UV-Vis, Circular dichroism (CD), extent of fibril formation, shrinkage temperature (using Differential scanning calorimetry, DSC). The experimental procedures has been adopted from our earlier works (7).

3. Results and Discussion

3.1 Characteristic features of Cr_2O_3 nanoparticles

FTIR Analysis: Stable chromium complexes were formed with urea. The urea molecules may coordinate with the Cr ions through the nitrogen as well as the oxygen atoms. When the coordination is through the oxygen, the IR bands are shifted to lower frequencies, whereas when the coordination to Cr is through nitrogen present in the complex, then the stretching frequencies of the H-N is shifted to higher frequencies. The FTIR of the Cr_2O_3 nanoparticles synthesized in this study is presented in **Figure 1.**

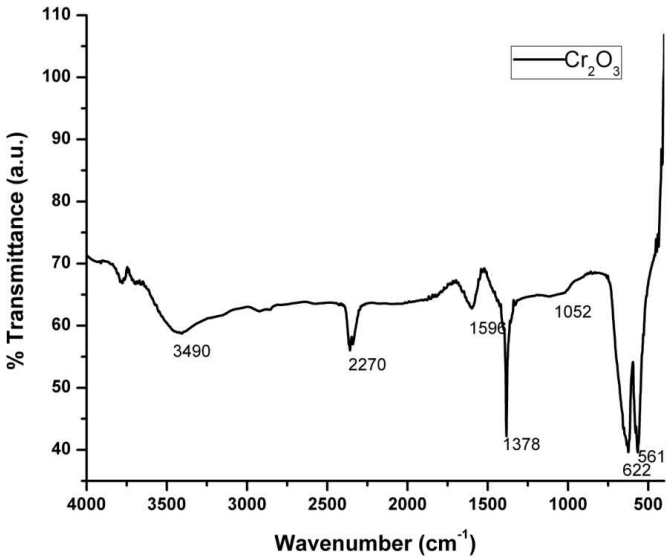

Fig 1. IR spectra of Cr_2O_3 nanoparticles synthesized through urea decomposition route

The strong band at 561 cm^{-1} corresponds to the characteristic vibrational mode of a symmetric CrO_6 system. The peak at 622 cm^{-1} is attributed to the structural ordering of A_{2u} model of Cr_2O_3 in amorphous phase. The peak around the region of 1052 cm^{-1} corresponds to the chromyl (Cr=O) vibrations. The coordinated $–NH_2$ groups shows some characteristic peaks such as, at 1596 cm^{-1} for N-H bond stretching, at 2270 cm^{-1} for N=C=O stretching. The peak at 1378cm^{-1} is due to the deformation on NH_4^+ ion. The bands in the region at 3400-3787 cm^{-1} indicates the presence of –OH arising from the chromium hydroxide intermediate.

UV-Vis spectra analysis: The UV- absorption spectra for the Cr_2O_3 nanoparticles was obtained after coating the nanoparticles on a white board using linseed oil as medium. The spectra showed two broad

peaks at 460 nm and 600 nm (**Figure 2**). The possible transitions of the Cr(III) ion in the crystal fields are

$^4A_2g \longrightarrow {}^4T_2g$ ----------► 1

$^2A_2g \longrightarrow {}^2T_1g, {}^2E_2g$ ----------► 2

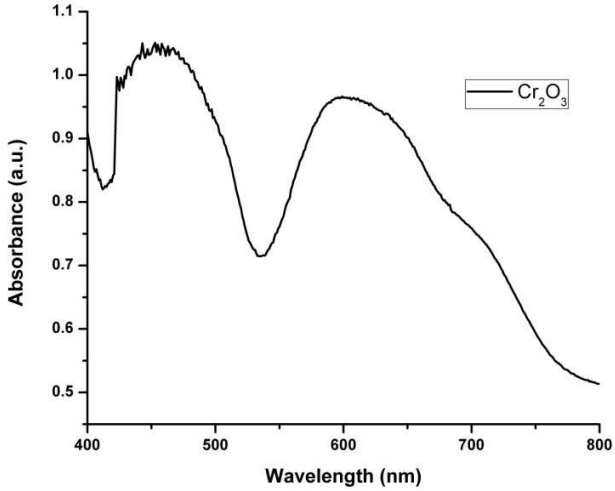

Fig 2. UV-Vis spectra of Cr_2O_3 nanoparticles coated as film

The transition stated in equation 1 show absorption band around the region of 460 nm and it corresponds to the octahedral geometry. The transition in equation 2 shows the band in the region of 590 nm and it corresponds to the six-coordinate geometry.

Morphological studies: Scanning Electron Microscopy images of the synthesized Cr_2O_3 nanoparticles is presented in **Figure 3.** From the SEM images the nanoparticles of Cr_2O_3 seem to have rice shape morphology with average size ranging from 50 - 72 nm. The nanoparticles are more or less uniform in size and discrete without much aggregation.

PS-b-PAA is a soluble amphiphilic block copolymer consisting of two blocks with different polarity, which is covalently bonded together. When dissolved in solvent, it can self-assemble into a variety of three dimensional arrays owing to the microphase-separation of the binding and solvating moieties (14). The styrene block can be designed to interact strongly with the appropriate inorganic crystals and surfaces, in this instance the chromium oxide nanoparticles and the -COOH block would be available for interaction with collagen.

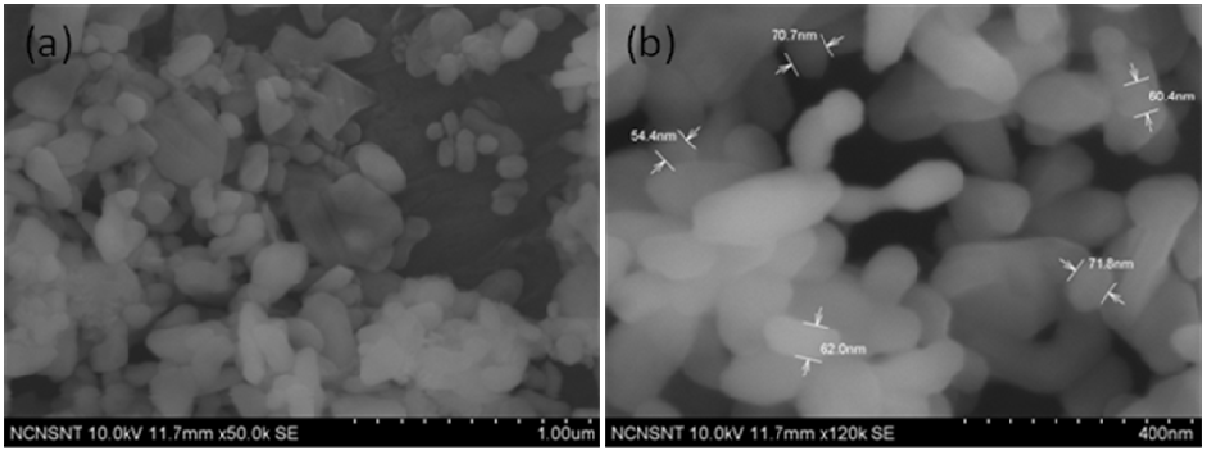

Fig 3. SEM images of Cr_2O_3 nanoparticles at two different magnifications

3.2 Understanding the nature of interaction of collagen with PS-b-PAA and Cr-PS-b-PAA

Absorption spectra analysis: The absorption spectra of collagen treated with PS-b-PAA and Cr-PS-b-PAA shows clear absorption spectrum with absorption maximum at 280nm (data not shown). It is possible that collagen with PS-b-PAA and Cr-PS-b-PAA system could form adhesive–collagen like gels or matrices by incorporation of PS-b-PAA and Cr-PS-b-PAA in concentration dependent manner.

Influence of PS-b-PAA and Cr-PS-b-PAA on collagen structure: The CD measurements provide ample information on the nature of transformation occuring in collagen upon crosslinking with PS-b-PAA and Cr-PS-b-PAA respectively. CD spectra of collagen solution treated with two different concentrations of PS-b-PAA and Cr-PS-b-PAA is presented in **Figure 4** respectively. A negative peak at 197nm and positive transition at 220nm is characteristic of collagen. The ratio of positive peak intensity over negative peak intensity (Rpn) is used in establishing helical conformation of collagen in solution. Rpn ratio for collagen treated with different concentrations of PS-b-PAA and Cr-PS-b-PAA is given in **Table 1**. Rpn values (~0.1) are indicative of conformational stability of collagen in the presence of PS-b-PAA and Cr-PS-b-PAA. The deviations in the molar ellipticity at 220 nm for collagen treated with PS-b-PAA and Cr-PS-b-PAA are neglible and do not suggest possible conformational changes or denaturation.

Fibril formation: Collagen molecules form fibrils or fibril bundles in tissues and can be extracted as soluble solution by treatment with acid. Acid soluble collagen molecules self assemble and form fibrils under physiological conditions. Fibril formation was monitored by the turbidity of solution of native collagen upon interaction with PS-b-PAA and Cr-PS-b-PAA.

The $t_{1/2}$ was found to be 93s, 99s, 147s for native, PS-b-PAA and Cr-PS-b-PAA respectively. An increase in the $t_{1/2}$ when treated with nanoparticles is a measure of the degree of fibril formation. This was further confirmed by the determination of shrinkage temperature of collagen using DSC. In the presence of Cr-PS-b-PAA the fibres showed a marginal increase in shrinkage temperature indicative of the ability of the functionalized nanoparticle to provide stability to collagen.

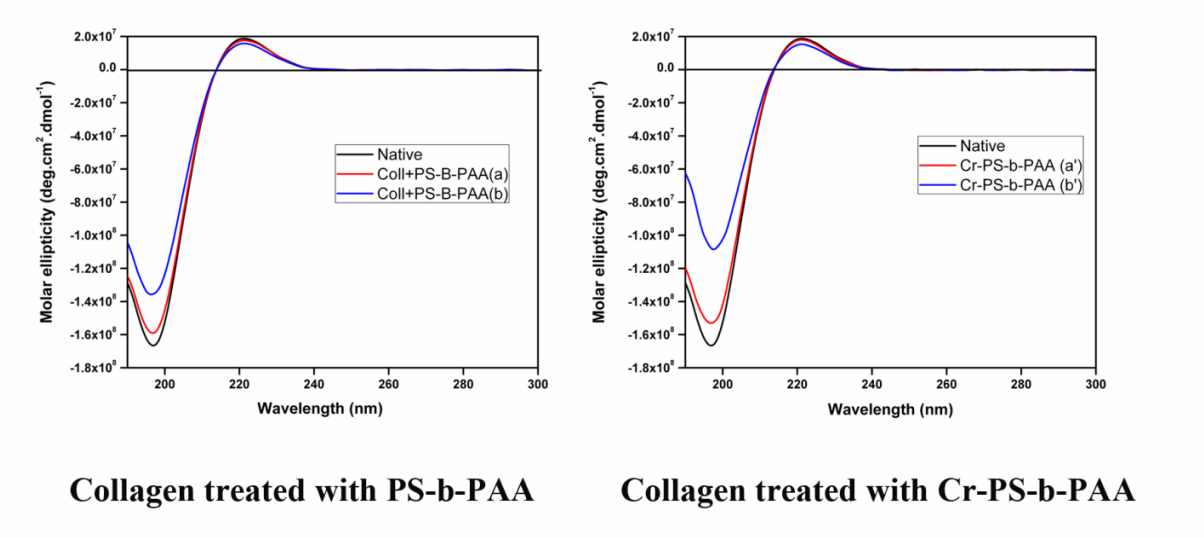

Fig 4. CD spectra of collagen treated with PS-b-PAA and Cr-PS-b-PAA

Table 1: Rpn values for collagen treated with PS-b-PAA and Cr-PS-b-PAA

Sample	Wavelength (nm)	Molar ellipticity ($deg.cm^2.dmol^{-1}$)	Rpn ratio
Native collagen	220	15166335	0.14
	197	-107893091	
PS-b-PAA (a)	220	18066534	0.113
	197	-159016207	
PS-b-PAA (b)	220	18635201	0.111
	197	-166579471	
Cr-PS-b-PAA (a')	220	18066534	0.118
	197	-152647142	
Cr-PS-b-PAA (b')	220	19203867	0.115
	197	-166579471	

where (a) Coll:PS-b-PAA – 297 µL : 3µL (b) Coll:PS-b-PAA – 270 µL : 30 µL (a') Coll:Cr-PS-b-PAA – 299 µL : 1µL and (b') Coll:Cr-PS-b-PAA – 291 µL : 9µL

Polarizing optical micrographs: Highly concentrated solutions of purified collagen molecules spontaneously form ordered assemblies characterized by polarizing optical microscopy. The polarizing optical micrographs of native and PS-b-PAA and Cr-PS-b-PAA crosslinked collagen fibrils are given in **Figure 5**. The thickness and texture of collagen fibrils crosslinked with PS-b-PAA and Cr-PS-b-PAA do not present any major change when compared to the native fibres. However, the Cr-PS-b-PAA treated fibres were shorter in length and this needs further investigation.

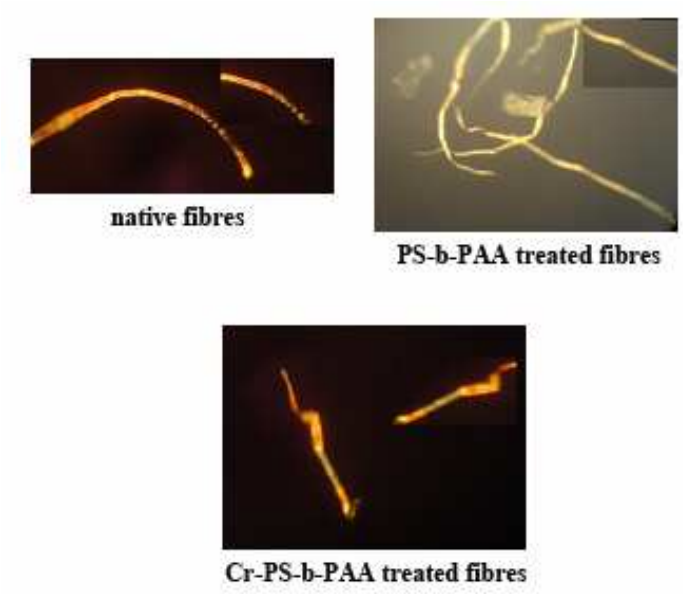

Fig 5. Polarizing optical micrographs of native fibres treated with PS-b-PAA and Cr-PS-b-PAA

4. Conclusion

Chromium(III) oxide nanoparticles functionalized using polymers such as PS-b-PAA have been found to stabilize collagen. While the extent of stability needs further improvement, the methodology offers ample scope for successful development of modified collagen with potential applications in biomaterials and tanning.

5. References

1. Han, B., J. Jaurequi, B.W. Tang, and M.E. Nimni, Proanthocyanidin: A natural crosslinking reagent for stabilizing collagen matrices, *Journal of Biomedical Materials Research Part A* 65A:118-124 (2003).
2. Damink, L., P.J. Dijkstra, M.J.A. vanLuyn, P.B. vanWachem, P. Nieuwenhuis, and J. Feijen, Cross-linking of dermal sheep collagen using a water-soluble carbodiimide, *Biomaterials* 17:765-773 (1996).
3. Rault, I., V. Frei, D. Herbage, N. AbdulMalak, and A. Huc, Evaluation of different chemical methods for cross-linking collagen gel, films and sponges, *J. Mater. Sci.-Mater. Med.* 7:215-221 (1996).
4. Castaneda, L., J. Valle, N. Yang, S. Pluskat, and K. Slowinska, Collagen Cross-Linking with Au Nanoparticles, *Biomacromolecules* 9:3383-3388 (2008).
5. Duan, X., and H. Sheardown, Crosslinking of collagen with dendrimers, *Journal of Biomedical Materials Research Part A* 75A:510-518 (2005).
6. Jastrzebska, M., J. Zalewska-Rejdak, R. Wrzalik, A. Kocot, I. Mroz, B. Barwinski, A. Turek, and B. Cwalina, Tannic acid-stabilized pericardium tissue: IR spectroscopy, atomic force microscopy, and dielectric spectroscopy investigations, *Journal of Biomedical Materials Research Part A* 78A:148-156 (2006).

7. Usha, R., A. Rajaram, and T. Ramasmi, Stability of collagen in the presence of 3,4-dihydroxyphenylalanine (DOPA), *J. Photochem. Photobiol. B-Biol.* 97:34-39 (2009).
8. Gaidau, C., M. Giurginca, T. Dragomir, A. Peticac, and W. Chen, Study of collagen and leather functionalization by using metallic nanoparticles, *J. Optoelectron. Adv. Mater.* 12:2158-2164 (2010).
9. Deeken, C.R., D.B. Fox, S.L. Bachman, B.J. Ramshaw, and S.A. Grant, Characterization of bionanocomposite scaffolds comprised of amine-functionalized gold nanoparticles and silicon carbide nanowires crosslinked to an acellular porcine tendon, *J. Biomed. Mater. Res. Part B* 97B:334-344 (2011).
10. Zhang, J.J., M.M. Gu, T.T. Zheng, and J.J. Zhu, Synthesis of Gelatin-Stabilized Gold Nanoparticles and Assembly of Carboxylic Single-Walled Carbon Nanotubes/Au Composites for Cytosensing and Drug Uptake, *Anal. Chem.* 81:6641-6648 (2009).
11. Deeken, C.R., M.J. Cozad, S.L. Bachman, B.J. Ramshaw, and S.A. Grant, Characterization of bionanocomposite scaffolds comprised of amine-functionalized single-walled carbon nanotubes crosslinked to an acellular porcine tendon, *Journal of Biomedical Materials Research Part A* 96A:584-594 (2011).
12. Zhong, S.P., and L.Y.L. Yung, Enhanced biological stability of collagen with incorporation of PAMAM dendrimer, *Journal of Biomedical Materials Research Part A* 91A:114-122 (2009).
13. Suehiro, T., C. Kojima, S. Tsumura, A. Harada, and K. Kono, Higher Order Structure of Short Collagen Model Peptides Attached to Dendrimers and Linear Polymers, *Biopolymers* 93:640-648 (2010).
14. Yue, L., and D. Jin, The synthesis of spherical calcium carbonate composite in amphiphilic PS-b-PAA solution and its thermal dynamic characteristic, *Chinese Science Bulletin* 49:235-239 (2004).

6. Acknowledgements

The authors thank the Director CLRI for the support extended. Authors also acknowledge the research funding from the Council of Scientific and Industrial Research, Govt. of India through EMPOWER project on Collagen stabilization using functionalized nanoparticles. The travel grant support made available to KJS by DST, CLRI (CSIR) and CICS is thanked. MN thanks the CSIR for the SRF. SS thanks CSIR Network program on ZERIL for the research fellowship.